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Antioxidant Activity and Soluble Protein Content of Tempeh Gembus Hydrolysate

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ABSTRACT

Tempeh gembus is fermented soy-pulp product contains high protein and its bioactive peptide components has potential antioxidant activities. In this study bromelain enzyme was applied in tempeh gembus to break up peptides bond and released bioactive peptides and amino acids. The aim of this research was to analyse antioxidant activity and soluble protein of tempeh gembus hydrolysate. Experimental research with 4 bromelain enzymes were applied in tempeh gembus as 0 ppm, 5000 ppm, 8000 ppm, and 10000 ppm. Antioxidant activities were measured by ABTS and DPPH radicals test. Whil e soluble protein content was measured by Bradford test. In general, antioxidant activity of tempeh gembus was higher when measured by ABTS radical (63.14±1.16 – 92.85±2.28%) compared to DPPH radical (52.21±5.76 – 65.70±5.89%). Antioxidant activity of tempeh gembus hydrolysate in ABTS test differed significantly between treatment groups (p=0.001), but not on DPPH test (p=0.110). While soluble protein content of these protein hydrolysate were 0.58±0.05 – 0.78±0.11% and significantly differed between treatment groups (p=0.019). Antioxidant activity was significantly higher when measured by ABTS radical compared to DPPH radical with different protein soluble content.

Keywords: *tempeh gembus, hydrolysate, antioxidant activity, soluble protein*

Free radicals are the molecule which have one or more unpaired electrons.[1] It is known that many disease related to oxidative stress is caused by excess of free radicals.[2] Diseases such as cancer, cardiovascular disease like hypertension, atherosclerosis, and neurological disease, all show strong evidence that ROS (reactive oxygen species) is involved in their pathophysiological process.[3] Human body actually has defense mechanism against the free radicals called antioxidant. Antioxidant will neutralise free radical by giving one of its free electrons, thus stop the chain reaction done by free radicals.[1]

Soybeans contain high antioxidant content, belong to *Leguminosae* family.[1] The use of soybeans as local food have been various, some of them are tempeh, oncom, tahu, and tempeh gembus. Tempeh gembus is food fermented product made from soy pulp which left over after soybean curd making process. The microorganism used to fermented this soy pulp is same with the microorganism used to fermented tempeh. In Japan, this soy pulp is known as *Okara* which is the residue of tofu or soy milk making process.[4, 5]

The nutrition components of tempeh gembus are similar with tempeh although the contents of tempeh gembus nutrition are less than tempeh. This happens because tempeh gembus is made by soy pulp from soybean curd residue making process so the nutrition contents inside have been diminished. Tempeh gembus contains nutrition contents, such as essential fatty acids, unsaturated fatty acids, protein, carbohydrate, fiber, calcium, and iron.[6, 7] The content of tempeh gembus energy is about 50% of the tempeh energy, the protein and lipid contents are less than tempeh as well, while the fiber content is on the other hand, it is three times (4.69%) richer than tempeh (1.40%). Protein content in tempeh gembus is about 3.41 gr/100 gr wet weight of tempeh gembus or 4.07 gr/100 gr dry weight of tempeh gembus that contains seven essential amino acids and eight non-essential amino acids.[4, 8] Another study conducted to analyse nutritional composition during tempeh gembus processing found that the total content of amino acids decreased from 34.95% in soybean to 6.7% in tempeh gembus without any changes on amino acids composition.[9] The composition of amino acids in tempeh gembus is complete enough so tempeh gembus can be a potential bioactive peptides source. Lipid content of tempeh gembus is low, it is about 0.23 gr/100 gr dry weight of tempeh gembus, but it contains essential fatty

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acids that is linoleic acid (21.51%), oleic acid (16.72%), and linolenic acid (1.82%). In addition, tempeh gembus also contains several minerals, ergosterols, and isoflavonoids.[4, 8]

Bioactive peptides are specific protein fragments that are inactive within the sequence of the parent protein and may show their physiological function when they are released from the parent protein.[10, 11] One of their physiological functions is their ability to be antioxidant. Bioactive peptides have good ability in free radicals scavenging activity because of their good ability to effectively react with the free radicals.[12] In a study, antioxidant activity showed by bioactive peptides from fermented *Okara* using *B. subtilis* B2, reaches 4.1 and 34.8 gr trolox eq/g dry *Okara* using DPPH and ABTS tests, respectively.[13]

Functional ability of protein can be modified via enzymatic hydrolysis under controlled condition. Many bioactive peptides are inactive within the parent protein and can be released during the enzymatic hydrolysis.[14] Enzymatic hydrolysis result in peptide bonds breaking so the bioactive peptides inside may release. Bromelain is one of proteases that can be used in enzymatic hydrolysis process. Bromelain is produced from pineapple fruit that spread over Indonesia. Bromelain is chosen because of its advantages that bromelain is easily obtained in Indonesia and be available from the beginning of the pineapple fruit growth till the fruit is ripe, although there is fluctuation in its proteolytic activity. Protease from other fruits such as tin and papaya are only found when the fruits are ripe. In addition, bromelain also active both in pure enzyme form and in pineapple juice form.[15] The use of bromelain for producing bioactive peptides from tempeh gembus has never been reported yet.

The intention of the present study was to determine the antioxidant activity and the soluble protein content of the tempeh gembus hydrolysate which possess anti-oxidative properties. Antioxidant activity of tempeh gembus hydrolysate in this research was measured by ABTS and DPPH tests. Both methods were chosen to be used because ABTS and DPPH are simple enough, the most used, and the most popular tests to analyse the antioxidant total capacity.[16] Besides, both ABTS and DPPH have same principle test that ABTS and DPPH undergo decolorisation to identify antioxidant activity which scavanges the ABTS and DPPH radicals.[17] Whereas, the soluble protein content was measured by Bradford test. Bradford test was chosen to confirm the process of protein hydrolysis becomes peptides or amino acids. In this method, amino acids or peptides are not able to form a complex with CBB dye so the blue color will not appear.[18]

METHOD AND MATERIALS

This was an experimental study with complete randomized experimental design using four treatments. Samples were tempeh gembus hydrolysate obtained from hydrolysis process using bromelain enzyme with concentrations were 0 ppm, 5000 ppm, 8000 ppm, and 10.000 ppm. Based on the concentration of bromelin enzyme used to hydrolyse, the tempeh gembus hydrolysate was categorised into four treatment groups. Tempeh gembus was obtained from local home industry of tempeh gembus in Semarang Indah, Central Java, using soy pulp of the soybean curd residue from Grobogan Village. The data were analysed by ANOVA.

Tools and Materials

The tools were blender, spoons, test tube, small cup, water bath incubator, stative, stirring bar, freeze dryer, kuvet, vortex, sentrifuge, magnetic stirer, pipet, pH meter, erlenmeyer, and beaker glass. The materials were tempeh gembus, aquadest, bromelain enzyme, dextrin, NaCl, NaH_2PO_4, Na_2HPO_4, ABTS, DPPH, metanol, pottasium persulfate, and Bradford reagent.

Process of making tempeh gembus hydrolysate

Tempeh gembus (300 gr) was steamed for 10 – 15 minutes, then the steamed tempeh gembus was blenderised and 450 ml aquades were added. Next, the blenderised tempeh gembus was divided into four groups, each groups was added bromelain enzyme as much as 0, 5000, 8000, and 10000 ppm, then each groups was incubated in water bath incubator for 1.5 hours at 55^{o}C. After being incubated for 1.5 hours, each groups of blenderised tempeh gembus was added dextrin and NaCl, each of them were 1.5 gr then the groups were heated to 70^{o}C for 10 minutes. After 10 minutes, tempeh gembus that had been thick due to the process was freeze dryed until dry.[19, 20]

Determination of antioxidant activity using ABTS test

ABTS kit solution was made by reacting 7 mM of ABTS solution with 2.45 mM of pottasium persulfate (1/1, v/v) then the mixture was dark incubated for 16 hours at room temperature. After being incubated, the mixture was diluted with metanol to obtain an absorbance of 0.700 ± 0.05 at 734 nm. Samples were tempeh gembus hydrolysate that have been dissolved before (100

µl) reacted with 900 µl of ABTS kit solution, shook for a moment, then the absorbance of samples was measured at 734 nm.[21]

Determination of antioxidant activity using DPPH test

DPPH kit solution was prepared by dissolving 7.88 mg of DPPH into 100 ml metanol to make DPPH solution with concentration was 0.2 mM. The absorbance of DPPH solution was measured at 517 nm by reacting 600 µl metanol in a kuvet then added by DPPH solution until the volume were 3 ml. Closed and shook the mixture until homogen, before measuring the absorbance. Samples were made by dissolving 2 mg of tempeh gembus hydrolysates into 4 ml of metanol, after being dissolved, samples (600 µl) were reacted with 3 ml of DPPH solution. Closed and shook until homogen, then the absorbance of samples was measured at 517 nm.[22]

Determination of soluble protein content using Bradford test

Bradford reagent was made by mixing 10 mg of Coomassie Briliant Blue (CBB) into 50 ml of metanol, then the mixture was dissolve into 100 ml of phosphate acid. After dissolving, aquades were added following 1:2 ratio. Stored in a dark closed bottle. Samples were prepared by reacting 20 µl of tempeh gembus hydrolysate (the tempeh gembus hydrolysate has been dissolved before) with 1 ml of Bradford reagent, then the mixture was incubated for 1 hour. The absorbance of samples was measured at 595 nm.[23]

RESULTS

Antioxidant activity

All groups of tempeh gembus hydrolysate showed radical scavenging activity against ABTS and DPPH radicals presented as antioxidant activity percentage (%) and shown on Table 1. There was significant different over the four treatment groups assessed by ABTS test (p=0.001) but not significant to DPPH test (p=0.110). Antioxidant activity of tempeh gembus hydrolysate assessed by ABTS test showed increase as the concentration of bromelain enzyme used was increased, while assessed by DPPH test, its antioxidant activity decreased with the same treatments. Tempeh gembus hydrolysate showed the highest of antioxidant activity when bromelain enzyme used reached 10000 ppm concentration (92.85 ± 2.28%) using ABTS test. Whereas using DPPH test, the highest antioxidant activity of tempeh gembus hydrolysate (65.70 ± 5.89%) achieved when no bromelain enzyme was added (0 ppm).

Soluble protein content

Table 2 shows the soluble protein content of tempeh gembus hydrolysate. It was found that the soluble protein content of tempeh gembus hydrolysate showed significant decrease as the use of bromelain enzyme was increased (p=0.019). The highest value was 0.78 ± 0.11 when the concentration of bromelain enzyme used in tempeh gembus hydrolysate was 5000 ppm.

DISCUSSIONS

Antioxidant activity of tempeh gembus hydrolysate

Antioxidant activity of tempeh gembus hydrolysate significantly increased based on ABTS test but decreased in DPPH test. Enzymatic hydrolysis process which used bromelain enzyme as hydrolisis agent may cause antioxidant activity increased through several mechanisms. It was clear that hydrolysis brokedown peptide bonds of tempeh gembus protein and may cause the bioactive peptides of tempeh gembus released. Bioactive peptides were considered to be responsible to the increase of antioxidant activity of tempeh gembus hydrolysate. Bioactive peptides which had antioxidant activity generally contained 5 – 16 amino acids residue which may had different characteristics in composition, structure, and hydrofobicity.[11] A study stated that radical scavenging activity of β – conglycinin and glycinin increased 3 – 5 times after both of them enzymatically digested.[10, 24, 25]

Some studies mentioned amino acids such as tyrosine, methionine, histidine, lysine, cystein, and tryptophan have been generally accepted as antioxidant amino acids.[24] A study conducted to identified nutrition and composition of tempeh gembus stated that the highest amino acids existed in the tempeh gembus were threonine, tyrosine, and histidine (0.95%, 0.72%, and 0.60%, respectively).[8] The others stated that tyrosine, histidine, phenilalanine, lysine, and leucine amino acids showed and improved the peptide antioxidant actvity.[15, 26]

Certain amino acids have structural characteristics that may enhance the antioxidant activity in the tempeh gembus hydrolysate. During the soybean protein hydrolysate process, the structure of soybean protein underwent changes and amino acids in R group will be more actively exposed, resulted in the higher of antioxidant activity of soybean peptides produced from the hydrolysis process than the intact protein.[11, 27] Amino acids with aromatic residue such as tyrosine, typtophan, and phenilalanine amino acids were known could enhance antioxidant activity because of their capability to

easily release their proton to the radical that lacked of electron and directly played a role in radical scavenging activity. Histidine also indicated strong enough of radical scavenging activity and could act as metal – ion chelators, hydrogen donation, and oxygen active and radical hydroxyl scavenging.[24, 26, 28] It happened because histidine decomposed its imidazol ring during reacting.[28, 29] Cystein has sulfihidril (-SH) residue that allowed cystein to act as antioxidant activity as well by directly reacting with the radical.[28]

Hydrophobicity was the important factor that played a role in antioxidant activity of peptides. It was a tendency of amino acids to not interact with water at physiological pH.[30] Antioxidant activity of peptides was more related to the total hydrophobic amino acids content than peptides size.[15] A study conducted about refinery and characterisation of antioxidant peptide from a soy protein hydrolysate indicated that the content of hydrophobic amino acids increased 7% to 60% while the hydrophilic amino acids extremely decreased from around 59% to 9%. Hydrophobic amino acids were known to play a role in the inhibition of peroxidation by increasing peptide solubility in fat and acting as an antioxidant which increases peptide solubility in non-polar environments so as to facilitate the interaction of amino acids (especially in hydrophobic amino residues) with radical compounds by reducing the radical activity better.[31-33]

These three things may lead to the improvement of antioxidant activity of tempeh gembus hydrolysate.

Whereas the decrease of antioxidant activity of tempeh gembus hydrolysate assessed by DPPH test in the authors' result seemed to be correlated to several literatures. DPPH radical was relatively more stable than ABTS radical so it was harder to be neutralised. Some antioxidant agent reacted more quickly when assessed used other tests and reacted slower to DPPH resulting in a slower reaction range.[15] ABTS test was known more sensitive to assess radical scavenging activity done by peptides and amino acids because it was more reactive to peptides and amino acids.[32]

A study stated that DPPH test indicated the insensitivity of DPPH may caused antioxidant activity assessed by DPPH test was smaller. Only cystein having SH-group with strong reduction capability was able to show DPPH radical scavenging activity compared to the others amino acids assessed by DPPH test.[34] When DPPH test was compared to ABTS and ORAC tests to assess antioxidant activity, tyrosine and tryptophan indicated the strongest antioxidant activity and were thought to be responsible for the antioxidant activity of the peptides. Dipeptides that contained cystein showed DPPH radical scavenging activity around 0.14 – 0.25 µmol TE/mol, while all peptides containing tyrosine or tryptophan showed very weak DPPH radical scavenging activity with TE value around 0.00 – 0.02 µmol TE/mol. Whereas when assessed by ABTS and ORAC tests, dipeptides containing cystein indicated moderate radical scavenging activity and very strong radical scavenging activity to dipeptides containing tyrosine or tryptophan. Try – Gly dipeptides showed TE value up to 5.05 µmol TE/mol using ABTS test.[34] This insensitivity may allow DPPH test showed smaller result of antioxidant activity of tempeh gembus hydrolysate. As mentioned before, amino acids found high in the soy protein hydrolysate were tyrosine, histidine, isoleucine, phenilalanine, lysine, leucin, and valine.[26, 31] Whereas amino acids found high in tempeh gembus were threonine, tyrosine, histidine, glutamic acid, and aspartic acid, while cystein were found in very low levels.[4, 8] DPPH radical were unable to react quickly towards this such of peptides and amino acids resulted in decrease of antioxidant activity.

Soluble protein content of tempeh gembus hydrolysate

The soluble protein content of the tempeh gembus hydrolysate tended to decrease as the concentration of bromelain enzyme used increased. Bromelain is an endopeptidase enzyme which has sulfihidril group (-SH) on the active site so the bromelain is an cysteine protease.[15, 35] Bromelain break the protein on the carbonyl end of lysine, alanine, tyrosine, and glycine amino acids. Bromelain produces protein hydrolysate that contains mix of bioactive and non-bioactive peptides. [15] Hydrolysis peptide bonds and polypeptides by bromelain generated peptide with small size and amino acids. Hydrolysis will reduce the molecular weight of protein and enrich the number of polar groups.[20] Actually, the hydrolysis of protein changes non soluble protein into soluble protein due to the decrease in molecular weight of the protein so the solubility increase, but further hydrolysis will expose the hydrophobic protein that used to be interior protein which makes the solubility decrease.[19, 20] This literature explained the fluctuation of results of the soluble protein content of tempeh gembus hydrolysate. Hydrolysing using 5000 ppm of bromelain enzyme showed the highest soluble protein content because protein hydrolysis process changed the non soluble protein into soluble protein, thus the solubility and its value increased, but the results of soluble protein

content using 8000 ppm and 10000 ppm of bromelain enzyme decreased. The decrease was most likely caused by the further hydrolysis that produced the hydrophobic protein which made the solubility and its values decreased as well.

High free amino acids, peptides, and proteins with low molecular weight contents of the tempeh gembus hydrolysate that less than 3000 Da were unable to form complex with CBB *(Coomassie Brilliant Blue)* dye so the blue color did not appear resulted in the soluble protein content of tempeh gembus hydrolysate read as low.[18, 36] In addition, the probability of the appearance of hydrophobic protein due to further hydrolysis also could decrease the soluble protein content of tempeh gembus hydrolysate. So, this result was correlated with the result of the antioxidant activity of tempeh gembus hydrolysate that showed as high in the result of ABTS test.

The use of protein hydrolysate has been various. Protein hydrolysate is generally used for flavor-lasing, but now its usage starts shifting becomes functional food product.[7]

A study mentioned the use of tempeh hydrolysate to develop a sport drink formula as a beverage to recover the athletes' muscle damage. The high antioxidant content in the hydrolysate was useful in the development of sport drink, to prevent free radical activity causing lysis of muscle cell membrane, which was produced during and after strength exercise.[37] This study may open the opportunity for tempeh gembus hydrolysate to be developed as a basic or fortification ingredient in sport drink to increase the peptides and amino acids antioxidant content there in.

A study conducted on rats fed an atherogenic diet successfully found that giving of tempeh gembus with additional bromelain enzyme to the rats fed an atherogenic diet had the lowest mean value of homocysteine level. This result was due to a higher antioxidant content produced from protein hydrolysis process was able to protect the endothelium from oxidative stress as the endothelium produced nitrit oxide optimally that would detox homocysteine induced by the atherogenic diet. High level of homocysteine can cause oxidative stress due to excessive ROS and is considered as factor of independent risk for the cardiovascular disease and not constituent of normal diet.[7] This study may lead to the use of tempeh gembus hydrolysate as functional food to prevent the cardiovascular diseases. In addition, antioxidant content in tempeh gembus hydrolysate also can be used to inhibit the oxidation process in high fat foods and stabilize food products which need stabilisation.[38]

Tempeh gembus also has another functional properties. Tempeh gembus known to be potential source of microbial fibrinolytic protease that could be used to produce functional food or medicine to treat cardiovascular diseases. A study stated that *B. pumilus* confirmed as microbial fibrinolytic from isolate of tempeh gembus known to be have proteolytic and fibrinolytic activities that might be able to act as antithrombotic.[39, 40]

In conclusion, there were significant different on antioxidant activity using ABTS test and soluble protein content of tempeh gembus hydrolysate among the four treatment groups (0 ppm, 5000 ppm, 8000 ppm, and 10.000 ppm). ABTS test indicated higher antioxidant activity of tempeh gembus hydrolysate than DPPH test. Antioxidant activity of tempeh gembus hydrolysate increased as the concentration of bromelain enzyme increased based on ABTS test but not on DPPH test. The soluble protein content of tempeh gembus hydrolysate tend to decrease as the concentration of bromelain enzyme increased.

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Table 1. Antioxidant activity of tempeh gembus hydrolysate

Tempeh Gembus Hydrolysate (TGH)	Mean ± SD of Antioxidant Activity	
	ABTS test (%)	DPPH test (%)
TGH 0 ppm	63.14 ± 1.16^{a}	65.70 ± 5.89
TGH 5000 ppm	75.56 ± 9.43^{b}	58.58 ± 7.43
TGH 8000 ppm	88.56 ± 4.13^{c}	55.87 ± 9.25
TGH 10.000 ppm	92.85 ± 2.28^{c,d}	52.21 ± 5.76
p value*	0.001	0.110

*Based on One Way ANOVA test.

$^{a, b, c, d}$Different letters in the same column showed significant differences based on the LSD post hoc test ($p < 0.05$)

Table 2. Soluble protein content of tempeh gembus hydrolysate

Tempeh Gembus Hydrolysate (TGH)	Mean ± SD of Soluble Protein Content	p value*
TGH 0 ppm	0.60 ± 0.05[a]	0.019
TGH 5000 ppm	0.78 ± 0.11[b]	
TGH 8000 ppm	0.58 ± 0.10[a]	
TGH 10.000 ppm	0.58 ± 0.05[a]	

*Based on One Way ANOVA test.

[a, b, c, d]Different letters in the same column showed significant differences based on the LSD post hoc test ($p < 0.05$)

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Effect of Remote Ischemic Preconditioning on the Periprocedural Myocardial Injury Events during Elective Percutaneous Coronary Intervention

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ABSTRACT

Background: Periprocedural myocardial injury (PMI) occurs in at least a third of patients underwent elective percutaneous coronary intervention (PCI). Effect of remote ischemic preconditioning (rIPC) on the PMI and infarction remains elusive. Purpose of this study was to know the effect of rIPC on the PMI and infarction during PCI in patient with coronary artery disease (CAD) underwent elective PCI. **Method**: Forty-two patients with stable coronary artery disease underwent elective PCI were randomized into rIPC group (n= 20) and control group (n=22). RIPC protocol was 4 cycles of inflation-deflation using a blood pressure cuff 20 mmHg above the systolic blood pressure in one of the upper arm. Assessment of PMI was determined by increase of cardiac enzyme CK-MB at 18 to 24 hours post PCI. **Result**: The levels of CK-MB post PCI was significantly lower in the RIPC group than control, 25.15±5.46 vs. 40.59±21.16 μg/mL, respectively (p=0.003). Evidence of PMI was significant lower in the RIPC group than that of the control, 2.3% vs.19.04% (p=0.022), while that of the infarction was not significant difference between both groups, 0% vs. 2 (4.76%), respectively (p=0.489). **Conclusion**: Remote ischemic preconditioning may reduce periprocedural myocardial injury in patient with CAD underwent elective percutaneous coronary intervention.

***Keywords**: Periprocedural myocardial injury, remote ischemic preconditioning, creatine kinase myocardial band, percutaneous coronary intervention.*

It is known that periprocedural myocardial injury (PMI) is a common complication in patient undergoing percutaneous coronary intervention (PCI).[1] The incidence of PMI in patients underwent elective PCI is 15 to 40%.[2,3] PMI is any elevation of creatine kinase myocardial band (CK-MB) above the upper normal limit post PCI.[4] Periprocedural myocardial injury in elective PCI is also correlated with an elevated mortality risk, risk of recurrent infarction and need for revascularization in the future. Although the blood flow at epicardial level is in the range of normal and PCI is considered successful, a disturbance in microcirculation level evidenced by a slight increment in CK-MB level is correlated with a long term cardiovascular prognosis.[5-7]

There are several strategies to avoid a periprocedural myocardial injury. They are including strategy to prevent side branch occlusion, strategy to prevent distal embolization and microvascular coagulation, and strategy to protect myocardium.[3] Remote ischemic preconditioning (rIPC) is a method applying sub-lethal ischemic to target organs remote from the heart, such as small bladder, kidney, and the most non-invasive organ are the lower and upper extremities. rIPC decreases ischemic complication in both percutaneous coronary intervention and cardiac surgery procedure.[8,9] This procedure is safe, physiologic, non-invasive, simple, and inexpensive non-pharmacological method and potentially reduce periprocedural myocardial injury event. Patients with coronary artery disease (CAD) underwent PCI have risks for periprocedural myocardial injury and infarction, and this study found that remote ischemic preconditioning reduce periprocedural myocardial injury in patient PCI. However, the effect of rIPC remains elusive, and this study found that this rIPC reduce periprocedural myocardial injury but not infarction.

METHODS

Subject and design of study

This study was quasi-experimental, pre-posttest with control group design, in one center (Dr. Kariadi General Hospital, Semarang, Indonesia). Subjects of study were allocated into either intervention or control group by a simple randomization. Informed consent for participation in this study was obtained and the investigation was approved by the institutional ethics committee of human research in the

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Faculty of Medicine Diponegoro University and conformed with the principles outlined in the Declaration of Helsinki.[10]

The eligible subjects for inclusion in the study was patients with stable CAD underwent elective PCI. Patients with an increase of CK-MB level prior the PCI, acute coronary syndrome, chronic total occlusion (CTO) lesion, unstable hemodynamic (systolic blood pressure <90 mmHg and/or pulmonary edema), severe renal disease (creatinine serum level above 2.5 mg/dl), peripheral vascular disease, myo-skeletal injury or disease, malignancy, ore more than 70 years old were excluded from this study.

The baseline serum CK-MB was taken prior the PCI.[11] In the intervention group, a one hour rIPC prior PCI was performed by using a blood pressure cuff in the arm without intravenous line. The blood cuff was inflated 20 mmHg above patient's systolic blood pressure for 5 minutes, and then deflated for 5 minutes. This intervention was done for 4 cycles of ischemia-reperfusion.[12] The subject was dropped out if the PCI was postponed for more than 3 hours post rIPC protocol.[13] Angiographic characteristic and procedure, including lesion type, blood vessel target, pre-dilatation pressure and duration, length of stent, type of stent, thrombolysis in myocardial infarction (TIMI) flow post stent insertion, and complication post PCI were noted during PCI. Stent was inserted properly based on the clinical practice. All patients received bolus intravenous injection of unfractionated heparin with dose 70-100 *IU*/kg body weight. The second bolus heparin was given to maintain activated clotting time >250 seconds. Angiographic success was defined as residual stenosis <20% at the end of angiographic based on visual estimation. Procedural success was based on angiographic data and no major complication during hospitalization (acute myocardial infarction, need for emergency coronary artery bypass surgery or death). Complication after PCI was recorded. Those complication included side branch occlusion, dissection, distal embolization, arrhythmia, emergency coronary artery bypass surgery, acute myocardial infarction or death during hospitalization. Assessment of CK-MB level was done at 18 to 24 hours post PCI.[14,15]

Statistical analysis

SPSS software version 20.0 (Polar Engineering and Consulting, USA) was used for the statistical analysis. Mean differences between CK-MB level prior and post PCI were analyzed using the independent *t*-test or Mann-Whitney test as appropriate for continuous variables. The χ^2 test or Fisher's exact test was used as appropriate to compare categorical variables. The data were expressed in mean±SD. Differences with a p value of <0.05 were considered statistically significant.

RESULT

Baseline Characteristics

A total of 68 patients with CAD underwent elective PCI were enrolled. Nineteen patients were excluded and 7 patients were dropped out, and thus 42 patients were included for further analysis. They were randomized into intervention group (20 patients) and control group (22 patients). RIPC protocol was done successfully in all patients in the intervention group with no clinical evidence of arterial embolism, vein thromboembolism, or another complication. Clinical characteristics of subjects were shown in Table 1. The average of age was 58.47±5.96 years old. The most patients (78.5%) were male.

1.1 Angiographic and Procedural Characteristics

Most of patients had a single vessel stenting, including 13 (59.1%) patients in control group and 11 (55%) patients in intervention group (Table 2). The B type lesion based on the American Heart Association/ American College of Cardiology (AHA/ACC) criteria was found frequently in both groups. The number of *drug eluting stents* (DES) used was higher than non-DES, and they were placed mostly at left anterior descending artery (LAD). Almost of the stent installment procedure were preceded by pre-dilatation steps. There were no significant differences in angiographic characteristic and procedure between the intervention and control groups.

Table 1. Baseline Characteristics

Variables	Control (n=22)	rIPC (n=20)	*p*
Age	57.73±5.57	59.30±6.4	0.400
Male, n (%)	19 (86.3)	14 (70.0)	0.197
Body Mass Index ± SD	22.93±2.66	24.08 ± 2.91	0.186
LVEF (%), mean ± SD	56.60±9.19	56.25 ± 9.25	0.893
Traditional Risk factors			
Hypertension, n (%)	20 (90.9)	17 (85)	0.656
Diabetes Mellitus, n (%)	10 (45.5)	14 (70)	0.108
Active smoker, n (%)	5 (22.7)	3 (15)	0.767
Family history of premature CAD, n (%)	1 (4.5)	3 (15)	0.333
Hypercholesterolemia, n (%)	2 (9.09)	4 (20)	0.400
Pharmacological Therapy			
ACE-*inhibitors*, n (%)	12 (54.5)	10 (50)	0.768
Beta-*blockers*, n (%)	7 (31.8)	8 (40)	0.580
Nitrates, n (%)	17 (77.2)	13 (65)	0.379
Statin, n (%)	22 (100)	18 (90)	0.221
ARB, n (%)	5 (22.7)	2 (10)	0.269
Trimetazidine, n (%)	1 (4.54)	1 (5)	0.598

Statistical analyses were done using Mann-Whitney, except for age, body mass index and LVEF were using independent *t*-test.

Table 2. Angiographic and Procedural Characteristics

Variables	Control (n=22)	rIPC (n=20)	*p*
Target vessel revascularization, n (%)			
1 vessel	13 (59.1)	11 (55)	0.787[c]
2 vessels	7 (31.8)	8 (40)	
3 vessels	2 (9.1)	1 (5)	
Type of lesion (AHA/ACC)[16], n (%)			
Type A	3 (13.6)	1 (5)	0.591 [c]
Type B	14 (63.6)	15 (75)	
Type C	5 (22.8)	4 (20)	
Revascularized vessel, n (%)			
LAD	15 (68.18)	17 (85)	0.201 [c]
LCx	5 (22.7)	6 (30)	0.592 [c]
RCA	11 (50)	5 (25)	0.096 [c]
Others	4 (18.18)	3 (15)	0.782 [c]
Multi-vessels stenting, n (%)	10 (45.5)	9 (45)	0.976 [c]
Stenotic percentage, %, mean±SD	84.7±7.47	81.5±7.45	0.180[b]
Total stent length, mm, mean±SD	53.7±29,75	51.1±27.46	0.641 [b]
Overlapped stenting, n (%)	11 (50)	9 (45)	0.746[c]
Drug Eluting Stents, n (%)	10 (45.5)	14 (70)	0.108[c]
Fluoroscopic time, minute, mean±SD	24.83±16.69	24.03±12.74	0.862[a]
Direct stenting, n (%)	3 (13.6)	2 (10)	0.716[c]

[a]Independent t-test, [b]Mann-Whitney, [c]Chi-square

Table 3. Levels of CK-MB pre and post-PCI

Incidence	Control	rIPC	*p*
Periprocedural Myocardial Injury, n (%)	8(19.04)	1 (2.38)	0.022[a]
Periprocedural Myocardial Infarction, n (%)	2 (4.76)	0	0.489[a]

[a]Fisher's exact test

rIPC, remote Ischemic Preconditioning; LAD, Left Anterior Descending; LCx, Left Circumflex; RCA, Right Coronary Artery; AHA/ACC American Heart Association/ American College of Cardiology

1.2 Incidence of Periprocedural Myocardial Infarction and Injury

Table 3 shows that the level of CK-MB prior PCI was no significant difference between two groups, but its level post PCI was lower significantly in the intervention group than that of the control, 40.59±21.16 vs. 25.15±5.46 µg/mL, respectively (p=0.003). Thus, the delta level of CK-MB pre and post PCI was significant lower in the intervention group than that of the control, **6.15±3.88 vs. 22.0±20.0 µg/mL, respectively** (***p*=0.001).**

Table 4. Incidence of Periprocedural Myocardial Infarction and Injury

CK-MB	n	Mean ± SD, iu/L		*p*
		Control	rIPC	
Pre-PCI, mean±SD	42	18.59±3.17	19.00±2.90	0.665[a]
24 hour post-PCI, mean±SD	42	40.59±21.16	25.15±5.46	0.003[a]
Δ CKMB Pre-Post, mean±SD	42	22±20	6.15±3.88	0.001[b]

[a]Independent *t*-test, [b]Mann-Whitney

Furthermore, we observed periprocedural myocardial infarction and injury events in both groups. The periprocedural myocardial injury events were lower significantly in the intervention group than that of the control, 1 (2.38%) vs. 8 (19.04%), respectively (p=0.022), while the periprocedural myocardial infarction was not significant difference between the intervention and control groups, 0% vs. 2 (4.76%), respectively (p=0.489) (Table 4).

DISCUSSION

Remote ischemic preconditioning may potentially reduce periprocedural myocardial infarction and injury. The data of this study showed that four cycles of 5-minutes ischemia, followed by 5-minutes reperfusion in upper limb reduce the event of periprocedural myocardial injury in patient with CAD underwent elective PCI. Effects of remote ischemic preconditioning on myocardial injury have been reported by several studies, but its effectiveness remains elusive. Several factors affect the results, including the protocol used for rIPC, marker used to measure the outcome, subset of patients, and the use of cardioprotective agents such as statin.

Cardioprotective effect of rIPC was also showed by four other reports. [9,17-19] All of these studies used the same protocol for rIPC by performing three cycles of 5-minutes inflations of a blood pressure cuff to 200 mmHg around the upper arm, followed by 5-minutes intervals of reperfusion. They showed a similar outcome that rIPC prior to PCI attenuates the release of cardiac troponin at 16 to 24 post elective PCI. Our data in this study showed that rIPC prior to PCI reduce CKMB level at 18 to 24 hours post PCI, a finding that concur with result from a prior study by Liu et al.[17], while Ahmed et al.[19] reported that rIPC did not affect the CKMB level.

On the other hand, three studies reported that rIPC fails to show a significant effect on the level cardiac troponin release post elective PCI.[20-22] Yılmaztepe et al.[20] used one cycle of 5-minutes inflating the blood pressure cuff up to 200 mm Hg

on the non-dominant arm which may not reach an optimal ischemic preconditioning for myocardium. While Xu et al.[21] performed a 3 cycles of 5-minute pneumatic medical cuff inflations to 200 mm Hg in elderly patients with CAD having DM. These subset of patients may not get benefit from rIPC prior elective PCI since they could have mitochondrial dysfunction leading to failure in response to myocardial preconditioning.[23] Iliodromitis et al.[22] also used a three cycles of 5-minutes ischemia-reperfusion with blood pressure cuff inflated to 200 mmHg, but at both upper limbs, which was likely to induce inflammation reaction, shown by increase of level of C-reactive protein (CRP).

In regard with rIPC protocol, most of studies used a blood pressure cuff inflation to 200 mmHg for 5 minutes [9,17-19,22] ;however, in our clinical experience, this pressure is quite high and causes discomfort to the patients. Thus, the inflating blood pressure cuff 20 mmHg higher than systolic blood pressure in one arm was used in this study based on the rIPC protocol from McDonald et al.[24] which adjusted pressure according to the systolic blood pressure.[12] Both myocardial injury markers troponin and CK-MB have been used in the measurement of effectiveness of RIPC. We used CK-MB in this study because of it is more relevant and could be used to determine a prognosis implication when compared to troponin.[25]

The evidence of periprocedural myocardial infarction in this study was not significantly different between the intervention and control groups. The same finding was also reported by Ahmed et al.[19]. While Luo et al.[18] found that rIPC reduce the evidence of myocardial infarction related PCI. The statistical significance could be resulted from the different number of participants involved in those studies.

The mechanism how rIPC attenuate myocardial injury related PCI has been proposed in hypothesis. A current study found that rIPC induces an intracoronary increase of nitric oxide levels associated with a decrease in myocardial damage measured as no increase in cardiac troponin I with electrocardiographic increases in the sum of R waves, suggesting an improved myocardium after elective PCI.[26]

CONCLUSION

Remote ischemic preconditioning may reduce periprocedural myocardial injury in patient with coronary artery disease underwent elective percutaneous coronary intervention. Several prior reports,[9,17-19,22] corroborate the finding that rIPC as a safe and effective strategy to protect myocardium during elective PCI. It could be applied routinely in clinical practice since it is non-invasive, easy, cheap, and safe procedure, with no complication event.

STUDY LIMITATION

Limitation of this study is that the patients who used intracoronary glyceryl trinitrate during PCI were not excluded. This agent is known to have a preconditioning-mimetic effect in both experimental group and control group.[27] The CK-MB level was measured in a single blood sample obtained 18 to 24 hours post PCI rather than defining the CK-MB release profile every 4 to 6 hours. The resultant value may not be the maximum plasma concentration, although it is generally accepted that the maximum concentration occurs between 16 and 30 hours after myocyte necrosis.

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P-Selectin as Predictor Venous Thromboembolism in Cancer Patients Undergoing Chemotherapy

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ABSTRACT

Background: Venous thromboembolism (VTE), consisting of deep vein thrombosis (DVT) and pulmonary embolism (PE), is the second-leading cause of death in patients with malignancy. P-selectin, the member of the selectin family of cell adhesion molecules, is found in the alpha granules of platelets and the Weibel-Palade bodies of endothelial cells. It is express on the cell surface on activation, mediates the adhesion of leukocytes, platelets, and cancer cells in inflammation, thrombosis, and cancer growth and metastasis. Recently, P-selectin has been investigated as a novel predictor for DVT. **Objectives:** To investigate the role of sP-selectin as predictors of DVT in cancer patients undergoing chemotherapy. **Patients and Methods:** This prospective cohort study was conducted in Dr. Kariadi hospital, Semarang, Indonesia, on 40 newly diagnosed cancer patients. Venous blood samples were drawn prior and after initial chemotherapy, for sP-selectin measurement with ELISA method. These patients were observed for the possibility of developing VTE during three-months period. **Results:** DVT occurred in 5 (12.5%) patients after a median period of 8 weeks. The most frequent cancer type was colorectal cancer (45%) and cervical cancer (15%). The cut-off point sP-selectin pre-and post- chemotherapy were 106,7 ng/ml and 111,7 ng/ml respectively. The median levels of sP-selectin in DVT patients pre-chemotherapy was 121.0ng/ml (IQR 107.5-230.6) and post-chemotherapy was 204.4ng/ml (IQR 110.9-278.3). In other hand, the median levels of sP- selectin pre-chemotherapy and post- chemotherapy in DVT negative patients were 82.0ng/ml (IQR 31.3-230.6) and 92.5 (IQR 40.9-278.3), respectively. With cut-off point sP-selectin level 111,7 ng/ml, the relative risk of DVT event was 8,7 (95% CI 1,017-74,39). **Conclusion:** In this study, high plasma levels of s P-selectin are predictive for venous thromboembolism in cancer patients undergoing chemotherapy.

Keywords: venous thromboembolism, sP-selectin, cancer, chemotherapy

Venous thromboembolism (VTE), consisting of deep vein thrombosis (DVT) and pulmonary embolism (PE), is the second-leading cause of death in patients with malignancy. The risk for VTE increased in hospitalized cancer patient and in those on active therapy. VTE results in a requirement for long-term anticoagulation, risk of bleeding complications, risk of recurrent events even with anticoagulation, delay or discontinuation of chemotherapy, consumption of healthcare resources, and a potential impact on patient quality of life.(1)(2)

The pathogenesis of VTE in cancer patients appears to be multifactorial. The most important clinical determinants for the risk of VTE are: (i) cancer-related factors (primary site, extensive disease, time interval from cancer diagnosis); (ii) patient-related factors (raised body mass index, reduced mobility, comorbidities, sepsis, previous VTE, abnormalities in blood counts); and (iii) treatment-related factors (chemotherapy, hormonal and biological therapy, surgery, indwelling catheters, erythropoietin stimulating agents).(3)

Among tumor sites, the very high risk for thrombosis were pancreas and stomach, the high risk were lung, lymphoma, gynecologic, bladder and testicular; and the lower risk were breast and prostate cancer.(4) There also appears to be a time-dependent variation in the VTE risk. During the first 3 months after cancer diagnosis, patients are at highest risk of VTE.(5)(6)

Virchow's triad consisting of stasis, endothelial injury and hypercoagulability has long been known as the major risk factor predisposing patients to VTE. This model is particularly illustrative for cancer and thrombosis. Elevation in coagulation activation and thrombin generation (prothrombin fragment 1+2, thrombin-antithrombin complex, and D-dimer), alteration in fibrinolysis (plasmin-antiplasmin complexes, PAI-1), decreased inhibitors (protein C, protein S, antithrombin) and activated protein C resistance are considered as markers of hypercoagulability in cancer. Venous stasis in cancer may due to decreased patient mobility, extrinsic compression by tumor or node and venous invasion by tumor. Endothelial injury in cancer may as a result from tumor invasion,

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surgery, radiation, central venous catheter and chemotherapy.(7)(8) Recent advances in the epidemiology, pathophysiology and management of thrombosis in cancer patients.

Several biomarkers have been identified as potentially predictive for VTE. Pre-chemotherapy elevation in leukocyte and platelet count and low haemoglobin levels are all predictive for chemotherapy associated VTE.(9) Other biomarkers, including soluble P-selectin, prothrombin fragment 1,2, factor VIII, C reactive protein and D-dimers have been studied as well.

P-selectin (CD62) is localized in the alpha granules of platelets and the Weibel-Palades bodies of endothelial cells, and belong to the selectin family of cell adhesion molecules. P-selectin mediates binding to specific carbohydrate-containing ligands, like PSGL-1 (P-selectin glycoprotein ligand-1) which is present on the majority of leukocytes and in smaller amounts of platelet. Soluble P-selectin (sP-selectin) is a circulating form of P-selectin, which results from shedding of the extracellular domain and maintains the requirements for ligand binding.(10) Plasma levels of sP-selectin are elevated in acute deep vein thrombosis. Furthermore, high levels of sP-selectin were associated with increased risk for recurrence of DVT(11)(12) and in cancer patients, high plasma levels of sP-selectin were predictive for VTE[13]. It is very interesting that cancer cells are able to enhance P-selectin expression on monocytes, macrophages, endothelial cells, and platelets. In the other hand, cancer cells also express CD24 on their surface which was identified to be a receptor for P-selectin. The interaction between P-selectin and CD24 on cancer cells allows their interaction with platelets, and their adherence to endothelium in the process of metastatic spread.(10)

The aim of the study is to investigate the role of sP-selectin as predictive parameter for the occurrence of VTE in cancer patients undergoing chemotherapy.

METHODS

Study setting

This cohort prospective study was performed in Dr. Kariadi hospital, the university hospital of Diponegoro University, Semarang, Indonesia. The research protocol was approved by the Review Board of the Dr. Kariadi hospital. Written informed consent was obtained from patients.

Patients and data collection

Between November 2016 and February 2017, 40 consecutive patients with active cancers were enrolled in the study. All patients were informed about the details of the study in an individual interview. The inclusion criteria for the study were as follows: (i) patients with newly diagnosed of cancer (ii) histological confirmation of the diagnosis (iii) age over 18 years (iv) willingness to participate and (v) written informed consent. Exclusion criteria were overt bacterial or viral infection within the last two weeks, venous or arterial thromboembolism within the last three months and continuous anticoagulation with vitamin K-antagonists or low molecular weight heparin (LMWH), and surgery or radiotherapy within the last two weeks.

Patients underwent a structured interview on their medical history, and data on tumor site, tumor histology and tumor stage were documented. Patients were given detailed written information about symptoms of VTE and were asked to report immediately to our center, if such symptoms occurred. A blood sample for determination of laboratory parameters was drawn before and after initial chemotherapy. The observation period started at the time of blood sampling. Patients were contacted every month via telephone to get information about the clinical course of their disease regarding occurrence of VTE. Observation period ended after 3-mo nths period, or until the occurrence of VTE, death, loss of follow-up or withdrawal of consent (which one of these came first).

Diagnosis of deep vein thrombosis

Color duplex sonography was performed at the Division of Radiology of Dr. Kariadi Hspital, Semarang, Indonesia. Patients with clinically suspected DVT and the pretest probability (Well scores $\geq$ 2) were performed color duplex ultrasonography. To avoid investigator-related variations of the results, color duplex sonography was performed in each patient by the same investigator. Diagnosis VTE was established when patient presented symptoms of VTE and positive findings either in duplex sonography or venography.

Blood sampling and laboratory analysis

Venous blood specimens were collected by sterile and atraumatic antecubital venipuncture, and collected in in citrate vacutainer tubes SST 5ml, containing 0.5ml of liquid anticoagulant. To obtain platelet-poor plasma, the citrated blood was centrifuged at 1000g (3000 rpm) for 15 min. Plasma aliquots were stored at-20 °C until they were assayed for the determination of sP-selectin plasma levels in series. Samples were coded prior to laboratory analysis. P-selectin levels were measured using a recombinant human P-selectin

Immunoassay/CD62P catalog number ADP3 (R&D Systems, Inc. 614 McKinley Place NE, Minneapolis, MN 55413, USA) following the manufacturer´s instructions.

Blood samples were scheduled to be drawn at the following time-points: (i) baseline, before initial chemotherapy, and (ii) the day after initial chemotherapy completely administrated.

Statistical analysis

Continuous variables were summarized with mean (SD) or medians (25^{th}-75^{th} percentile), whereas categorical data were described by absolute frequencies and percentages. The correlation between two continuous variables were evaluated with Spearman rank correlation coefficient. Fisher exact test was performed if the number of cells with expected frequency less than 5. Independent t-tests were done to compare numeric variables between DVT and non DVT patients. The assumption of normality test of the data was checked before the t-test. Mann-Whitney were done when the data were not normally distributed. The cut-off-point of significance was p=0.05 with 95% confidence interval. The median follow-up time was calculated with the reverse Kaplan Meier method 33. A log rank test was used to compare the time until first thrombosis in these two groups.

RESULTS

Between November 2016 and February 2017, 40 patients with newly diagnosed cancer were enrolled in the study. One patient died before given chemotherapy on 6^{th} weeks. DVT occurred in 5 (12.5%) patients after a median period of 8 weeks. Positive findings in duplex sonography or venography established the diagnosis of DV

Table 1. Clinical characteristics of patients. n=40

Variable	N	%
Age		
<41	11	27.5
41-59	25	62.5
>59	4	10
Sex		
Male	22	55
Female	18	45
Body mass index		
Underweight	11	27.5
Normal	29	72.5
Site of cancer		
Rectal	10	25
Colon	8	20
Cervical	6	15
Pancreas	3	7.5
Lung	2	5
Gaster	2	5
Haematological	2	5
Others	7	17.5
Stage of cancer		
Localized	23	57.5
Distance metastatic	15	37.5
Unclassified	2	5

The mean age of patients was 47±11. The most frequent cancer type was colorectal cancer (45%) and cervical cancer (15%).

Plasma concentrations of sP-selectin in cancer patients with and without DVT

The plasma concentration of sP-selectin in cancer patients without DVT, pre- and post-chemotherapy administration (median, IQR) were: 82ng/ml, IQR (31.3-230.6) and 92.5ng/ml, IQR (40.9-278.3), respectively. The plasma concentrations sP-selectin in cancer patients with DVT, before and after chemotherapy (median, IQR) were: 121.0 ng/ml, IQR (107.5-230.6) and 204.4ng/ml, IQR (110.9-278.3), respectively. (Normal value sP-selectin: 0.99-47.7ng/ml).

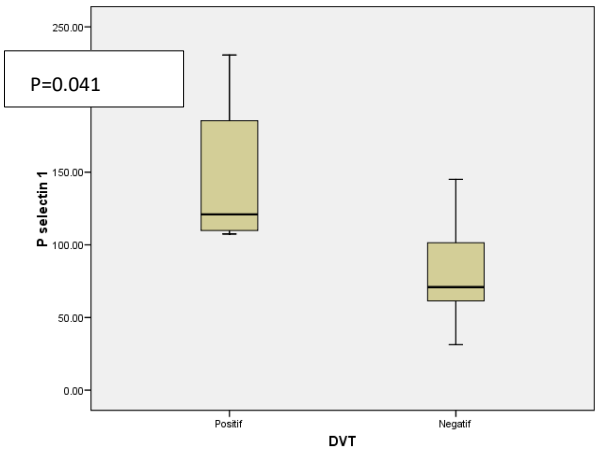

Figure 1.The plasma concentrations of sP-selectin levels pre-chemotherapyin 40 cancer patients with and without DVT. The difference is significant (p=0.041). Normal value sP-selectin: 0.99-47.7ng/ml.

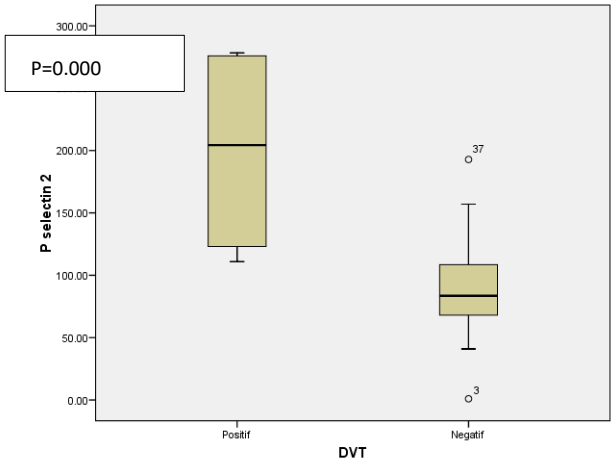

Figure 2.The plasma concentrations of sP-selectin levels post-chemotherapy in 40 cancer patients with and without DVT. The difference is significant (*p=0.000*). Normal value sP-selectin: 0.99-47.7ng/ml.

The cut-off point sP-selectin pre- and post-chemotherapy were 106,7 ng/ml and 111,7 ng/ml respectively. With the cut-off point P-selectin level > 111,7 ng/ml, the relative risk of DVT event was 8,7 (95% CI 1,017 – 74,39). (Table 2)

Table 2. Risk Estimate for the DVT occurrence with cut-off plasma levels sP-selectin111.7ng/ml in 39 cancer patients undergoing chemotherapy

	Value	Lower (95% CI)	Upper (95% CI)
Relative risk for sP-selectine 2(>111.7/≤111.7)	12.000	1.078	133.603
For cohort DVT = positive	8.700	1.017	74.390
For cohort DVT = negative	.725	.480	1.094
N of valid cases	39*		

The relative risk for DVT for 39 cancer patients undergoing chemotherapy with plasma levels sP-selectine >111.7ng/ml was 8.7 (95% CI 1.017-74.390). *one patient was died during the 6th week follow-up.

DISCUSSION

In the present study, we investigate the role of sP-selectine as a novel predictor DVT in cancer patients undergoing chemotherapy. Our finding demonstrates that plasma levels sP-selectine >111.7ng/ml, the relative risk for DVT is 8.7 (95% CI 1.017-74.390). Therefore, it means that high levels of sP-selectin are predictor for DVT in cancer patients undergoing chemotherapy.

Result from the Vienna CATS (Cancer And Thrombosis Study) demonstrates that elevated sP-selectin (cut-off level, 53.1ng/ml, 75th percentile of study population) was a statistically significant risk factor for VTE after adjustment for age, sex, surgery, chemotherapy, and radiotherapy (hazard ratio=2.6, 95% confidence interval, 1.4-4.9, p=.003).(13)

Our data indicate that, the levels of sP-selectin were high in all cancer patients, either in DVT or non-DVT, and, either pre- or post-chemotherapy. Virchow's triad (stasis, endothelial injury, and hypercoagulability) has long been used to illustrate the major risk factors predisposing patients to VTE. In our study, the increased levels of sP-selectin in all cancer patients reflected the hypercoagulability (prothrombotic) condition, which increased risk for thrombosis in cancer patients.

Moreover, in all cancer patients, either withDVT or without DVT, the levels of sP-selectin in post chemotherapy were higher than those in pre-chemotherapy, and the difference was significant. This finding indicated, that chemotherapy may increase the levels of sP-selectin, and therefore increase the risk for thrombosis.

In a population-based study of patients with a new diagnosis of VTE, there was a significantly increased risk of VTE in those who were receiving chemotherapy [OR 6.5, CI(2.11-20)].(14) Our study, which involving plasma levels sP-selectine >111.7ng/ml as cut-off point, the RRwas 8.7 (95% CI 1.017-74.390). Other study reported that chemotherapy increased the risk of VTE by 2-fold to 6-fold, with doxorubicin containing regimens.(3) The mechanism behind the risk of thromboembolic events with chemotherapy treatments are poorly understood. Many of these chemotherapies induce vascular damage, either directly or indirectly, thereby promoting local activation of the coagulation process. A number of more recent reports have demonstrated the role of P-selectin in hemostasis and thrombosis, including the demonstration that overexpression of P-selectin can induce a procoagulant state.

One out of 40 patients died during follow-up. The clinical presentation of this patient were shock and sudden death. This patient died possibly due to pulmonary embolism.

In conclusion, our study demonstrated that: (i) high plasma levels of soluble P-selectin are predictive for venous thromboembolism in cancer patients; (ii) the levels of sP-selectin were high in cancer patients, and the levels in post-chemotherapy were significantly higher than those in pre-chemotherapy. Further studies are needed whether combination of high levels of sP-selectin and predictive model for calculating risk of chemotherapy associated thrombosis (e.g. Khorana predictive model) increase the benefit of prophylactic anticoagulation for cancer patients undergoing chemotherapy.

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The Impact of Antenatal *Coping Skill Training* (ACST) towards Cortisol and IgG Serum Level among Pregnant Women

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ABSTRACT

Introduction: Stress coping skill is beneficial to make better outcomes of pregnancy and childbirth. The purpose of this study was to examine the impact of antenatal coping skill training on Cortisol and IgG levels. **Method:** This study used a randomized pre-test post-test control group design in which the ages of primigravida (24-34 weeks) in Semarang City Public Health Center were selected randomly. The mothers were randomly assigned to be an experiment group (N=31) and a control group (N=31). There were two pregnant women who dropped out because of giving birth. The experiment group was given the standard antenatal education and antenatal coping skill training while the control group was given the standard antenatal education only. The data collection was conducted in 4 weeks. Cortisol and IgG serum level were taken at the first week before the intervention and fourth week after the intervention. Cortisol and IgG serum level were measured by using ELISA method. The data analysis employed dependent sample t-test and independent sample t-test. **Results:** There was a significant change over Cortisol serum level for the intervention group ($p<.01$), but not in the control group. However, there was a significant change in the decrease of IgG serum level in the control group ($p<.01$). **Conclusion:** Antenatal coping skill training is predominantly effective to reduce Cortisol and enhance IgG serum levels. Thus, it is important for pregnant women to join antenatal psychoeducational training.

Keywords: *coping skill, cortisol, IgG serum, primigravida*

During pregnancy, women are more susceptible to experience stress than during postpartum.[1] The mother has a chance to increase stress following period of pregnancy.[2] These stress conditions can produce cortisol which suppresses the immune system that is immunoglobulin G (IgG). The IgG is an immunoglobulin produced by plasma cells derived from mature B lymphocyte proliferation. When the immune system is depressed then pregnant women are susceptible to be infected.[3] Pregnant women who experience stress and possess a lack of coping resource during pregnancy are more likely to encounter at least one pregnancy complication namely preterm birth (PTB) or shorter gestation and low birth weight (LBW).[4,5] According to Riskesdas (2013), LBW birth rate in Indonesia is still quite high, that is 10.2%.[6] Stress that occurs early in pregnancy will also cause the onset of hypertension in pregnancy and preeclampsia in the future because of excessive stimulation of the autonom nervous system.[2] While the psychological condition of the mother during pregnancy affects the output of labor and postpartum, mothers who have psychological problems during pregnancy tend to have problems in the output of labor and postpartum.[7] Thus, it is immediately clear that stress during pregnancy will affect a golden period in the first 1,000 days of life namely 370 days of gestation period and 730 days after birth which happens up to two years of life.

Antenatal education (AE) is one of the efforts to prevent problems and complications during pregnancy in pregnant women, in which an increased knowledge and a good preparation for childbirth are the main concerns. However, conventional AE is focused on delivering knowledge rather than preparing pregnant women for strengthening and identifying the sources of coping and self-confidence to face the delivery. There is also a mismatch of AE implementation in which the material is presented with the

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information needed by mother as expectant parents.[8,9] The adaptability of the mother during pregnancy is strongly associated with the mother's coping skills that distinguish the effects of psychological and physiological stress.[10] stress coping skill is the mother's ability to cope with and adapt to stress. Stress coping skill during pregnancy is meant to influence better outcome of pregnancy and childbirth, so that it can minimize or prevent the negative effects of emotional, behavioral, cognitive, and physiological responses to stress. Coping skills serve to select and implement appropriate efforts to cope with stress. In addition, it poses as an important resource bastion of pregnant women and children over the effects of the potential dangerous exposure of pregnancy stress.[11] Based on the aforesaid background, it is imperative to examine the impact of antenatal coping skills training (ACST) on the cortisol and IgG serum level in pregnant women. In addition to the urgency of the examination of the impact of antenatal coping skills training, there has been no research on antenatal coping skills training to pregnant women, so that it is justifiable to conduct the research to disclose the impact of antenatal coping skill training (ACST) on the cortisol and IgG serum level in pregnant women.

MATERIALS AND METHODS

Participants and Procedure

This study was a randomized study with pre-test post-test control group design. The Experiment aimed to examine any changes over Cortisol and IgG serum level from the intervention of ACST. The study was conducted at sixth Public Health Centers (PHC) of Semarang City, in which 37 health centers in Semarang City were chosen randomly. There were three PHCs as a control group and three PHCs as an experiment group. Six PHCs were randomly chosen to be three PHC control group and Each PHC held one standard of AE or ACST with 10 – 11 pregnant women for each PHC. The subjects in this study were 62 pregnant women *primigravida* who encountered the end of second trimester and early third trimester of pregnancy (24-34 weeks). In addition, the subjects also experienced normal pregnancy which possessed the productive age ranging from 20 up to 35 years old. Furthermore, the subjects were also demanded to possess exclusion criterion namely no history of psychological problems. In addition, the subjects must not come from single parent families. Subjects were coming from each health center over six PHCs were contacted and informed to participate in this study voluntarily. Subjects who met the criteria and were approved to participate were randomly chosen as the experiment group (N=31) and the control group (N=31). During the study there were two mothers from the control group who dropped out at the third week because of giving birth. Thus, at the end of the study, there were 60 subjects. Each PHC applied one AE class which consisted of 10 – 11 pregnant mothers. Control group was given AE standard that is developed by Health Ministry program provided in PHC. Intervention of ACST in the experiment group was done by giving AE standard and ACST that were given in the same session of antenatal education classes.

Measures/Instruments

Tools and materials in this study were the AE module package and the antenatal coping skill training (ACST). AE module has been standardized as an AE guideline in Indonesia issued by the Ministry of Health. ACST module was developed through literature reviews of stress coping skills. ACST module was also reviewed and validated by a psychologist. It was also validated through several stages of testing and refinement. At each meeting, there is a tool in the module consists of post-test knowledge, and evaluation of the ACST that have been used to revise the module.

The stress coping skills module consists of three parts. The first material contains material regarding stress, which is elaborated based on the definition of stress, sources of stress, factors that affect stress, psychological and physiological reactions to stress, stress on health, and stress in pregnant women as well as sources to support pregnant women. The second material discusses coping skills in pregnant women including problem-focused coping and emotion-focused coping. The material of the third meeting discusses about the principle of intervention coping, types of intervention coping, and the coping skills practice.

There were two kinds of books employed for the study that is facilitator's book and participants' book. Facilitator's book contains a guidance of delivering the materials and the goals while participant's book contains goals for each session, materials and exercise

at home and during AE class. The length of time for each meeting of the AE standard group was 120 minutes and the group of ACST was 190 minutes.

Interventions at each PHC were carried out by six trained midwives who have been certified as AE facilitators by the government. Each midwife held one class in each PHC. Three facilitators who held experiment group was also trained by certified psychologist to deliver the ACTS. Three midwives were given certified ACTS training. In other side, three midwives also delivered ACTS classes as a trial before implementing ACTS module.

The level of cortisol and IgG serum was measured by using ELISA method. Subjects were measured two times in the form of Cortisol and IgG serum level with venous blood sample. The venous blood sample was taken from the subjects at 09.00 – 10.00 a.m. Cortisol and IgG serum level (pretest) were measured at the time of the first contact (week one) with a gestational age of 24 weeks - 34 weeks before AE and ACST intervention. Posttest Cortisol and IgG serum level were measured at week four after intervention.

Ethics

Ethical clearance was proved by ethical committee of medical faculty of Diponegoro University and Kariadi Hospital number 767/EC/FK-RSDK/2016. All participants that involved in this study were given informed consent to participate in this study voluntarily.

Statistics

Bivariate analysis using a dependent t test was done to determine the differences of Cortisol and IgG serum level before and after the treatment within group, and the independent t test was done to determine the difference between control and treatment groups,[12] which the p value < .05 showed significant differences. T-test were employed because the values were normally distributed, which the p value >0.05.

RESULTS

Cotisol serum Level

The study results clearly revealed that the experimental group which received ACST encountered sharper decrease in cortisol levels (-47.132) compared to the control group who received AE (-14.247). Cortisol levels were measured by ELISA with normal levels of 50-230 ng / ml. Changes of serum cortisol levels before and after the intervention are shown in the Table 1 and Figure 1 below.

In the experimental group, the difference in cortisol levels before and after the ACST were analyzed by using paired samples t-test which resulted in a value of p <.01. The findings showed that there were significant differences between the mean of cortisol levels before (314.83) and after (267.70) at experimental group. The mean of cortisol changes levels amounted to -47.132 with standard deviations of 85.92.

In the control group, cortisol levels before and after the AE were analyzed by using paired samples t-test and found p value = 0.461. Underlying the aforesaid result, it can be concluded that there was no difference in cortisol levels in the control group before and after AE was being given despite the change in the average levels of cortisol amounted to -14.247 with a standard deviation of 102.65.

Conversely, different test results of mean change (delta) in cortisol levels between the two groups showed that there was no significant difference in cortisol levels (Δ=-47.132 and Δ = -14.274); p <0.181) in the experimental group who received the ACST and the control group that received AE.

IgG serum Level

The findings of this study demonstrated that the experimental group who received ACST posed significant increase in mean of IgG (.654) compared with the control group who received AE. In contrast, the control group experienced a mean decrease in IgG levels (-1.563).

IgG serum levels were measured by ELISA with a normal level of 4.4 - 21.3 mg / ml. The changes of IgG serum levels before and after treatment are shown in Table 2 and Figure 2 below.

In the experimental group, differences in levels of IgG serum before and after the intervention of ACST are tested using a paired sample t-test and p value = 0.234. By looking at the study result, it is immediately clear that there was no difference in the levels of IgG before and after the intervention of ACST. Evidence to this is the fact that the mean levels of IgG before (9.33) and after

intervention of ACST (9.98) was changed at 0.654 with standard deviations of 3.054.

In the control group, the level of IgG before and after being given AE was tested by using paired sample t-test. The results showed that the value of p <.01 indicates that there was significant difference between the mean IgG levels before and after being given AE. The mean levels of IgG before (9.94) and after (8.38) being given AE was changed at -1.563 to 2.193 of standard deviations.

Furthermore, differences between the mean changes in the two groups were tested by using independent sample t-test. Different test results showed differences in the mean change in IgG levels significantly (Δ=.654 and Δ = -1.563); p <0.01) in the experimental group and the control group.

The mean change in IgG in the experimental group who received ACST amounted to 0.654 with standard deviations of 3.054. The experimental group had experienced an increase of IgG levels. In contrary, the mean changes in levels of IgG in the control group that received AE was -1.563 and the standard deviation of 2.193. Considering the aforesaid facts, it can be stated that the control group experienced decreasing levels of IgG.

Table 1. Cortisol serum level in pre and post intervention within and between group

Cortisol	Group		
	ACST (n=31) mean±SD	AE (n=29) mean±SD	*p*§
Pre	314.83 ± 126.87	290.47 ± 84.91	.389
Post	267.70 ± 96.37	276.21 ± 84.91	.761
p¥	.005	.461	
Δ Cortisol	-47.132± 85.92	-14.247± 102.65	.183

¥ pre vs post: *paired t-test*

§ *Independent t-test*

Tabel 2 IgG serum Level at pre and post intervention between group

IgG serum Level	Group		
	ACST (n=31) mean±SD	AE (n=29) mean±SD	p
Pre	9.33 ± 2.64	9.94 ± 2.95	.355
Post	9.98 ± 3.62	8.38 ± 2.55	.051
p¥	.243	.001	
Δ IgG	.654± 3.054	-1.563± 2.193	.002§

¥ pre vs post; *paired t-test*

§ *Independent t-test*

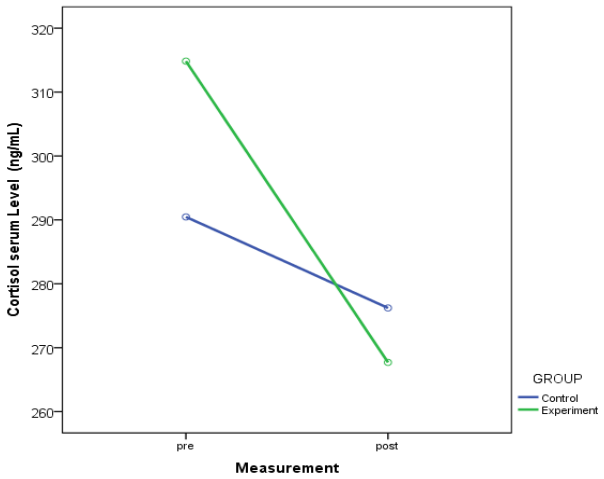

Figure 1. Cortisol serum level pre and post intervention between groups

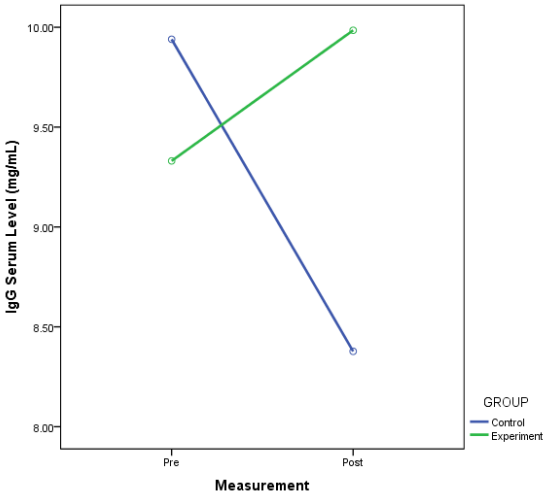

Figure 2. Pre and Post IgG serum Levels Between groups

DISCUSSION

The study that was aimed to examine whether ACTS is more influential to decrease cortisol levels compared to AE is clearly proven as the study result showed the differences in changes significantly in cortisol levels before and after being given the ACST than the pregnant women who was given AE only. Both groups equally encountered the decreased cortisol levels. However, the decreased cortisol levels in the experimental group who were given intervention ACST is greater than the control group who were given AE.

In pregnancy there is an increase of cortisol 2- 3 times of normal adult levels of cortisol that is 50-230 ng / ml. Cortisol levels in pregnant women are influenced by the role of the HPA axis in response to stress and corticotrophin releasing hormone (CRH) which regulate the dispensing of adrenocorticotropic releasing hormone (ACTH) from the pituitary. In addition, is the Cortisol levels also influenced by the feedback from synthesis and expenditure of cortisol. The role of the estrogen and progesterone hormones, especially in pregnancy, also poses significant contribution in increasing cortisol level during pregnancy. Estrogen contributes to decrease cortisol excretion in urine and decrease cortisol ties with transcortin. Progesterone also contributes to decrease cortisol bound by transcortin through the

mechanism of bonding with transcortin competition where transcortin has a higher affinity for binding to progesterone compared to Cortisol, so that the amount of free cortisol will increase. Another factor affecting the level of Cortisol is the adrenal glands of the fetus, of which 0.5% fetal body weight at 20 weeks increases up to 20 times of the weight of adrenal adults and the adrenal glands will return to normal after the birth.[13,14]

On the other hand, Cortisol plays a major role in helping lung maturity of the infants during pregnancy. The cortisol level will rise in gestational age of 34-36 weeks. However, high levels of cortisol show important predictor of preterm pregnancy or preterm pregnancies. One of the reasons for the increased levels of cortisol is a perceived stress which then triggers the beginning of delivery process.[15,16]

It can be stated that intervention of ACST is proven to reduce stress levels for the pregnant mothers who then leads to a significant contribution to decrease cortisol levels in pregnant women. The decreased stress levels will lead to activate the HPA axis response and neuroendocrine system which are affecting cortisol production. Intervention of ACST presented materials which contain knowledge about pregnancy, childbirth, postpartum and newborn care, as well as other relevant materials deal with coping skills to stress during pregnancy. Pregnant mother who was given those materials may become a provision for the mothers to find out more about the development of labor until the time of parturition and figure out how to cope with stress during pregnancy. Maternal knowledge which was acquired through ACST supplied mothers to learn more about the condition of pregnancy and what needs to be prepared at the time of pregnancy and afterward. The readiness of mother after finding out about the condition of pregnancy, childbirth and postpartum and newborn care affect the psychological condition; reduce stress, and impact on the levels of cortisol.

The decreased cortisol in the experimental group who received ACST was greater than the control group who was only given AE course. This fact proves that the material which was presented and drilled through the ACST has significant impact for the mother to lower the stress levels. This fact is acceptable because the ACST material trains the mother to possess an ability or skill of interventions coping. The mastery of the types of coping are trained to the pregnant women in the experimental group which was aimed to make the mother to be able to change the source of stress and to challenge and restore homeostasis in pregnant woman's body. In such circumstances the mother is able to change the state of stress into eustress that actually benefits mothers to achieve satisfaction and well-being of pregnant women.[17]

The findings of the study were in line with the research over the interventions performed on pregnant women through psychoeducation intervention and cognitive-behavioral intervention in which the interventions were given 8 times with 90 minutes for the length of time for each meeting. The study showed that pregnant women who received the intervention encountered a decrease in cortisol levels after the intervention but there was no difference in a change of cortisol after 3 months postpartum.[18] Increased levels of cortisol-induced stress in pregnancy affects the risk of premature birth, and BBLR.[19,20] An increased cortisol during pregnancy also poses as a strong predictor of the outcomes in infants which discloses that the babies born will have a low Apgar score values, and affect the development of children.[21,22]

The study aimed to examine whether ACST is more beneficial to increase in IgG serum levels compared to AE was clearly proven. The results showed that an increase in IgG serum levels were greater in the experimental group which received the intervention ACST compared with a control group who was given AE. The experimental group experienced an increase in IgG serum levels, in contrast to the control group which experienced a decline in the average levels of IgG serum. The decreased IgG serum levels

in pregnancy are common, not only IgG, but also other types of immunoglobulin like IgA and IgM serum level. During pregnancy, the mother experiences a suppression of the immune system in order to receive and retain the conceptus for avoiding rejection from the body. The decreased level of immunology in the body is maintained until the age of pregnancy. In such circumstances, pregnant women are more susceptible to massive infection exposure.[23] The factors that influence IgG serum levels in the body beside of pregnancy is nutrition and the presence of massive infection.[3]

The other condition that affects the levels of immunoglobulin or immune system is the psychological condition. One of the most paramount psychological conditions which affect the levels of immunoglobulin or immune system is maternal stress. Maternal stress will stimulate the increase of cortisol levels through the mechanism of the HPA axis. Increased levels of cortisol are resulted from stress condition of the pregnant women which then affects the immune suppression that is immunoglobulin G (IgG). The IgG is an immunoglobulin produced by plasma cells derived from mature B lymphocyte proliferation. Cortisol in the blood stream will suppress the immune system by suppressing the lymphocyte B cells that do not produce immunoglobulin.[3] IgG antibodies pose a predominant role as the bastion of a mother in combatting either viral infection or bacterial infection. The mothers who have lower levels of IgG are then more vulnerable to viral and bacterial infections. It also affects the health of the mother and baby during pregnancy because the IgG antibody is the only antibody that can go across the placenta in pregnant women and protect the fetus from an excessive infection exposure.[24]

The ACST intervention through emotion focused coping (EFC) and problem focused coping (PFC) trainings in pregnant women allow the mothers to change the stress into eustress. Those trainings also allow mothers to master the ability to suppress the symptoms of stress. Furthermore, the trainings give major impacts to maximize the immune function through the mechanism of cytokines circulation and suppression of the inflammation response.[25] It is advantageous for mothers to improve one immune system especially IgG which has significant role in protecting the mother and infant from the exposure of viral and bacterial infections. This is consistent with the findings that pregnant women who received ACST have elevated levels of serum IgG, while pregnant women who did not receive ACST pose decreased IgG, although they possess maternal serum IgG levels within normal limits ranging from 4.4 -21.3 mg / dl.

CONCLUSION

Compared to AE, ACST is more beneficial and influential to decrease Cortisol levels. The decrease of the cortisol levels in the experimental group after the intervention of ACST is sharper than the decrease of the Cortisol levels in the control group after being given AE.

Compared to AE, ACST is more influential to increase IgG levels. The experimental group experienced an increased IgG levels after the administration of ACST while the control group encountered decreased levels of IgG after being given AE.

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The Dose Dependence Analysis of the Water Fraction of *merremia mammosa (lour.)* Extract on Diabetic wound Healing Enhancement

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ABSTRACT

Introduction: Diabetic wounds or ulcers happened in Indonesia's hospitalized diabetes patients range from 17.3 to 32.9%. The high cost of treatment, the high risk of amputation and the difficulty of handling diabetic wounds, make it necessary to look for alternative medicine derived from plants e.g. *Merremia mammosa (Mm)*. This study aimed to analyze the potential dose of the water fraction of *Mm (Lour.)* extract on diabetic wound healing enhancement. **Method:** This study used fifty-seven male wistar rats that were made diabetic by intraperitoneal injection of 40 mg/kg body weight streptozotocin. Rats divided into six groups equally, which consist of positive control (gentamicin 0.1%), negative control (aquadest) and water fraction of *Mm (Lour.)* extract dose 12.5 mg, 25 mg, 50 mg and 100 mg. Wound was made by Morton method and treatment applied on the wound every other day for 21 days. Wound healing process were observed by percent wound healing and histopathological changings on day 0, 3, 10 and 25, representing each healing phase. **Results:** The percentage of reduction in wound size comparison at day 10 showed no significant different when compared with positive control started from dose 50 mg. This result is consistent with the histopathological changings parameter (angiogenesis, macrophage, fibroblast and collagen density). **Conclusion:** Water fraction of *Mm (Lour.)* extract was dose-dependently enhanced the process of wound healing in diabetic rat model and the most effective dose was 100 mg, which looks similar with positive control. Therefore, it is potential to be developed further as a topical drug.

Keywords: *Merremia mammosa (Lour), wound healing, diabetic ulcers*

The process of wound healing in Diabetes Mellitus (DM) patients are longer than normal injuries due to disruption of all processes of wound healing [(1)]. Continuous hyperglycaemic conditions, pro-inflammatory environments, peripheral arterial disease, and peripheral neuropathy simultaneously cause impaired immune function, ineffective inflammatory responses, endothelial cell dysfunction, and neovascularization disorders [(2).] Increased sugar levels in collagen synthesis, worsening epithelization, decreased angiogenesis in the proliferation phase and fibroblasts caused extracellular matrix was not formed maximally because the circulatory and oxygen distribution to the region were disrupted [(3)]. Diabetic injuries that do not treat well will rapidly expand to bacterial infections and in further circumstances will cause diabetic gangrene. Diabetic gangrene is a form of tissue in patients with DM due to reduced or cessation of blood flow to the tissue.

The management of diabetic injuries is complex because it requires comprehensive and multidisciplinary handling. Wound care in patients with DM is done with the aim to prevent the occurrence of infection, accelerate wound healing and reduce the risk of amputation. The effectiveness of wound care can be seen through changes in wound area, repair of wound severity, wound healing time and complete wound healing [(4).] Management of diabetic injuries include overcoming comorbid diseases (hypertension and atherosclerosis), reducing the burden (offloading), keeping the wound always moist (moist), handling infections and debridement. Skin moisture is needed to accelerate the process of tissue re-epithelization through stimulation of

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proliferation and epithelial cell migration and growth factor growth [5].

Mm (Lour.) is one of medicinal plants from Indonesia that can be found in Meru Betiri National Park. Derived from Convolvuraceae family, it can be used as an anti-inflammatory, analgesic, wound healer, treating snake bites, cancer, leprosy, syphilis, typhoid, diphtheria, inflammation and diabetes [6, 7]. Upper luminous tubs contain polysaccharides, flavonoids and glycoside resin compounds such as meremocides A, E, J, mammosa that have activity as antibacterial by hollowing the cell membrane [8]. Agil et al, 2011 and Sugijanto et al, 2012 reported research on extract of the *Mm* (Lour.) showed an ability to inhibit the growth of pathogenic bacteria of Microbacterium tuberculosis, Salmonella typhi, Staphylococcus aureus (research report). It is also capable on lowering blood sugar levels in diabetic rats with impaired glucose tolerance (Tilaqza, 2009, unpublished script).

The same genus with *Mm* namely *Merremia tridenta* has been shown to have activity as wound healer, [9] anti-diabetic, anti-hyperlipidemic, antioxidant and anti-inflammatory [10, 11]. Our preliminary study with the *Mm* (Lour.) extract has shown that healing process significantly enhance at day 7 of the treatment and water fraction was the most effective fraction of *Mm* (Lour.) extract among the ethyl acetat, n hexane and water fraction on diabetic wound healing enhancement (unpublished data). Using chemotaxonomic approach with the plant in the same family or genus of similar compounds and the possibility of having almost the same efficacy and empirical usage of *Mm* (Lour.), this research aims to analyze the potential dose of the water fraction of *Mm* (Lour.) extract on diabetic wound healing enhancement.

MATERIALS AND METHODS

Experimental animals

Albino Wistar rats of male sex in early adulthood weighing between 150-200 g were used in the present study. Rats kept in an individual cage with a standard feed of ad libitum food and water. Mice adapted to the condition for 1 week. The mice were induced using Streptozotocin (STZ) dissolved in 0.05 mol / L buffer citrate (pH 4.5) with a single dose of 40 mg / kg body weight. The mice had diabetes when blood glucose levels exceeded 300mg/dL on the fifth day after STZ injection [12]. Blood glucose levels were measured using Glucose meter (Easytouch, Taiwan) once a week. The experimental protocol was approved by Institutional Animal Ethics Committee and animals were maintained under standard conditions in an animal house approved by Ethic Committee.

Formulation of water fraction of Mm (Lour.) extract

The viscous ethanol extract of *Mm (Lour.)* was fractionated by partition using 3 different solvents of polarity i.e. n hexane, ethyl acetate and water. 50 g of condensed extract added with 100 mL of water and stirred until homogeneous. This water fraction is subsequently in a successive partition using n hexane and ethyl acetate with a ratio of 2: 3. The fractionation is performed by 3 repetitions. The water fraction is then concentrated by a freeze dryer.

Effect on excision wound

Animals were anesthetized using ketamin (50 mg/kg) and xylazine (10 mg/kg) injected intramuscular. An impression was made on the dorsal thoracic region 1 cm away from vertebral column on the anaesthetized rat. Particular skin area was shaved and the skin of impressed area was excised to the full thickness to obtain a rectangle wound area of about 25x25 mm.[13] Animals were then grouped (n = 9 per group) and treated topically as follows: Group C(+): Control positive with gentamycin 0.1%, Group C(-): Control negative with aquadest, and the rest are with water fraction of *Mm (Lour)* extract dose 12.5 mg (T1), 25 mg (T2), 50 mg (T3) and 100 mg (T4) every other day for 21 days. The wound was left undressed to the open environment. Wound area was measured by tracing the wound on a millimeter scale graph paper. Percentage of wound healing was calculated as original wound size as for each animal of the group on predetermined days i.e, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 days post-wounding.

Histopathology

Histopathological parameter observed on days 0, 3, 10 and 25 (n=3 per group per day) representing the wound healing phase (inflammation, proliferation and remodeling). The mice were sacrificed on those days with cervical dislocation and excision was performed to obtain tissue for histopathologic examination with hematoxylin-eosin stain. The tissue was

observed under a light microscope (Olympus trinocular type 31X) at 5 large fields (400X) and photographed to assess angiogenesis, macrophage, fibroblast density and collagen density degree and expressed in the scoring system. The scoring of the parameters mentioned above was determined as absent (0) when it was between 0-10%; mild (1) when it was between 10-40 %; moderate (2) when it was between 40-70% and severe (3) when it was between 70-100%.[5] In addition, extracellular matrix (ECM) represent collagen fiber density was also being assessed using ImageJ software, described as percentage [13].

Statistical analysis

The data obtained were analyzed using unpaired Student's t-test to identify significant differences between group using Excel software from Microsoft Office Profesional Plus 2016. Values are shown as the mean ± standard error (SE).

RESULTS

Body weight and non-fasting blood glucose

The data obtained showed that each group has similar body weight changing pattern. As shown in Figure 1A, after induction the rat body weight increase for a week, slightly decrease on day 3 of the excision and return to gain weight until the day of sacrificed. As shown in Figure 1B, the non-fasting blood glucose increased to more than 300 mg/dl five days after injection of STZ and maintain above 300 mg/dl until the day of sacrificed.

Wound healing

The average measurement of percent wound healing from day to day on a macroscopic basis among the wound given gentamycin 0.1%, aquadest and the water fraction of *Mm (Lour)* extract several doses can be seen in the table 1 where the percent wound healing on day 10 showed no significant different with positive control starting from dose 50 mg. For a clearer result, Figure 1C showed the percent wound healing measured every two days of the rats kept until day 25 (n=3).

Histopathology

Histopathological parameter in all experimental group during the experimental period summarizes in table 2. It showed that on day 10 and 25 the collagen fiber have significant different in all group except T1 on day 10 when comparing with C(-) and no significant different when comparing with C(+). Description of histopathological parameter by photomicrograph in each healing phase can be seen in figure 2 which there were no significant different yet on day 0 and 3 of wound excision.

DISCUSSIONS

Wound healing is a complex process that involves interactions between cells, ECM, growth factor, and cytokines. Wound healing process consists of three stages i.e. inflammation, angiogenesis (proliferation) and remodeling [14]. Diabetic wound possibly developing in difficult-to-heal wounds (wounds still proceeding through the wound healing stages but at an exceedingly slow rate), although such wounds have not been well studied [11, 15]. This study demonstrated that water fraction of *Mm (Lour)* extract enhances excisional diabetic wound healing, as defined by a more rapid of healing phase showed by faster percent wound healing and faster formation of ECM that mostly consist of collagen. In particular, the assessment of histopathological parameter revealed an advantage for the groups primed with water fraction of *Mm (Lour)* extract in comparison to the control animals. The time needed to achieve 50% closure of the wound decreased from 7.7 ± 1.0 days in control negative to 5.6 ± 0.6 in water fraction of *Mm (Lour)* extract dose 100 mg (data not shown). This is consistent with our previous study on *Mm (Lour)* extract.[8] Therefore, the findings in this study allow the presumption that local *Mm (Lour)* treatment may be a novel and clinically applicable treatment for enhancing wound healing in diabetic wounds.

A number of studies have reviewed the use of natural plants as local treatment for wound healing but to our knowledge this is the first to be done with water fraction of *Mm (Lour)* extract [8, 16, 17]. The ethanol extract used in the previous study was still contained all the components of *Mm (Lour)* which gave effect as well as those not on wound healing. The amount of extract required is still large (200 mg) because the active components are still mixed with other components that are not active. Fractionation or stratified purification of uptake extract performed to increase the yield of the active component and decrease the amount of non-active material contaminant in the extract. Non-active material removal is also expected to facilitate in the formulation phase to improve

the acceptability of the preparation. It can also be used to minimize unwanted effects from impurities. In an immunomodulatory preparation contained active *meniran* extract, fractionation is proven to be able to eliminate the unwanted effects of diuretics.

Wound healing percentage showed that the effective dose started from 50 mg (no significant different with the positive control at day 10, n=3) and the effect increased by increasing the dose into 100 mg. But when we examine histopathology parameter, it is described that dose 25 mg already showed wound healing enhancement by evaluating the ECM enhancement on day 10. There is no significant different can be seen in other histopathology parameters probably due to the limited number of samples and the data were not fully quantitative. While the other groups showed low density of all examined histopathology parameters, negative control group showed a still high density of ECM, at day 25, that significantly different with all other group. This is because the negative control has a prolonged phase of healing, relevant with the result of Ackermann et al study on diabetic wound.[1] However, although showed trend of delayed healing process at day 10, negative control wound healing percentage was not reached any significant different (n=3) when comparing with all other group, including the positive control (p=0.08), probably due to high variation in control negative data that shown by higher standard error. This variation may be the result of individual non-treatment inflammatory response differences that related to various factors, i.e. epidermal barrier function, growth factor production, etc [(1)].

Some of the content of *Mm (Lour)* are polyphenols such as flavonoids. Flavonoids have antioxidant effects that accelerate the inflammatory phase by capturing free radicals and prevent oxidation reactions by increasing the activity of the enzyme Superoxide dismutase (SOD) and glutathione transferase (Subandi, et al., research report). In addition, flavonoids have anti-inflammatory activity that inhibits the important phase in biosynthesis that is on the path of cyclooxygenase and an antibacterial activity by inhibited bacterial DNA gyrase function so that the replication and translation of bacteria is inhibited (Gunawan, 2009, unpublished script). Flavonoids with their anti-inflammatory activity can stimulate cells such as macrophages to produce growth factors and cytokines such as EGF, TGF-ß, IL-1, IL-4, IL-8 to accelerate the proliferation and other wound healing phase. The content of flavonoids in *Mm (Lour)* can also stimulate cellular immunity by proliferating lymphocytes and production of reactive oxygen intermediate macrophages (Farizal, 2012, unpublished script).

Diabetic rats model in this study were maintained to have severe diabetes because the STZ dose administered at 40 mg/kg body weight and the non-fasting blood glucose higher than 300 mg/dL through all the experiment period (Figure 1) [(12)]. Body weight in all group showed a slightly decreased right after STZ injection and then increased gradually, this is relevant with DM pathogenesis [(18, 19)].

Although the researcher has tried maximally to control every step in conducting this research, there were still few limitations such as the method in measuring wound size and grading the density of angiogenesis, macrophage, fibroblast and collagen that was done by manual measurement. The usage of ImageJ software also has a limit to only differentiate the ECM and cannot be specific in collagen structure.

CONCLUSIONS

Diabetic wound possibly developing in difficult-to-heal wounds, therefore, it need a special treatment. Based on the result of data analysis, water fraction of *Mm* (Lour.) extract was dose-dependently enhancing the process of wound healing in diabetic rat model and the most effective dose was 100 mg, which looks similar with positive control. When comparing with negative control there is no group significantly different including the positive control, probably due to high variation in control group related to severity variation of DM. According to the purpose of this research, it is suggested to develop the effective dose of *Mm* (Lour) extract water fraction as a topical drug in diabetic wound or other prolonged wound healing conditions. However, to overcome the limitation of this study, it is suggested to conduct future research with larger number of samples and a more specific parameter on wound healing phase.

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We declare that we have no conflict of interest.

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The Efficacy of Education with the WHO Dengue Algorithm on Correct Diagnosing and Triaging of Dengue-Suspected Patients; Study in Public Health Centre

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ABSTRACT

Background: Correct diagnosing and triaging dengue fever remains clinical, but is difficult because of unspecific flu-like symptoms. Best tool at the moment is the easy–to-use 2009 WHO guidelines. ***Objective*:** To investigate the efficacy of educational intervention with the (adapted and translated) algorithm from the 2009 WHO dengue guideline to healthcare providers in the Indonesian primary health care setting of Central Java. ***Methods:*** Quasi-randomized intervention study implemented in two Public Health Centres (PHCs), one being intervention and the other control. Intervention consisted of educational actions on healthcare providers with a presentation, hand-outs and posters. All patients with fever seen in policlinic or emergency department were included. Data were collected with a participatory observation using the WHO algorithm as a guidance. ***Results:*** Pre-intervention, a total of 88 patients (n=38 intervention group; n=50 in the control group), and post-intervention, a total of 231 patients (n=105 in the intervention group; n=126 in the control group) were included. Pre-intervention, correct diagnosing and triaging was not significantly different (63.2% vs 64.0% ; *p=0.935*), while post-intervention, the intervention group scored higher (75.2% vs 62.7% ; *p=0.041*). However, in both pre- and post-interventional phase, more than 50% of the cases in 19/22 domains were not investigated by the intervention group. ***Conclusion:*** Statistical analyses showed a significantly better outcome in correct diagnosis in the intervention group. However, results are considered inconclusive due to incompleteness of relevant information, which most probably leads to many false positive correct diagnoses and triaging.

Keywords: *DHF, WHO guidelines, primary care setting*

Dengue fever, is a mosquito-borne viral infection that has now spread to most tropical and subtropical regions of the world including Indonesia, and continues to increase in incidence and severity.(1) In endemic areas, diagnosis of Dengue Fever is usually made clinically and based on reported symptoms, physical examination and at times a full blood count (haematocrit, WBC and platelets). The actual WHO-guideline from 2009 has been recognized as an authoritative reference worldwide. Different studies have proven the effectiveness of the triaging-system of the guideline especially in recognizing Severe Dengue, and showed clinical and epidemiological usefulness, especially when there are no laboratory tests available.[i-3] The WHO algorithm provides a probable diagnosis of Dengue and triages patients into group A (can be sent home), group B (referred for inpatient care), or group C (referred for emergency treatment in hospital). Points for improvement suggested by most studies was re-assessment of warning signs as predictors for severe disease progression.(1-3) At the moment, there is no national Indonesian dengue guideline available in the English language. The existing guideline from the Indonesian Ministry of Health also is intended for medical doctors only(2).

Preeliminary result of an observational cross-sectional unpublished study about the diagnosis, triaging and management of Dengue Fever in the Public Health Centre (PHC) compared to the 2009 WHO dengue guidelines indicated incomplete history taking and physical examination in 63.9%

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cases. Wrongful triaging happened in 18.5% (group B/C instead of group A) and 25.0% of the cases (group A instead of group B/C) respectively. In interviews with the healthcare providers they confirmed their awareness of the WHO guidelines, however this was not objectified in the study.

Although a complex disease in its manifestations, accurate triaging and management is relatively simple, applicable and inexpensive. In primary healthcare settings where patients are first seen and evaluated, early detection and proper triaging and management are critical in determining better clinical outcomes. This not only prevents unnecessary hospital admissions but also saves lives and helps identifying outbreaks. The local authorities can benefit from having a proper insight into the prevalence, determinants and treatment of Dengue Fever, which facilitates improving health policy (e.q.fair distribution and spending of finances and materials). Aim of this study was to investigate the effectiveness of educational intervention with the (adapted and translated) algorithm from the 2009 WHO dengue guideline to healthcare providers in the primary healthcare setting (PHC).

METHODS

The design of this study was a quasi-randomized controlled trial because concealed randomization was unfortunately logistically not possible in the setting of the research. Two PHCs, were included in this study, one serving as intervention and the other as control. Both PHCs are located in small rural villages of the Jepara regency, Central Java province, Indonesia. Randomly allocation of intervention and control group was done by flipping a coin. In the intervention and control group there are four and three general practitioners respectively and several nurses. According to the WHO guideline, both PHCs have ability to diagnose and triage dengue-suspected patients adequately.

Intervention consisted of education in the form of a 45-minute presentation to all healthcare providers, based on a shortened version of the adopted and translated algorithm of the 2009 WHO guideline for dengue (leaving out the management part). During the presentation it was intensely emphasized that complete history taking and physical examination is crucial for correct diagnosing and triaging of dengue-suspected patients. The presentation included five illustrative example cases in the end that had to be solved (diagnosis and triaging into group A/B/C) by the group to test the gathered knowledge. All cases could be correctly solved by the group. After the presentation, handouts of the algorithm were distributed to all healthcare providers and A3-sized posters were hung up clearly visible in all policlinics and the emergency department.

Picture 1. *Poster of WHO guidelines put in Emergency department*

Data collection was separated into a pre-interventional and post-interventional phase. Patients were selected at their moment of presentation in the policlinic or emergency department and were informed and asked permission about anonymous use of their data with written informed consent or patient´s legal guardian if patient was underage. Healthcare providers in both PHCs were also asked permission to be observed with written informed consent.

In the pre-interventional phase all doctors from both groups were interviewed about their motivations in decision-making for dengue-suspected patients. Data retrieving was done by closely following, observing and interviewing healthcare providers while seeing patients. For laboratory interpretation, national reference values of the PHC were used. Twenty two (22) variables that resemble relevant symptoms and signs for correct diagnosing and triaging of Dengue Fever according to the WHO guideline were observed.

RESULTS

In the pre-interventional phase, 38 patients from the intervention and 50 patients from the control group were included in the study. There was no statistically significant difference in days of fever (*p=0.195*) or in the distribution of setting whether in policlinic or emergency department (*p=0.344*) between the two groups of study. In the post-intervention phase, 105 patients in the intervention group and 127 patients in the control group were included. One patient from control group was excluded because he did not return from laboratory.

Table 1. Completeness of history taking, physical examination and laboratory information *Pre-intervention*

Domain	**Intervention Group** n=38 % of cases NOT investigated	**Control Group** n=50 % of cases NOT investigated	***p-value***
History taking			
1) Anorexia and nausea	26.3	14.0	0.147
2) Rash	94.7	62.0	0.000
3) Aches and pains	84.2	56.0	0.005
4) Abdominal pain	71.1	24.0	0.000
5) Persistent vomiting	26.3	6.0	0.008
6) Mucosal bleeding	100.0	94.0	0.255
7) Respiratory distress	100.0	98.0	1.000
8) Coexisting condition	94.7	98.0	0.576
9) Social circumstance	97.4	96.0	1.000
Physical examination			
10) Temperature measurement	71.1	20.0	0.000
11) Tachycardia or bradycardia	78.9	82.0	0.719
12) Hypotension	14.3	44.4	0.025*
13) Respiratory distress	100.0	100.0	n/a
14) Abdominal tenderness	84.2	28.0	0.000
15) Liver enlargement	100.0	78.0	0.002*
16) Clinical fluid accumulation	76.3	20.0	0.000*
17) Mucosal bleeding	97.4	70.0	0.001*
18) Lethargy/restlessness	100.0	98.0	1.000
19) Tourniquet test	100.0	100.0	n/a
20) Petechiae or Rash	92.1	56.0	0.000*
21) CR>2s or cold and sweaty extremities	100.0	98.0	1.000
22) Laboratory information[0]	61.1	20.8	0.000*

Pre-intervention

Out of the 22 domains that were analysed for evaluation of completeness of history taking, physical examination and laboratory examination, in the intervention group only the performance in one single domain was significantly better compared to the control group (investigation of hypotension). There was no statistically significant difference in performance between the groups in 10/22 domains, and in the remaining 11/22 domains the control group scored significantly higher, 4 were in the section of history taking, 6 in physical examination, and 1 in laboratory testing. More than 50% of the cases in the intervention group, 19/22 domains were not investigated, while in the control group this was 14/22 domains (Table 1)

Post-intervention

Table 2 showed the comparison between pre and post intervention in the intervention group. The performance of this group after intervention was better in 9/22 domains, but significantly better in only one single domain (physical examination of mucosal bleeding). Whereas, in 3/22 domains, their performance was significantly better in the pre-interventional phase (investigation of temperature measurement, tachy- or bradycardia, and laboratory testing), and in the remaining 18/22 domains showed no statistically significant difference. In this post-interventional phase, also the intervention group did not investigate in more than 50% of the cases in 19/22 domains.

Table 2. Completeness of history taking, physical examination and laboratory information in Intervention group, Pre- and Post-intervention

Domain	Intervention Group Pre n_2=38 % of cases NOT investigated	Intervention Group Post n_4=105 % of cases NOT investigated	*p-value*
History taking			
1) Anorexia and nausea	*26.3*	*38.1*	*0.192*
2) RASH	94.7	97.1	0.609
3) ACHES AND PAINS	84.2	67.6	0.051
4) ABDOMINAL PAIN	71.1	58.1	0.159
5) Persistent vomiting	*26.3*	*36.2*	*0.269*
6) Mucosal bleeding	100.0	100.0	n/a
7) Respiratory distress	100.0	100.0	n/a
8) Coexisting condition	*94.7*	*99.0*	*0.172*
9) Social circumstance	*97.4*	*100.0*	*0.266*
Physical examination			
10) Temperature measurement	*71.1*	*91.4*	*0.002**
11) Tachycardia or bradycardia	*78.9*	*92.4*	*0.035**
12) HYPOTENSION$^+$	14.3	12.7	1.000
13) RESPIRATORY DISTRESS	100.0	97.1	0.565
14) ABDOMINAL TENDERNESS	84.2	79.0	0.492
15) Liver enlargement	100.0	100.0	n/a
16) CLINICAL FLUID ACCUMULATION	76.3	59.0	0.058
17) MUCOSAL BLEEDING	97.4	66.7	0.000*
18) Lethargy/restlessness	100.0	100.0	n/a
19) TOURNIQUET TEST	100.0	97.1	0.565
20) Petechiae or Rash	*92.1*	*94.3*	*0.700*
21) CR>2s or cold and sweaty extremities	100.0	100.0	n/a
22) Laboratory information	*61.1*	*77.9*	*0.049**

Upper Letter: better performance after intervention

Italic : lower performance after intervention

Table 3 showed there was no any statistically significant difference in correct diagnosing/ triaging between intervention and control group in the pre-intervention phase or between pre- and post-intervention phase within the intervention group. However, there was a significant better performance in correct diagnosing and triaging of the intervention group compared to the control group in the post-intervention phase (p=0.041). When this analysis was further stratified by type of healthcare provider (doctor and nurse) between the groups, we calculated a significant better performance only by the nurses of the intervention group compared to the nurses of the control group.

Table 3. Correct diagnosing and triaging in Intervention and Control group Pre & Post interventional

		Intervention Group	**Control Group**	***p-value***
Pre	% of cases correctly diagnosed and triaged	63.2 (n= 38)	64.0 n= 50	0.935
Post	% of cases correctly diagnosed / triaged (total)	75.2	62.7	0.041*
	by doctors	69.8	70.0	0.986
	by nurses	81.0	62.2	0.033*
	p-value	0.215	0.445	
		(n=105)	(n=126)	

Interviewed about motivation of dengue diagnosis and triaging

All doctors from both groups were interviewed about their motivations in decision-making for dengue-suspected patients in the pre-interventional phase. The result of this interviews was concordant between all doctors: patients are first assessed for flu-like symptoms, being fever, weakness, headache, and myalgia or arthralgia. If these symptoms persist already more than three days at time of consultation, patients are referred for laboratory analysis. In the other cases they are sent home for eventual re-evaluation if there is no improvement of symptoms within one day.

Only major indicator for referral to inpatient care is thrombocytopenia. Awareness of relevant clinical signs for diagnosis, warning signs or other admission criteria suggested by the WHO guideline could not be confirmed.

DISCUSSION

The result of this study showed no difference in correct diagnosing between the groups pre-interventional. However in the post-interventional phase, in the intervention group scores of correct diagnosing and triaging were statistically significant better. Comparison by sub-stratification of doctors and nurses between the groups post-interventional revealed significant better performance of doctors in both groups, particularly in domains of physical examination, while the nurses of the intervention group scored significantly better than the nurses in the control group. Thus, the result of this study indicated that the nurses´ better performance in the intervention group compared to the control, which probably made the total better performance in correct diagnosing and triaging.

Overall performance on completeness of relevant information was very poor in both groups, pre- as well as post-interventional with a major part of the relevant domains not investigated in more than 50% of the cases. Therefore, this study can not proved that completeness of relevant informations have correlation with correct diagnosing and triaging, which is most likely attributed to the generally poor performance on completeness of relevant signs and symptoms from history taking, physical examination

and laboratory information. It could also be a source of false positive correct diagnoses and triaging. This is also why the results from evaluation of correct diagnosing and triaging should be regarded inconclusive and should be interpreted with suspicion.

Before intervention, there was no knowledge about the WHO guideline, and thrombocyte count is the main indicator for diagnosis and triaging of Dengue-suspected patients. Based on interview, all doctors indicated that there was no awareness of relevant clinical signs for diagnosis, warning signs or other admission criteria suggested by the WHO guideline. The results of this research revealed huge deficits in relevant history taking and physical examination in both groups, quantitatively as well as qualitatively. This will have negative effects on clinical practice and will lead to wrong diagnosing and thus wrong management of Dengue Fever. To prevent this, very basic history taking and physical examination needs to be improved. Different studies have proven the effectiveness of the triaging- and management-system of the guideline especially in recognizing Severe Dengue, and showed clinical and epidemiological usefulness[1-3], especially when there are no laboratory test available. The failing of this intervention to have a positive effect on measured outcomes can be caused by the limited time of education (only 45 minutes powerpoint presentation), which can be considered as not enough to make sure the understandable of the audiens. Besides, this intervention did not followed by test of knowledge about 22 domain before started with post-interventional data collection.

There are also several factors in this study that might lead to over- or underestimation of certain outcomes. Factors that could lead to overestimation in performances of the healthcare providers in general are the Hawthorne effect, and the dubious manner of execution of certain physical examination techniques, which were not reliable but were often considered as investigated (blood pressure measurement, investigation of rash/ petechiae, investigation of clinical fluid accumulation). Furthermore, on the survey when signs or symptoms were filled in as "investigated", it is not possible to differentiate between if patients reported the symptoms themselves or if the healthcare provider asked actively. When only very few signs and symptoms were investigated by a healthcare provider, this could quickly lead to a false positive labelling in the analyses. The latter two could thus also lead to an overestimation in performance of healthcare providers. Moreover, factors that might underestimate the performance of healthcare providers are the slightly alternated use of thrombocytopenia and increased haematocrit as warning signs, which is gives possibly more diagnosed patients and more patients triaged into group B.

Considering the fact that the previous study (unpublished) as well as this study concluded that basic relevant history taking and physical examination are very deficient for Dengue diagnosis in this setting, further research should primarily focus on investigating and improving that point before concentrating on other clinical outcomes, like correct diagnosing and triaging or management. A longer timeframe for data collection is recommended as this not only gives more samples cohort but also can be used to measure the consistency of healthcare providers behaviour.

In conclusion, although statistical analyses showed a significantly better outcome in correct diagnosis and triaging in the intervention group, the results of this study are considered inconclusive due to the incompleteness of several relevant informations, which most probably leads to many false positive correct diagnoses and triaging.

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The Effect of Green Tea Epigallocatechin-3-Gallate on Spatial Memory Function, Malondialdehyde and TNF-α Level in D-Galactose-Induced BALB/C Mice

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ABSTRACT

Introduction : Neuroinflammatory process and oxidative stress play an important role in the mechanism of brain aging and neurodegenerative diseases such as Alzheimer. Epigallocatechin-3-gallate (EGCG) have antioxidant, anti-inflammatory, and neuroprotective effects. This study aims to prove the effect of green tea EGCG on spatial memory function, malondialdehyde (MDA), and TNF-α level in Balb/c mice induced by D-galactose. **Method :** An experimental study using "post test only control group design". The samples were 18 male Balb/c mices, aged 6-8 weeks, divided into 3 groups. Negative control group (N-C) was induced by subcutaneous injection of D-galactose (150 mg/kg) once daily for 6 weeks. EGCG-2 and EGCG-6 were induced by D-galactose and orally administered by 2 and 6 mg/kg EGCG once daily for 5 weeks. The indicator of examination were spatial memory function using morris water maze, MDA and TNF-α level using Elisa. One-way Anova, Kruskal-Wallis, and Pearson were used for statistical analysis. **Results :** Means of % escape latency time and path length in the EGCG-2 {42,02(SD=5,9);43,47(SD=5,97)%} and EGCG-6 {40,45(SD=6,5); 41,78(SD=6,77)%} were significantly higher than N-C {28,68(SD=9,15), p=0,013; 32,98(SD=7,75)%,p=0,04}. MDA level in the EGCG-2 {587,79(SD=76,04)ng/ml} was significantly smaller than N-C {722,64(SD=134,78)ng/ml,p=0,037}. TNF-α level in all groups was not different (p=0,786). There was a significant and strong correlation between MDA level and spatial memory function (r=-0.551; p=0,018). **Conclusion** : EGCG may improve spatial memory function and oxidative stress in mice induced dementia, but it may not improve the status of neuroinflammation.

Keywords: *Green tea epigallocatechin-3-gallate, spatial memory function, MDA, TNF-a, D- galactose*

Dementia has become a serious problem in the global health. The 2015 World Report Alzheimer's estimates that 46.8 million people worldwide are living with dementia in 2015, with 9.9 million new cases each year (one new case every 3 seconds). This number is estimated will increase to 74.7 million cases by 2030 and 131.5 million by 2050. This estimation comes from a population-based study that examines the prevalence of dementia in different regions of the world[1,28,33,34]. According to The 2016 World Health Report, dementia contributed 11.2% causing disability cases in subjects aged over 60 years, greater than stroke (9.5%), musculoskeletal disorders (8.9%), cardiovascular disease (5%), and all types of cancer (2.4%)[33,34].

Alzheimer's disease (AD) is the main cause of dementia (50-75%) in the elderly[27]. AD is a progressive and irreversible neurodegenerative disease, characterized by decreased of cognitive and memory function, and degeneration of cholinergic neurons[3,12,15]. Several studies have shown that oxidative stress and inflammatory process play an important role in the pathogenesis of brain aging and neurodegenerative diseases such as Alzheimer[10,14,23,24,30].

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Oxidative stress is a condition characterized by an imbalance between pro-oxidant molecules and the antioxidant system[2]. MDA is formed by the degradation of free radicals OH^- from unsaturated fatty acids, converted to highly reactive free radicals[2,14]. In this study, MDA is examined because it is the final product of lipid peroxidation process that can represent oxidative stress processes in the central nervous system.

Spatial memory is one of the important indicators for assessing neurocognitive function. Hippocampus is an important part of the brain in mediating spatial and contextual memory functions. Morris water maze (MWM) is a standardized examination used to assess the hippocampal-dependent memory in experimental animal. The MWM plays an important role in the validation of rodent models for neurocognitive disorders such as AD[8,9,31].

D-galactose are known to be widely used in animal model for brain aging and neurodegenerative diseases. D-galactose is known to cause aging-related changes including the spatial memory impairment and destruction of nerve cells. D-galactose causes cellular metabolic damage by decreasing the activity of Na^+,K^+,ATPase enzymes and increasing oxidative stress through increased lipid peroxidation and decreased antioxidant enzyme activity[24].

Several studies have shown that epigallocatechin-3-gallate (EGCG) is a major polyphenol in green tea that has antioxidant, anti-inflammatory, anticancer, and neuroprotective effects[20-24]. In a cross-sectional study in Japan that examined the association between green tea consumption and cognitive function, it was mentioned that the consumption of green tea 2 cups or more per day (100 ml/ cups) was associated with a decreased prevalence of cognitive impairment[19]. However, in other study showed that the administration of EGCG with a dose of 50 mg/kgBW was not able to improve spatial memory function or repair brain nerve cells damage in repeated ischemic induced Balb/c mice[29]. The exact effect of EGCG on cognitive remains unclear. This study attempts to prove the effect of EGCG green tea on spatial memory function, oxidative stress, and neuroinflammatory status in D-galactose induced Balb/c mice. The use of multilevel doses is intended to obtain the most effective dose that can provide optimal results.

MATERIALS AND METHODS

Experimental animals and study design

This research was an experimental study with randomized, post test only control group design. The samples were 18 Balb/c males mices, aged 6-8 weeks obtained from the Integrated Research and Testing Laboratory, Gajah Mada University, Jogjakarta, Indonesia.

The sample was divided into 3 groups by simple randomization. N-C group was induced by subcutaneous injection of D-galactose (150 mg/kg) once daily for 6 weeks. EGCG-2 and EGCG-6 were induced by D-galactose and orally administered by 2 and 6 mg/kgBW EGCG once daily for 5 weeks. The mean of mice body weight before treatment in N-C group was 29,60 (SD = 0,99) gram, EGCG-2 group was 27,87 (SD = 1,21) gram, and EGCG-6 group was 28.52 (SD = 0.75) gram, thus qualify the normality assumption (*Shapiro Wilk* $p > 0.05$) and homogenity (*Lavene's* test $p = 0.412$).

Reagents and chemicals

The pure epigallocatechin-3-gallate (EGCG) green tea compound was obtained from the Sigma-Aldrich with E-4143 catalog code, as well as the D-galactose reagent obtained from the Sigma-Aldrich with G-0750 catalog code.

Morris water maze test

At the end of the 6th week of treatment and induction, spatial memory function was examined with morris water maze (MWM). The MWM is a circular pool filled with water (100 cm in diameter and 50 cm in height). Mices were trained to use extra-maze visual cues to locate an escape platform hidden just below the surface of the opaque water. In this procedure, the test was done through 3 phases: (1) Learning/ acquisition phase: a training process for mices to form their spatial memory. Mice were trained to find a hidden platform. The platform was placed in ± 1 cm below the surface of the water, in a predetermined quadrant. This phase was done for 4 days in a row with 4 times per day. Mice tested their ability to escape, characterized by the success of mice to find a hidden platform. The path length and escape latencies time to find the terminal platform for all mice in 6 days were measured and calculated; (2) Retention phase (probe trial): a test phase of spatial memory function. This

phase is carried out at least 24 hours after the last exercise in the acquisition phase, and made just 1 time. Platform removed, and the mice were given 60 seconds to locate the previous platform. The parameter measurement was % time and % distance in target quadrant; (3) Visual testing: to assess the sensorimotor and visual functions of the animals. Trial was done 3 times in 1 day. Mice were given 60 seconds per trial to find a visible platform. The path length and escape latencies were recorded[8,9,31].

Termination and preparation of sample

After MWM examination, mice subsequently terminated by cervical dislocation techniques. The hippocampal dissection procedure was based on the protocol of previous studies by Beaudoin, et al, 2012[6].

The mice hippocampal tissues were weighed and then homogenized together with phosphate buffered saline (PBS; pH 7.4) with a ratio of tissue weight and volume of PBS is 1:9. Then homogeneous solution was centrifuged at 8000 rpm for 15 min at 4° C, thus obtained supernatant of hippocampal tissue.

Measurement of malondialdehyde level

The supernatants were used for measurements of MDA level using MDA assay kits (Elabscience, USA). Data was read at 450 nm wavelength and MDA levels were expressed as ng/ ml protein.

Measurement of TNF-α level

The supernatants were used for measurements of TNF-α level using TNF-α assay kits (BioLegend, LEGEND MAX, California USA). Cytokines in tissue were expressed as pg/ ml protein.

Statistical analysis

One-way Anova, Kruskal-Wallis, and *Pearson* were used for statistical analysis. Statistical analyses were done using SPSS version 21.0 for Windows.

Ethical clearance

The study protocol has received ethical approval from the Medical Research Ethics Committee of Faculty of Medicine, Diponegoro University/ Dr. Kariadi Semarang with ethical clearance no. 462 / EC / FK-RSDK / 2015.

RESULTS

The visual testing phase of MWM showed that all mice can reach the platform before the time runs out (<60 seconds). The slowest escape latency time was in the EGCG-2 group (6.49 (SD=3.85)) seconds, while the fastest was in the EGCG-6 group (6.21 (SD=4.42)) seconds. The longest path length was in the N-C group (0.92 (SD=0.25)) meters, while the shortest was in the EGCG-6 group (0.83 (SD=0.41)) meters. The *Kruskal-Wallis* test showed that there was no differences between N-C, EGCG-2, and EGCG-6 group for escape latency time (p = 0.960) and path length (p = 0,587). This result showed that there was no difference of sensorimotor function of the mice in the N-C, EGCG-2, and EGCG-6 groups, so the acquisition trial and probe trial can be done.

The learning phase (acquisition trial) of MWM showed that mice in the N-C group took longer time and longer distance to find hidden platform on day 1, 2, 3, or 4. Mice on EGCG-2 and EGCG groups showed better results with faster escape latency time recording and shorter path length when compared to the N-C group especially at days 2, 3, and 4 (p <0.05). While between EGCG-2 and EGCG-6 group, the results were not different from the first day until the fourth day (p> 0,05).

In the probe trial phase it was found that the lowest % time was in the N-C group (28.68 (SD=9,15))%, while the highest was in the EGCG-2 group (42.02 (SD=5.90))%. The *One Way Anova* test showed a significant difference between the experimental groups (F = 5,593; p=0.015). The post hoc LSD test showed a significant difference between the N-C group and the EGCG-2 group (p = 0.007), and between the N-C group and the EGCG-6 group (p = 0.014). But there was no difference between EGCG-2 and EGCG-6 group (p = 0,716) (Figure 1). Mean of % path length in probe trial phase was lowest in the N-C group (32.98 (SD = 7.75))%, while the highest was in the EGCG-2 group (43.47 (SD = 5.97))%. *One Way Anova* test showed a significant differences between experimental groups (F = 4.028, p = 0.040). The post hoc LSD test showed a significant difference between the N-C group and the EGCG-2 group (p = 0.018), and between the N-C group and the EGCG-6 group (p = 0.042). But there was no differenc e between EGCG-2 and EGCG-6 (p = 0.677) (Figure 2). This result proves the hypothesis that EGCG in both 2 and 6 mg / kgBW doses

has an effect on the improvement of spatial memory function in dementia induced Balb/c mice using D-galactose.

In this study it was found that the lowest MDA levels were in the EGCG-2 group (587.79 (SD = 76.04)) ng / ml, while the highest was in the N-C group (722.64 (SD = 134.78)) ng/ ml. *Kruskal-Wallis* test showed no differences between experimental groups (p=0.141). The *Mann-Whitney* test showed a significant difference between the N-C and EGCG-2 groups (p=0.037). In contrast, no differences were found between the N-C and EGCG-6 groups (p=0.470), and between the EGCG-2 and EGCG-6 groups (p=0.336) (Figure 3). Thus, it can be concluded that EGCG 2 mg/ kgBW can affect decreased levels of dementia-induced hippocampus MDA mice. *Pearson* correlation test showed a significant correlation between MDA level and spatial memory function (p= 0,018; r=0,551). Negative correlation, indicating that higher MDA levels are associated with lower spatial memory function. The correlation diagrams of MDA level and spatial memory function are presented in Figure 4.

In this study it was found that EGCG orally at a dose of 2 and 6 mg/kgBW daily for 5 weeks did not provide changing levels of TNF-α (p=0.786). *Pearson* correlation test showed no correlation between TNF-α levels with spatial memory function (p = 0,313).

FIGURES

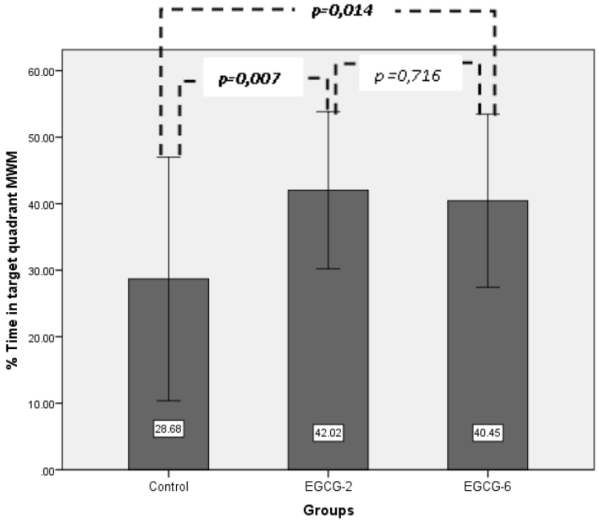


Figure 1. Mean comparison of % time in target quadrant on probe trial phase MWM; mean (SD)

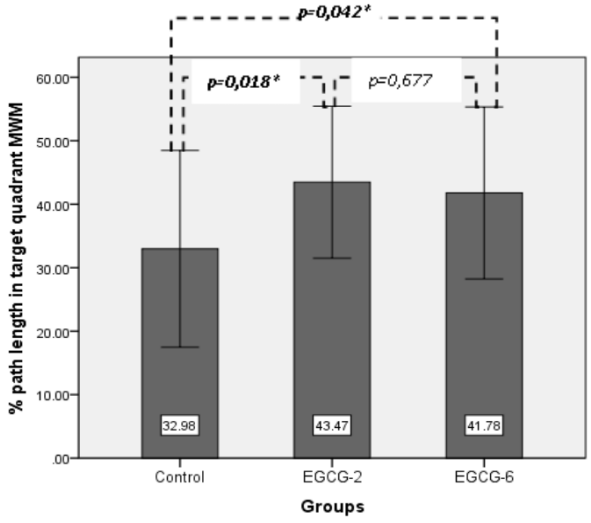


Figure 2. Mean Comparison of % path length in target quadrant on probe trial phase mean (SD)

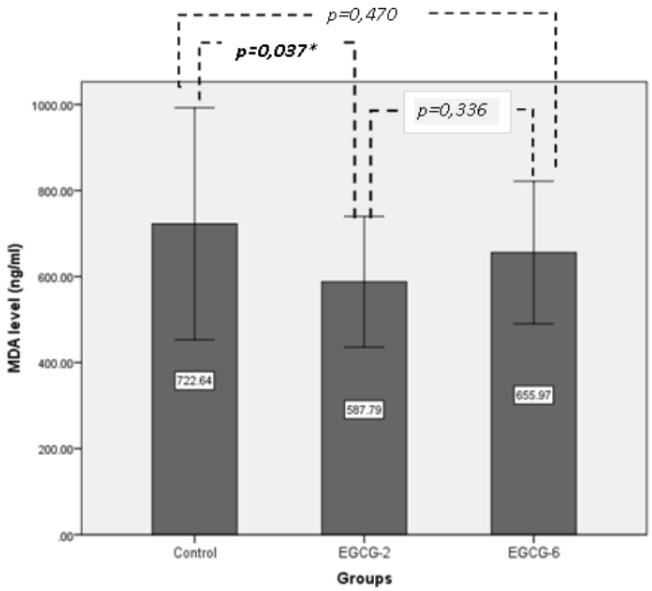
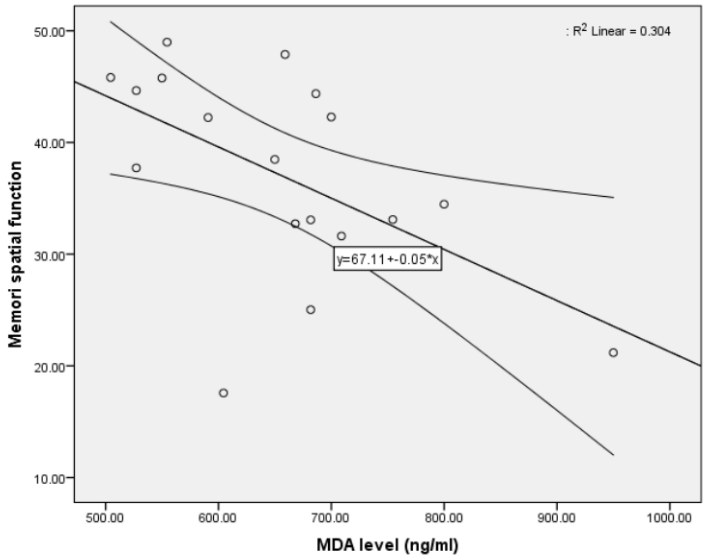


Figure 3. Mean comparison of MDA level (ng / ml) levels between groups; mean (SD)



Pearson r = -0,551 p = 0,018

Figure 4. Correlation between MDA level (ng/ml) and spatial memory function

DISCUSSION

The result of this study indicate that mice in EGCG-2 and 6 group showed better ability with faster escape latency time recording and shorter distance compared to control in learning phase of MWM. In addition, the mice in EGCG-2 and 6 group also showed better memory ability, with high percentage of time and path length in target quadrant compared with control group (p=0,015; p=0,040) in probe trial phase of MWM. This is in consistant with previous studies which stated that EGCG green tea can improve memory spatial function in experimental animals[24]. Other studies have suggested that administering EGCG 10 mg/kgBW daily for 4 weeks can improve the cognitive abilities of Wistar rats induced dementia by intracerebroventrikular streptozotocin[7]. However, the results of this study did not match the previous study which states that the administration of EGCG with a dose of 50 mg/kgBW is not able to increase spatial memory function or repair brain nerve cells damaged in repeated ischemic induced Balb/c mice[29]. These different results are likely due to different duration of EGCG administration. In the study by Pu et al,

EGCG was given only 2 times, ie 30 minutes before ischemic induction was done. This allows less optimum effects of EGCG to repair memory impairment due to ischemic induction. In this study, EGCG was administered in small doses for a relatively long time (5 weeks). This causes the accumulation of EGCG optimally and give better effect.

Spatial memory is an important indicator in assessing neurocognitive function of an individual. Spatial memory impairment is often an early symptom found in early dementia. Dementia is a major cause of disability in elderly. In the aging process, Alzheimer's disease is the main cause (50-75%) of dementia in the elderly[27].

The experimental animals induced by D-galactose are known to be widely used in brain aging and neurodegenerative diseases. The results of this study indicate that mice in N-C group take longer time and longer distance to find hidden platform when compared with EGCG-2 and 6 group. Mice in the N-C group also get difficulties in recalling the location of the platform, indicated by the low percentage of time and path length passed in the target quadrant of MWM. This is consistent with previous studies in which 150 mg/kgBW subcutaneous injections of D-galactose daily for 6 weeks may cause behavioral and memory impairment in mice[18,24,26]. D-galactose is also known to cause spatial memory impairment and nerve cell damage due to cellular metabolic damage by decreasing the activity of Na^+, K^+, ATPase enzymes and increasing oxidative stress through increased lipid peroxidation and decreased antioxidant enzyme activity[24]. Hadzi-Petrushev et al mentioned that administration of D-galactose intragaster 100 mg/kgBW for 6 weeks was able to increase levels of TNF-α and IL-1ß plasma and lipid peroxidation. This study proves that administration of D-galactose at concentrations exceeding normal can lead to the production of free radicals and advanced glycation end products (AGEs). AGE will bind to its receptors (RAGE) and trigger activation of the nuclear factor-κB (NF-κB) transcription factor that plays a role in the production of proinflammatory cytokines (TNF-α and IL-1ß) and free radicals[16].

Oxidative stress and inflammatory processes are known to play an important role in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease[10,14,17,23,24,30,32]. Several studies have shown that epigallocatechin-3-gallate (EGCG), a major polyphenol in green tea, has antioxidant, anti-inflammatory, anticancer, and neuroprotective effects[20-24]. However, other studies suggest that EGCG or green tea extract with a certain dose does not have a significant effect on cognitive function, oxidative stress, or neuroinflammatory status[13,29]. Excess production of reactive oxygen species and or decreased activity of antioxidant enzymes in the brain can lead to lipid peroxidation processes, mitochondrial DNA damage, and protein oxidation, leading to impaired neurocognitive function[24]. Malondialdehyde (MDA) is a dialdehyde compound which is the final product of lipid peroxidation in the body[2,14,17,25]. D-galactose induction in experimental animals is known to cause impaired cognitive functioning because it triggers cellular oxidative stress, increases MDA levels, decreases SOD and GSH-Px enzyme activity[11].

The results of this study indicate that EGCG 2 and 6 mg/kgBW given daily for 5 weeks can decrease MDA levels in D-galactose-induced mice hippocampus. *Pearson* correlation test showed that there was a significant correlation between MDA level and spatial memory function (p=0.018), with strong correlation strength (r=0.551), and negative correlation direction indicating that higher MDA level was associated with lower spatial memory function. This is in consistant with previous studies that mention the antioxidant effects of EGCG on the central nervous system[4,12,24]. Ejaz Ahmed et.al proved that 10 and 20 mg/ kgBW of green tea catechin hydrate orally for 3 weeks was able to increase the activity of SOD, GSH-Px, and catalase enzymes, also decrease MDA level of hippocampus Wistar rats induced by intracerebroventricular streptozotocin[12]. Other studies have suggested that administration of green tea extract can significantly lower MDA levels when compared with controls[4]. However, these results differ from research of Flores et al mention that green tea extracts containing EGCG 299.56 ug/ml administered over one month can not change hippocampus MDA levels in 10 months Wistar rats[13]. This difference is likely to be influenced by the age of the experimental animal, the duration of the intervention, the dose concentration, and the type of the experimental animal.

In this study it was found that EGCG 2 and 6 mg/kgBW did not provide changes in TNF-α levels. This is due to the use of young

adult animals in this study. Older experimental animals show an exaggerated and more prolonged neuroinflammatory response when compared to young adults. In a study conducted by Barrientos et.al who studied the proportionate responses of proinflammatory cytokines in mice (3 months) and old age (24 months) infected with *Escherichia coli*, it was found that both groups had an elevated IL -1ß hippocampus at 2 h post infection. IL-1ß levels in mouse hippocampus decreased significantly after 24 h post-infection in adult rats group, whereas in old age rats group IL-1ß levels continued to increase until the 8th day post infection. IL-1ß levels decreased as baseline after the 14th post infection day. This suggests a more durable neuroinflammatory response to the aging process.[5)] In this study, researchers used young adult rats (6-8 weeks) in which new TNF-α levels were tested on the 7th day after the last D-galactose injection. This allows the decrease of TNF-α levels to initial conditions. This became one of the limitations in this study. Further research on the effects of green tea EGCG on neuroinflammatory status should be performed using effective timing of TNF-α assay levels post-dementia induction.

From this research we can conclude that EGCG may improve spatial memory function and oxidative stress in mice induced dementia, but it may not improve the status of neuroinflammation.

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Muntingia calabura Leaves Extract, a Potential Gastroprotective Agent against Gastric Mucosal Damage Induced by Soft Drink and Alcoholic Beverages

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ABSTRACT

Introduction. Exposures of alcoholic beverages and soft drinks have been notorious for their deleterious effect on gastric mucosal cell, causing disturbances on gastric mucosa. The high content of antioxidant in *Muntingia calabura* leaves have potentials to counteract the degeneration of gastric mucosal cells due to exposure of both drinks. This study aimed to evaluate the effects of flavonoids in *Muntingia calabura* ethanolic leaves extract (MCELE) as gastroprotective agents against the alcoholic beverages and soft drinks induced gastric mucosal damage. **Method.** Twenty four male wistar rats were divided into four groups respectively treated with 1,8 ml/200grBW 40%-alcoholic beverages (K1), 50 ml/day soft drink (K2), pre-treated with 500 mg/kg MCELE one hour before oral administration of 40%-alcoholic drinks (P1) and soft drink (P2). All rats were treated for 30 days. On the 31st day, the rats were teminated and the histological degrees of gastric mucosal damage were determined by modified scale of epithelial mucosa integrity *Barthel-Manja.* **Results.** The K1 and K2 group exhibited severe gastric mucosal injury, with observed ulceration percentages of 33,3% and 23,3% respectively. Meanwhile, the pre-treated MCELE groups (P1 and P2) exhibited significant protection of gastric mucosa histologically ($p<0.001$), showing 0,0% of ulceration on both groups. **Conclusion.** Soft drinks have degenerating effect as strong as the alcoholic beverages. The treatment with MCELE prior to 40%-alcoholic beverages and soft drinks has significantly protected gastric mucosa as ascertained by significant reduction of gastric mucosal injury and increase in normal gastric mucosa.

Keywords: alcohol, soft drinks, gastric mucosa, *Muntingia calabura*.

A significant increase in the world's soft drink consumption occured within the past two decades.(1) A Randomized controlled crossover trial study suggested that sugar-sweetened drinks consumption in a moderate amount for three weeks could elevate inflammation biomarker levels, the high sensitivity C-reactive protein, up to more than 60%.(2)

Chronic alcohol consumption has been known as to cause gastrointestinal diseases such as peptic ulcer, gastroesophageal reflux disease (GERD), liver and kidney diseases, psychological disorders, and even cancer.(3) These conditions resulted from the chronic process of alcohol metabolism within the body, causing much carcinogenic agents such as Reactive Oxygen Species (ROS) to build up within certain organs particularly the gastrointestinal organs. ROS destroys cell components, alter NADH to NAD+ ratio, leads to tissue injury, metabolic alterations, causing cancer, and drugs interaction.(4)

The use of medicinal plants extraction has drawn such interest within the discovery of better treatment for unhealthy diet-related diseases such as gastric ulcer. Natural products has become such trends and that the use of natural source for drugs such as medicinal plants extracts is likely to be more favorable.

One of the potential medicinal plants to alleviate gastric ulcer is *Muntingia calabura*, commonly known as *talok* or *kersen* (Java, Indonesia), *kerukup siam* (Malaysia), or Jamaican cherry. *Muntingia calabura* is a

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perennial plant which can be found easily as a roadside trees in tropical Asian countries such as Indonesia.(5) Previous studies suggested *Muntingia calabura* as a great source of flavonoids which act as potent antioxidant agents, with the highest content of flavonoids lay within its leaves.(6)(7) *Muntingia calabura* leaves extract exhibited significant pharmacological effects such as antiulcer, antiinflammatory, antinociceptive, antimicrobes, and anticancer.(7) Despite its known pharmacological properties, there is little we know about how *Muntingia calabura* leaves extract alleviates the morbidity caused chronic consumption of soft drink and alcoholic beverages.

MATERIALS AND METHODS

This study is a quasi experimental with post test only control group study consists of four groups of male wistar rats receiving treatments for a whole 30 days course of treatments. The alcoholic beverage used was whisky, and the soft drink used was soft drink with the brand name initial "CC".

Plant specimen and extract preparation

Muntingia calabura leaves were obtained from Faculty of Medicine Diponegoro University, Indonesia and identified by the biologist of Faculty of Science and Math Diponegoro University. The dried leaves was then ground using electrical grinder. One hundred grams of the finest ground *Muntingia calabura* leaves were soaked in 500 ml 95% ethanol for 72 hours. After 72 hours, the mixture were filtered using muslin cloth followed by filter paper (Whatman No.1) and distilled under low pressure with 40°C temperature in an Eyela rotary evaporator (Sigma-Aldrich, USA). The dry extract was then dissolved in CMC (0,5% w/v) to be given orally to the experimental rats with the dose of 500 mg/kgBW (5 ml/kgBW) *Muntingia calabura* ethanolic leaves extract (MCELE).(8)

Experimental animals and inductions of gastric mucosal damage by soft drink and alcoholic beverage

The study required healthy male wistar rats with body weight ranged within 200–300 grams, 8-12 weeks old which were obtained from Integrated Research and Testing Laboratory of Gajah Mada University, Indonesia. The rats were placed in a separated cage and were adapted for one week before receiving any treatments. The rats were given pellets and water ad libitum. The study was approved by Ethics Committee for Medical Research Faculty of Medicine Diponegoro University, Indonesia. The experimental rats were chosen and treated according to Institutional Animal Care and Use Committee Guidebook from National Institutes of Health and World Health Organization's General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine.

The gastric mucosal damage were induced by the administrations of 50 ml/day soft drink ad libitum and alcoholic beverages with ethanol percentage of 40% (whisky) as much as 1,8 ml/200gBW given with oral gavage. The dosage was calculated in aim to reach the gastric mucosal damage level wanted within 30 days of treatments. Twenty four male wistar rats divided into four groups respectively treated with 1,8 ml/200grBW 40%-alcoholic beverages (K1), 50 ml/day soft drink ad libitum (K2), pre-treated with 500 mg/kg MCELE one hour before oral administration of 40%-alcoholic drinks (P1) and pre-treated with 500 mg/kg MCELE one hour before oral administration of soft drink ad libitum (P2). The rats were sacrificed on day 31st

Histological evaluation of gastric mucosal damage

Gastric tissue samples were obtained and sent to anatomical pathology laboratorium of Kariadi Central Public Hospital in order to process them into preparations using microtechnique and HE staining. Histological evaluation on gastric mucosal lesions were determined by using modified scale of epithelial mucosa integrity *Barthel-Manja.*

Statistical analysis

The data collected was then assessed for its statistical significance of differences between groups using Kruskal-Wallis statistical test followed by Mann-Whitney test. A value of $p \leq 0{,}05$ was considered significant.

RESULTS

Descriptive analysis

In the present study, there were no macroscopic bleeding found on the gastric mucosa. However, some of the gastric samples from K1 group had irregular shape when distended, which was caused by the

thinning of the gastric mucosa on several areas, notably at fundus and corpus area. Gastric samples obtained from K1 and K2 groups both had macroscopic mucosal thinning, erosions, and reduced rugae when the gasters were incised open along the great curvature of the gaster. Otherwise, the gastric samples from P1 and P2 groups showed better mucosal condition compared to K1 and K2 groups, with the rugae still noticeable and mucosal thickness similar to normal gastric mucosa of a rat.

Microscopic examination showed a remarkable changes on gastric mucosal integrity; from no pathologic changes (normal mucosa) to epithelial desquamation, epithelial surface erosion (gap 1-10 epithelial cells/lesion), and epithelial ulceration (gap >10 epithelial cells/lesion).

In K1 group, microscopically the gastric mucosa had epithelial surface erosion up to 46,7% and ulceration up to 33.3% (Figure 1a.). The K2 group which were treated with only softdrink, showed microscopic appearance of gastric mucosa with almost no distinct features than K1 group (Figure 1b.). Both groups had 0,0% normal epithelial found.

Otherwise, P1 and P2 groups were dominated by epithelial desquamation and normal epithelial with no ulceration found. In P1 group, the epithelial desquamation was observed up to 66,7% (Figure 1c.) while in P2 group it was observed that there was 53,3% epithelial desquamation (Figure 1d.). Both groups showed minimal epithelial surface erosion with percentage of only 6,7%. The frequencies and percentages of the gastric histopathological observation after treatment is shown in Table 1.

Inferential analysis

Kruskal-Wallis test was performed between experimental groups and it showed a significant difference between them (p<0,001). A Post Hoc test of Kruskal-Wallis test, Mann-Whitney test was perfomed following the previous mentioned test. Mann-Whitney test for K1 vs P1 and K2 vs P2 both showed significant differences with p value of p<0,001. Meanwhile, K1 vs K2 showed no signficant difference with p value of p=0,293 or p>0,05.

DISCUSSION

The exposures of destructive agents such as ethanol, water restraint stress, or ischemia followed by reperfusion on gastric mucosa will lead to pathological alterations in a form of inflammation process, haemorrhagic erosion, and acute ulcer. The basis of these alterations disturbs the protective mechanisms and impairs the gastric mucosal defense. The previous studies suggested that there are involvements of the impaired gastric blood flow, mucous secretion, and the role of prostaglandin and NO generation in the pathomechanism of gastric mucosal lesions formation.(9) Gastric lesions by ethanol are commonly associated with ROS generation, whereby these lesions produce an imbalance between oxidant and antioxidant cellular processes. This is evidenced by increased levels of malondialdehyde, a marker of increased lipid peroxidation.(6) The deleterious effect by ethanol manifests through either via direct reactive metabolites generation or contributing to other mechanisms that finally support oxidative damage.(10)

The imbalance of aggressive factors and protective factors of gaster triggers acute inflammation reaction. Interleukin-1 beta (IL-ß) and tumor necrosis factor alpha (TNFα) are the major proinflammatory cytokines which hold important roles in creating acute inflammatory reaction followed by neutrophile infiltration within the gastric mucosa. Neutrophile produces radical anion superoxide (O_2^-) which is one of the ROS kinds, and reacts with cellular lipids, resulting in the formation of lipid peroxides which are then metabolized into malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), two of the metabolytes indicating the presence of mucosal injury by ROS. The concentrations of 4-HNE and MDA in intact mucosa measured at low levels, whereas in the 100% ethanol-exposed mucosa they were found to be twice as high. The damage on the gastric mucosal integrity induced by ethanol may be caused by decrease in gastric blood flow, increase in inflammatory changes indicated at the increased level of IL-ß and TNFα and the ROS production as well as the endogenous antioxidant activity attenuation within the cells.(9)

Chronic consumption of soft drink are related to increased oxidative stress processes.(11) The high content of fructose and sucrose within soft drink triggers malabsorption and increases any inflammation event in the gaster which are associated with atrophic gastritis events and

paracellular leakage of gastric mucosa.(12)(13)Constant irritation from the acidity, gas content, high sucrose and fructose content, and the presence of bysphenol A (BPA) in a significant level within soft drink induce alterations of gastric mucosal integrity as a result of continuous oxidative stress process and decreased activity of endogenous cellular antioxidant which is caused by a decrease in the levels of antioxidant proteins.(11)(14) One of the previous study suggested that gastric cells exposed to nonylpolyphenols, a form of of BPA, in a certain dose with maximal cytotoxic dose of 10^{-7} M within 48 hours would have its cell cycle and apoptosis rate altered.(15)

Nitric oxide (NO) acts as both intercellular and intracellular messenger. It acts in two different ways in stomach; NO induces the activation of defensive factors of gaster, but when overproduced it causes inflammation process including ROS and NO generation which caused gastric ulcer, chronic gastritis, bacterial gastroenteritis, and other gastrointestinal diseases in the end.(16)

Present study showed an insignificant histopathological difference between K1 and K2 groups (p=0,293), which could be interpreted as the exposure of soft drink for 30 days with a dose of 36-39 ml/day in wistar rats generated deleterious effect on gastric mucosa as potent as 40%-ethanolic beverages exposure with the equal duration. The volume range of soft drink required to induce desquamation–ulceration lesions in human when consumed everyday for at least 30 days is 256–275 ml. Destructive effects of both soft drink and alcoholic beverages come from the generation of free radicals and ROS when the gastric epithelial cells are exposed to both drinks. The high level of ROS oxidizes the lipid within cell membranes directly and induces peroxidation, prolonged the degeneration on gastric cells and resulted in producing desquamation, erosion, evenmore ulceration on the gastric mucosa.(11)

Microscopic examinations on the wistar rat gaster which had been given MCELE 500 mg/kgBW prior to administration of 40%-alcoholic beverages 1,8 ml/200grBW suggested that there is gastroprotective effect exerted by MCELE, protecting the gastric mucosal integrity and help strengthen the mucosa. It is ascertained from the result of comparison between K1 group to P1 group with resulting p value of <0,001 and the proportions of normal gastric mucosal integrity in P1 group was much higher (26,7%) than in K1 group (0,0%). In K2 and P2 groups comparison, there was significant difference with resulting p value of <0,001 and again, the higher proportion of normal gastric mucosal integrity was found in P2 group compared to K2 group. The ulcerated gastric mucosa was found to be higher in K1 group than in K2 group (33,3% and 23,3% respectively) and none was found in P groups.

Various studies examining the active compounds within *Muntingia calabura* conducted from 1991 to 2013 had identified 86 bioactive compounds isolated from various parts of the plant, including leaves, barks, flowers, and fruits in various forms of extractions. The bioactive compounds mainly found were flavonoids and its derivatives, chalcones, other phytosterols, and some organic acids such as syringic acid and vanilic acid.(7) A study of phytochemical analysis of *Muntingia calabura* leaves conducted in Indonesia in 2014 showed that the highest concentration of flavonoid obtained from the extraction process using polar solvents such as ethanol, methanol, and water. The common bioactive compounds identified in a large quantities were epigallocatechin gallate (EGCG) and genistein, both of those constituents are part component of catechin, one of the most powerful antioxidant mainly found in tea that are thought to provide several health benefits.(17) The peak plasma concentrations of EGCG are reached in 1-2 h in healthy subjects with one oral dose in the morning after an overnight fasting period then diminish gradually to undetectable levels in 24 hours.(15)(18)

Antioxidant activity of flavonoids, including those residing within *Muntingia calabura* leaves, comes from its capacity as free radical scavenging, breaks the chain of free radical formation reactions, decreases the amount of peroxides, and activating various endogenous antioxidant proteins within its interaction on oxidative stress signalling pathway.(19) *Muntingia calabura* leaves extract in various fraction solution using different kinds of solvent at a concentration of 100mg/ml showed NO-inhibiting activity in macrophage-induced inflammation and help maintaining cell viability.(6) Zakaria et al (2007) conducted a study on antioxidant activity of aquoeus extract of *Muntingia calabura* leaves ($AEMC_L$) using DPPH free radical-and superoxide anion radical scavenging assays method, and it showed that it had 94,80 ± 1,14 and 83,70 ± 2,05% measured antioxidant

capacity.(20) Otherwise, the antiinflammatory activity of *Muntingia calabura* leaves had also been studied. $AEMC_L$ of 10% and 50% concentration (27 and 135 mg/kg consecutively) have much higher antiinflammatory activity observed at 3 and 4 hours intervals after administration compared to acetylsalycilate acid 100 mg/kg as a reference drug.(21)

Flavonoid compound consists of one or more aromatic ring which have one or more hydroxil groups, giving flavonoid its ability to neutralize free radicals by converting them into resonance-stabilized phenoxyl radicals.(1) Other than that, flavonoids found in *Muntingia calabura* leaves increase mucus production, which has been known as gastric mucosa defense mechanism against corrosive and oxidative agents.(22) Previous study using MCELE of 250 mg/kg and 500 mg/kg compared to omeprazole 20 mg/kg as a reference drug showed that MCELE has significant and dose-dependet antiulcer activity when compared to omeprazole. It is also suggested that MCELE reduces the acidity of gastric juice and enhances mucous production within ethanol-induced gastric ulcers in rats.(8) The other study conducted in 2013 used *Muntingia calabura* methanolic leaves extract (MCMLE) and ranitidine as reference drug on ethanol-induced gastric ulcer model suggested that MCMLE has significant and in dose-dependent manner antiulcer activity. Histological examination showed that MCMLE has a potential to reverse toxic effect of ethanol and helps regenerate mucosal structure to its normal condition as shown in ranitidine administration. These abilities may have related to mechanism involving NO modulation and endogenous sulfhydril compounds. The ability of MCMLE fractions to prevent ethanol-induced gastric ulcer suggests the involvement of local and non-specific mechanism called adaptive cytoprotection.(6)

Finally, we suggest that MCELE has a potential to be used as an alternative source of medicine to treat degenerative disease, especially gastric-related disease. Further studies are required to identify the exact bioactive compounds that may be responsible for the antiulcer properties of *Muntingia calabura*.

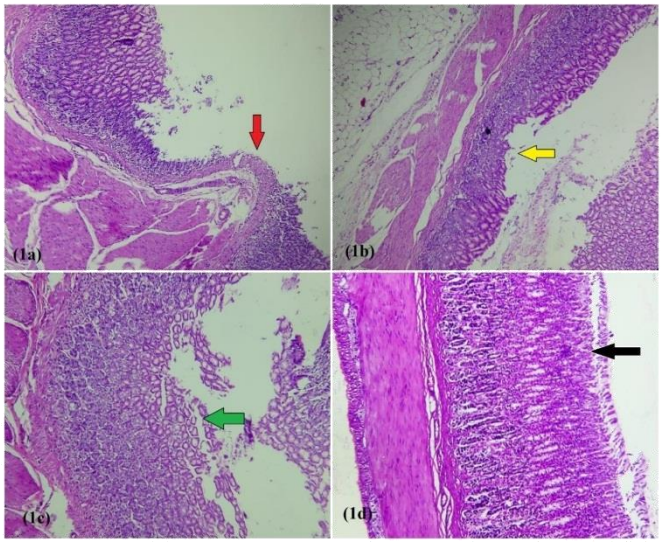

Figure 1. Histological examination of gastric mucosal integrity. The samples were examined using light microscope with the aforementioned magnifications. (1a.) HE staining (×100). The ulceration was shown (red arrow) and found most abundant in K1 goup. (1b.) HE staining (×100). The epithelial surface erosion was shown (yellow arrow), K2 group was dominated by this kind of lesion. (1c.) HE staining (×100). Black arrow points to the epithelial desquamation, which was dominating in P1 group, indicating gastroprotective effect exerted by MCELE given to this group. (1d.) HE staining (×40). P2 group showed the most abundant intact mucosa (red arrow) as indication to the gastroprotective effect of MCELE given to this group.

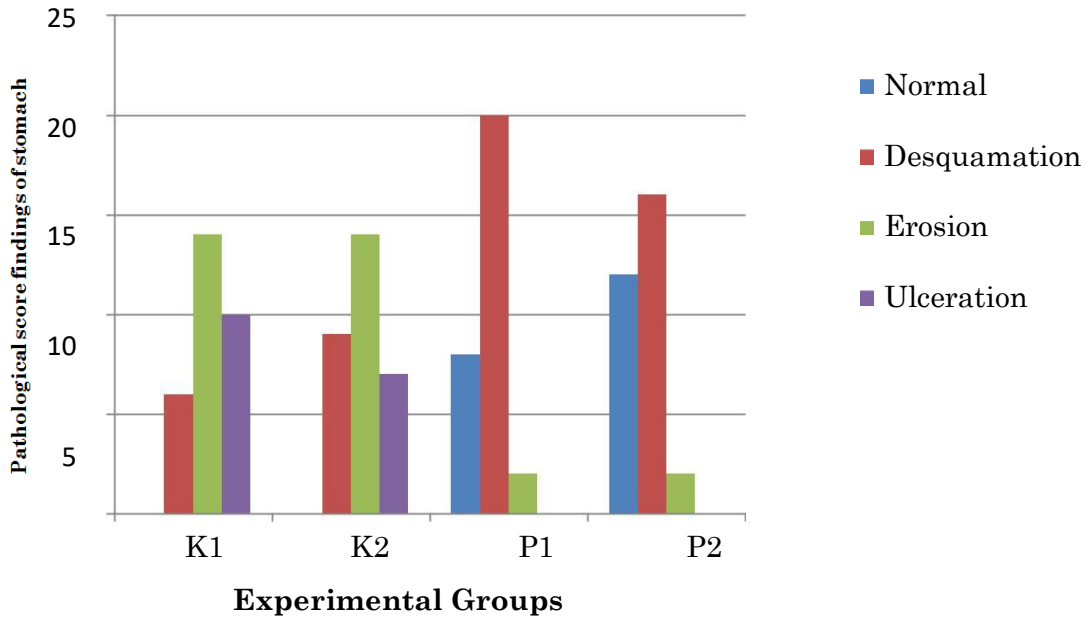

Figure 2. Result of pathological score findings of the experimental groups.

	Normal	Epithelial desquamation	Epithelial surface erosion	Ulceration	Total
K1	0.0% (0)	20.0% (6)	46.7% (14)	33.3% (10)	100% (30)
K2	0.0% (0)	30.0% (9)	46.7% (14)	23.3% (7)	100% (30)
P1	26.7% (8)	66.7% (20)	6.7% (2)	0.0% (0)	100% (30)
P2	40.0% (12)	53.3% (16)	6.7% (2)	0.0% (0)	100% (30)
Total	**16.7% (20)**	**42.5% (51)**	**26.7% (32)**	**14.2% (17)**	**100% (120)**

Table 1. Frequencies and percentages of the histopathologic examination on gastric experimented rats.

	***p* value**			
Group	**K1**	**K2**	**P1**	**P2**
K1	-	0.293	0.000*	-
K2	0.293	-	-	0.000*
P1	0.000*	-	-	0.338

Table 2. Mann-Whitney non-parametric test of the result on gastric mucosal integrity scoring and examination

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Effect of Mangosteen (*Garcinia mangostana)* PEEL Extract towards CD4+, CD8+ T LYMPHOCYTES, CD38 Expression, NK Cells, IL-2 and IFNγ in Hiv Patients with Antiretroviral Therapy

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ABSTRACT

Introduction: HIV/AIDS still being an emerging & epidemic disease in Indonesia. Humans infected with HIV have shown to be under chronic oxidative stress. Experimental studies have shown that obtained xanthones from mangosteen have remarkable biological activities as an antioxidant. This study aims to analyze the effects of Mangosteen Peel Extract (MPE) toward CD4+ T cells, CD8+ T cells, NK cells, CD8+CD38 expression, levels of IL-2 and IFN-γ, in HIV patients with antiretroviral therapy.

Method: This experimental study was designed using double-blind, randomized control group which randomized by the permuted table. Subjects were HIV-positive patients receiving antiretroviral therapy more than six months. Patients were divided into 2 groups; treatment group (n=20) and placebo group (n=20). The treatment group had been given MPE 2400 mg/day for 30 days the same as the placebo group. The variables were measured before and after treatment using FacsCalibur Becton-Dickinson flowcytometry.

Results: There was significant increase in the number of CD4+T cells (p=0.001). There was significant decrease in CD38 expression (p=0.001). There were no significant changes in CD8+T cells (p=0.601), NK cells (p=0.911), IL-2 (p=0.260) and IFN-γ (p=0.588).

Conclusion: Mangosteen peel extract increases the number of CD4+ T cells and decreases the level of CD38 expression, whereas the effect of CD8+ T cells, NK cells, IL-2 and IFN-γ in HIV patients with antiretroviral therapy were not significant.

Keywords: Mangosteen, CD4+, CD8+, CD38, NK cells, IL-2, IFNγ, HIV, Antiretroviral

The AIDS epidemic in Indonesia is one of the fastest growing in Asia. Indonesia will have almost twice the number of people living with HIV and AIDS in 2014 as compared to 2008, rising from an estimated 227,700 to 501,400.(1)(2)(3)

The hallmark of HIV infection is the progressive loss or depletion of CD4+ T lymphocytes.(4)(5)(6)(7)(8)(9) The changes in the CD4+ T lymphocytes counts are important indicators of the response to antiretroviral treatment.(10)(11)(12)(13)(14) Also, the analysis of CD38 expression on lymphocytes has become an important tool for monitoring patients during HIV-1 infection.(15)(16)(17) The role of CD38 expression in CD8+ T cells as a prognostic marker of virological failure. CD8 cells are also known to produce many cytokines(18)(19) including IFN-γ, TNF-α, and IL-2, in meticulous broadly related aspects of infection. Levels of IL-2 in HIV infection may be an indicator of the improved immune system in HIV infection.(20) The levels of IL-2 has an essential influence on the proliferation of CD4.(21) IL-2 have a major role to help the development of CD8+ T cytotoxic cells, CD4+ T cells, B cells, and increase the killing power of macrophages and regulate T cells multiplication.(22)(23) IFN-γ also produced by NK cells, it can stimulate dendritic cells which are non-specific and specific immune cell coordinator.(24) IFN-γ function prevents

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viral replication in infected cells and the surrounding cells that induce anti-viral environment. IFN-γ produced by NK cells, also has positive feedback on its own of NK cells, NK cells which can become more active in its function.(25)

Humans infected with HIV have been shown to be under chronic oxidative stress. Oxidant production could enhance HIV replication via activation of NFκB and indirectly through activation genes that further promotes oxidative stress and hence HIV replication.(24)(25)(26) Interestingly, HAART proved to have deleterious effects as a result of mitochondrial dysfunction, increase in oxidative status and it plays an important role in the occurrence of oxidative stress.(27) Antioxidant may have a role in the treatment of HIV infection.(28)

Experimental studies have shown that obtained xanthones from mangosteen have remarkable biological activities as antioxidant, antitumor, antiallergic, anti-inflammatory, antibacterial, antiviral activities, antiplasmodial, cytotoxic and potential cancer chemoprotective activities.(26) Moreover, some of the xanthones from mangosteen have been found to influence specific enzyme activities, such as aromatherapy, inhibitor kB kinase, quinone reductase, sphingomyelinase, topoisomerase and several protein kinases.(26) Several studies showed that xanthones from Garcinia mangostana act as an active constituent against HIV-1 protease.(29)(30)(31) A randomized controlled trial study in healthy adults showed that intake of a xanthone-rich mangosteen product elevated of the frequency of peripheral Th cells frequency 2.6% (SD 5.7%).(32)

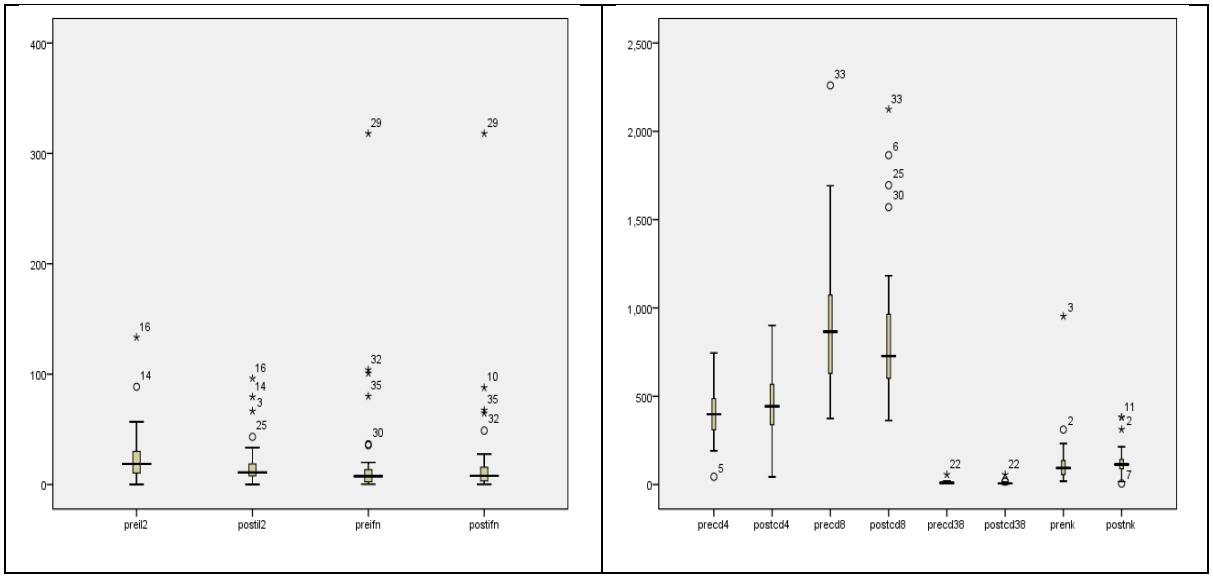
Although it has been many research related to Garcinia mangostana performed, research on the effects of mangosteen (*Garcinia mangostana)* peel extract towards $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, NK cells, IL-2, IFN-γ and CD38 expression in HIV patients has not been investigating. Thus not known whether mangosteen *(Garcinia mangostana)* peel extract affects the $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, NK cells, IL-2, IFN-γ and CD38 expression in HIV patients with HAART.

MATERIALS AND METHODS

This experimental study was designed using double-blind, randomized control group which randomized to the permuted table. Subjects were HIV-positive patients receiving antiretroviral therapy more than six months. Patients were divided into 2 groups; treatment group (n=20) and placebo group (n=20). A treatment group had been given MPE 2400 mg/day for 30 days the same as the placebo group. The number of $CD4^+$ cells, $CD8^+$ T cells, NK cells, $CD8^+CD38$ expression, the level of IL-2 and IFN-γ were measured before and after treatment using FacsCalibur Becton-Dickinson flowcytometry.

RESULTS AND DISCUSSION

Analyses of immune cells before and after treatment revealed significant increase in CD4+ T cells, but reduced another immune cells and cytokine shown in the Graphic 1. Boxplot pre-post test treatment group.



Graphic 1. Boxplot pre-post test treatment group

Changes in CD4⁺ T cells

The statistical analysis showed that the P value 0.000 (P<0.05) in each group. There is a significant increase in pretest and posttest group. Means treatment group 401.9±152.2 and placebo group 492.8±196.2 Mean value between pre-test and post value difference is larger, so the result means that there is a significant difference of Mean.

Changes in IL2

IL-2 level decreased both two groups. But the decreased in the treatment group was not significant (p=0.26). Delta decreased in the treatment group was 5.94 and 8.72 in the placebo group. It shows delta placebo group was 1.4 times higher than the treatment group.

Changes in CD8⁺ T cells

The frequency of peripheral blood CD8⁺ T cells decreased in both groups. But the decreased was not significant in extract group (p=0.601) neither on placebo group (p=0.135). Delta decline of the number of CD8⁺ T cells from extract group is 51.65 and 59 in placebo group which is delta decline of placebo group 1.14 times higher than the Delta of treatment group. There is an increase in Th/Tc ratio between baseline and post-treatment. In the extract group Th/Tc ratio increase from 0.44 to 0.51, and 0.45 to 0.54 in the placebo group.

Changes in CD38 expression

CD38 expression decreased both two groups. The decreased was significant in extract group (p=0.001) either in the placebo group (p=0.001). Delta decline of CD38 expression from extract group is 3.23 which is 1.035 times higher than the Delta of placebo group whose delta is 3.12.

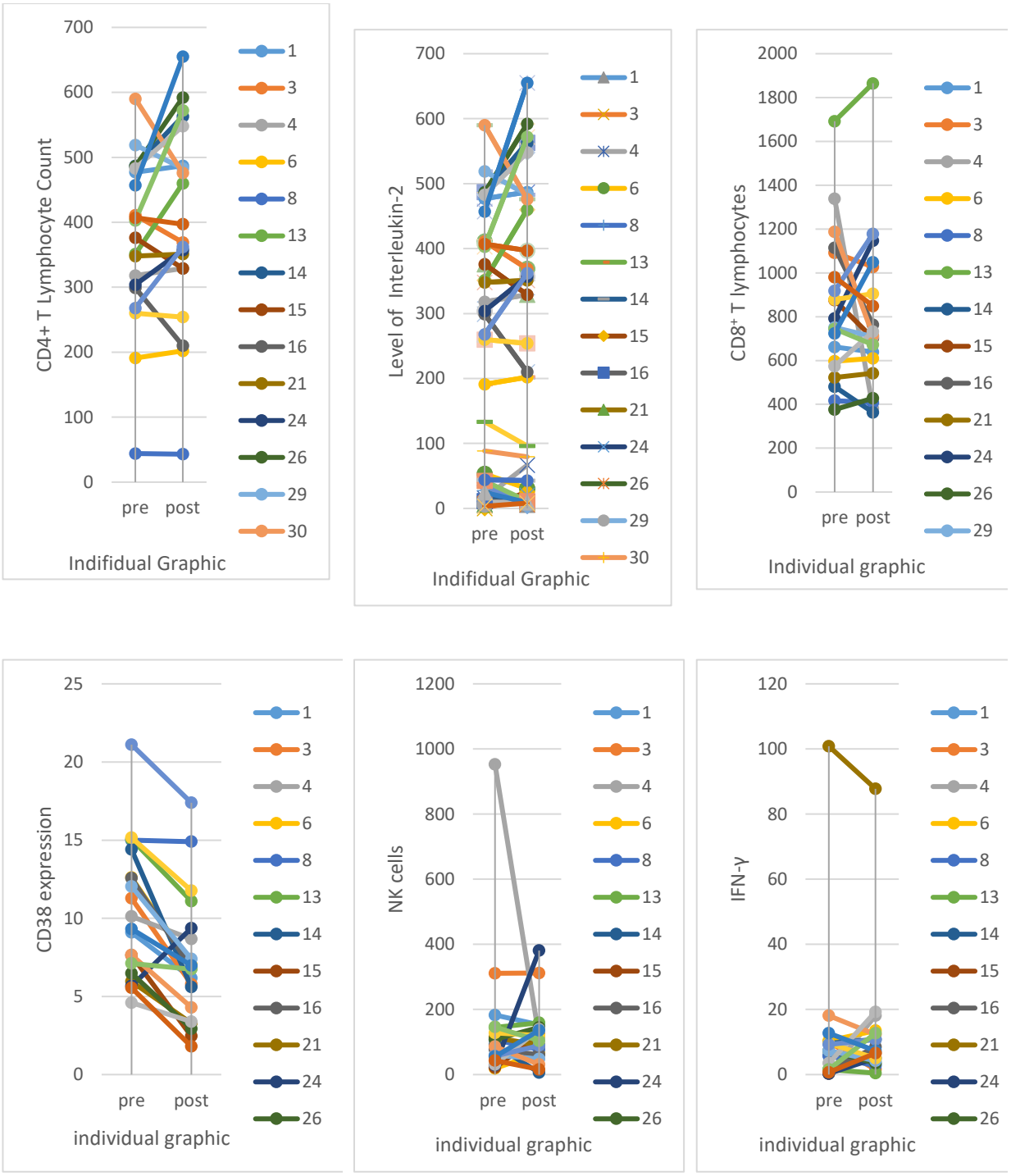
Changes in NK Cell

NK cells in the treatment group decreased but not significant (p = 0.911). The number of NK cells in the placebo group increased, but the increase was not significant (p= 0.121).

Changes in IFN-γ Level

There is no significant increase in IFN-γ levels in the treatment group (p = 0.588), and there is no significant decreased of the placebo group (p=0.444).

The other than that, the authors report the changes of each indicator in individual graphic



Graphic 2. Individual graphic treatment group

The authors report here that after the treatment of mangosteen peel extract, CD4+ T cells have shown a significant increase in the treatment group, resulting in decreased levels of IL-2. Otherwise, $CD8^+$ T cells decreased in line with a decrease of CD38 expression. In the other side, there were no significant changes in IFN-γ caused the decreased of NK cells.

The increasing IFN-γ and CD4+ Lymphocytes is closely related to the antioxidant content contained in Mangosteen Peel Extract/MPE. In this study, HIV patients had high levels of ROS, along with high viral levels and the side effect of consumption the drugs can increase the level of ROS. Antioxidants in MPE can inhibit the high levels of ROS (Reactive oxygen species)

in HIV patients. The mangosteen peel extract contains xanthones compounds which have a high antioxidant function that can be used to protect and reduce cell damage mainly caused by free radicals. These results indicate a role of MPE as an antioxidant which also suppresses apoptosis that would maintain the number of CD4+ T lymphocytes.

Decreased levels of IL-2 could be due to the role of cytokines that influenced easily soluble factor or a local effect on a particular cellular environment. It would affect T cell stimulation and the effect of fluctuations of the immune activation. In this study, increase the number of $CD4^+$ T cells not accompanied by elevated levels of IL-2 resulting in the balance of the immune system in HIV patients. The descent of IL-2 cytokines, which decrease the number of NK cell can happen because of several factors. It could be due to exhausted factors on the condition of HIV patients to increase so that the number of $CD4^+$ T cells not accompanied by elevated levels of IL-2 resulting in the balance of the immune system in HIV patients. Increase in IFN-γ levels was due to a decrease in CD8 cells due to the mechanism of HIV.[38] The work of IFN-γ above indicates that the results of this study are not enough to seek changes in IFN-γ levels, but still require research looking for the effectiveness of mangosteen peel extract on changes in function and work of IFN-γ. The function have done by working with NK cells; this cooperation occurs after NK cells identify microbes and perform its cytolytic function, then NK cells produce IFN-γ. The impairments of NK cell are associated with expansion of an "anergic" NK cell. In HIV infection, CD4 T cells induce IFN-γ and IL-2 in an attempt to suppress viral infections.

Exhausted factor mechanisms began in early infection (primary) where an increase in $CD4^+$ T cells and $CD8^+$ T cells, followed by the rate of infection cannot stop, and the production of IL-2 levels was high. This situation makes $CD4^+$T cells, and $CD8^+$T cells do not respond, characterized by the inability to produce cytokines, perforin, and granzyme (partial exhaustion I). Furthermore, the value chronic immune activation has been reasoned to be a significant contributor to disease progression in HIV-1 infected patients which has prompted the use of the expression of cell surface activation markers such as CD38 to monitor disease progression. Results from several such studies have documented a correlation between plasma viral load and the increased expression of CD38 as strong predictor of disease progression, and it may because of the response to ARV despite to mangosteen peel extract.

Variations in individual results can be due to differences in allele and Polymorphism in every individual which caused by the variation of the expression of DNA base composition and chromosome differences. Polymorphism in enzymes can increase the toxic effects of the drug. The involvement of the gene and the protein will affect the body's response to an adjuvant and drug products. Thus, the need for pharmacogenomics approach to that may explain individual variations in the response of each of the drugs given; this response is closely related to the genetic differences of each. Another factor that affects the immune response is different for every individual who is affected by immunogenetics factors, one of which is an HLA (Human Leukocyte Antigen) system on each that will be an expression of different characteristics. HLA's role associated with the number of CD4+ T lymphocytes. HLA associated with MHC class II or the so-called APC cells for antigen presentation to T CD4+ lymphocytes. MHC (Major histocompatibility complex) controls the immune response and antigen expression.

Bass(33) in his research stated that the correlation absence between the number of CD4+ T lymphocytes and the levels of IL-2 might be due to response disturbance of the mitogens proliferation which correlates with decreased expression of the IL-2 receptor and increased expression of HLA-DR. CD4+ T lymphocytes. Kawamura(34) added function decline in the role of Dendritic cells (DC) as immunopathogenesis in HIV disease that will affect the APC response in CD4+ T lymphocytes, which will stimulate CD4 cell proliferation and production of IL-2 production which is mediated by the binding of gp120 in HIV patients.The decrease result in this study, because the bioavailability of mangosteen xanthones is limited as it is for many phytochemicals, the gastrointestinal (GI) tract is exposed to high concentrations of these compounds and their metabolites.It may lead to improvement in patients defecation.

On the previous study about mangosteen, the study population was very homogeneous regarding nutritional status and other

lifestyle factors. They are representative of ordinary, generally healthy adults.It may take a different result dealing with this study, whereas study population was an HIV patient wherein HIV patients, there was progressive dysregulation of the immune response to progressive disease. Although they were accepted antiretroviral therapy, ART only partially corrects these deficits.

Another factor that affects the immune response is different for every individual who is affected by immunogenetics factors, one of which is an HLA (Human Leukocyte Antigen) system on each that will be an expression of different characteristics. HLA's role associated with the number of CD4+ T lymphocytes. HLA associated with MHC class II or the so-called APC cells for antigen presentation to T CD4+ lymphocytes. MHC (Major Histocompatibility Complex) controls the immune response and antigen expression. The literature presents equilibrium between oxidants and antioxidants is crucial to the body; it would be important to look into the products consumed, that have a protective effect on the organism and encourage other population to consume it for its beneficial effect against certain diseases such as the metabolic syndrome.

Correlation towards the negative in the extract group may be due to the of the increase mechanism in the number of CD4 + T lymphocytes. It can express CD38, and HLA-DR showed the activation of immune chronic affecting the deregulation of cytokines, resulted in increased production pro-inflammatory cytokines such as IL-1, TNF, IL-6 as well as a decrease in TH1, such as IL2 and IFN. Decreased IL-2 production will affect the endogenous IL-2 receptor. Unbalanced lymphocytes T CD4 + and CD8 number can damage certain antigens. (17)

However, this study did not analyze the viral load; further research needed with viral load as a gold standard. Other than that the results will be seen to variance with the patient in the early stages (primary), before the patient given anti-retroviral and herbal research done ± six months, so it can be seen how effective the concoction in the immune system and process deregulation immune balance. This study also report that MPE can be the adjuvant therapy for HIV patients, while consumption the anti-retroviral therapy.

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Effects of Physical-Cognitive Therapy (PCT) on Criticaly ill Patients in Intensive Care Unit

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ABSTRACT

The condition of Criticaly ill patients in Intensive Care Unit (ICU) can make heavier impairment physical and cognitive functions. The research objective is to prove that physical-cognitive therapy affects towards increasing physical and cognitive functions to critical patients in ICU. The research design was Randomized Controlled Trials (RCTs). The samples were critical patients in ICU of Kediri Baptist Hospital as many as 64 critical patients according to inclusion and exclusion criteria. The research has got ethical clearance from Commite Ethic Medical Faculty of Diponegoro University. The research instrument used Physical Function ICU Test (PFIT) Indonesian Version and Mini-Mental State Examination (MMSE) Indonesian Version. Differential test used Independent t-test on physical function and Mann-Whitney test on cognitive function towards intervention group and control group. The results showed that physical-cognitive therapy significantly affected increasing physical function (ρ = 0,000) with mean increased of 3.2 points and cognitive function (ρ = 0,000) with mean increased of 7.3 points. The difference test of influence between the intervention group and the control group was done by testing the posttest data on physical function (ρ = 0,000) and cognitive function (ρ = 0,000) in both groups. Effect size > 0.8 (Physical Function: 3,2; Cognitive Function: 1,9). In conclusion, there was affecting physical-cognitive therapy towards increasing physical and cognitive functions to critical patients in ICU.

Key words: Criticaly ill patient, Intensive Care Unit, Physical-cognitive therapy.

Critically ill patients are at risk of reversible dysfunction in one or more life-threatening organs and require care in the Intensive Care Unit (ICU). [1,2] Critical patients in the ICU prevalence continue to increase each year. The World Health Organization (WHO) in 2016 reports that deaths from critical illness to chronic illness in the world increase by 1.1 to 7.4 million people and there are 9.8 to 24.6 critically ill patients and treated in ICU per 100,000 population.[3] The prevalence of critical patients is large along with the various problems in the ICU to be resolved. Critically ill patients have various health risks that can arise during the results of preliminary study based on Medical and Medical Record Installation Data Baptist Hospital Kediri obtained the average of critically ill patients treated at ICU Baptist Hospital Kediri in May-August 2016 as many as 75 patients and 56 patients (74.7%) survive and exit ICU.[4] The results of previous research studies in ICU Baptist Hospital Kediri in July 2016 found 41 critically ill patients with 11 patients (26.82%) impairment physical function and accompanied by signs of tardive dyskinesia syndrome. Tardive dyskinesia syndrome is a collection of symptoms caused by bed rest response, immobilization and the provision of medical therapy and sedation. Patients had a decreased physical function of rapid movement of the arms and legs and 34 patients (82.92%) had cognitive impairment and poor sleep quality. [5]

Critically ill patients with impaired physical function have a picture of weakness of muscle quadriceps femoris, decreased strength and decrease in daily activities. Critically ill patients will experience

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mechanical unloading and decreased. neuromuscular activity. Patients critical during ICU neuromuscular activity. Patients critical during ICU will lose 20% of muscle volume, and 70% of protein for 1 week are admitted to ICU. The study also found 476,862 patients (60% -80% of the total critical patients in ICU) with 30% of them unable to return to work (nonproductive) due to loss of muscle strength of 1% -2% each day after patient out of ICU.[7–16] Critically ill patients with decreased physical and cognitive functioning are caused by various treatment measures and the accompanying illness. Patients with physical and cognitive impairment were caused by a history of using a mechanical ventilator (33%), infection or sepsis (50%), patients receiving treatment 2 days up to> 1 week in ICU (> 50%), delirium and critical illness or sepsis (70%), coronary heart disease (36.6%), CHD Unstable Angina (UA) (41.5%), Hypertension (19.5%), Supraventricular Tachycardia (SVT) (2.4 %). [5,7,8,17] The main causal factors causing it are long-term care (≥2 days) and minimal mobilization. Other causative factors include previous medical history (health status and previous disease history), acute illness, critical illness (delirium, hypoxia, hypotension, glucose dysregulation, respiratory failure, shock, CHF (Congestive Heart Failure), sepsis and others), severity diseases, inflammation, loss of muscle strength, sedation, and anxiety levels (communication dissatisfaction, sleep disturbances).[10,12,18–20] Critically ill patient decline in physical and cognitive functioning if not promptly prevented during ICU treatment may have an impact on increasing health problems when treated in the ICU and when out of the ICU. Critical patients with reduced physical and cognitive functioning if not promptly prevented during ICU, may have the effect of aggravating and weakening the function of other organs.

METHODS

This type of research is an experimental research with Randomized Controlled Trials (RCTs) research design. The intervention provided is physical-cognitive therapy. The intervention group received physical-cognitive therapy intervention, while the control group was not given physical-cognitive therapy. The sample criteria determinants are very helpful for the researcher to reduce the bias of the research result, especially if there are variables (control or confounding) which in turn have influence of the variables studied. Inclusion criteria include Patients who have been treated in ICU ≥ 24 hours, RASS -5 to +1, No visual disturbance, and hearing. Exclusion criteria include RASS +2, +3, and +4, Patients who change RASS values to +2, +3 and +4 when intervened or different days, Patients screening scores change during intervention, Patient forcibly return home or refer to another hospital, Patient dies, Initial assessment or ongoing intervention in patients is found with Cardiac Surgery, Neurodegenerative disease, Post cardiac arrest with suspected anoxic brain injury, Unstable fracture, long bones and open abdomen, Psychotic disorder. The population in the study were all critical patients treated at Kediri Baptist Hospital. Based on ICU RS. Baptist Kediri in May-June 2017 there were 267 patients treated in ICU. The samples were critical patients in ICU of Baptist Hospital Kediri as many as 64 critical patients according to inclusion and exclusion criteria. Independent variable in this research is physical-cognitive therapy. Dependent variable in this research is physical function and cognitive function. The research tool in this research is physical function measurement tool (PFIT) and cognitive function (MMSE). Data collection has been done after completing the research proposal. Researcher get ethical clearance from KEPK Medical Faculty of Diponegoro University, and Researcher apply research permission from Diponegoro University Semarang to Director of RS. Baptist Kediri. The Wilcoxon test was used to determine differences in cognitive-physical function before and after physical cognitive therapy in each group, whereas the Mann Whitney test was used to determine the posttest of cognitive-physical function between the intervention group and the control group. The value of confidence interval applied is 95% with significance level 5% ($\alpha = 0,05$).

RESULTS

Table 1. Respondent Characteristic

Characteristic	Group		Z	ρ Value (N=64)
	Intervention (n=32)	Control (N=32)		
Gender				
Male	19 (59,4%)	16 (50%)	0,74	0,455
Female	13 (40,6%)	16 (50%)		
Age ($\bar{x}$ ± SD)	59,9 ± 10,94	48,03 ± 11,4		0,000
12-16-Year-old	-	1 (3,1 %)	3,78	
26-35-Year-old	-	2 (6,4 %)		
36-45-Year-old	4 (12,5 %)	10 (31,3 %)		
46-55-Year-old	7 (21,9 %)	11 (34,4 %)		
56-65-Year-old	11 (34,4 %)	7 (21,9 %)		
> 65-Year-old	10 (31,3 %)	1 (3,1 %)		
Diagnose				
CHD -UA	5 (15,6 %)	10 (31,3 %)	1,01	0,312
CHD- OMI	11 (34,4 %)	7 (21,9 %)		
decomp cordis phase class III-IV	7 (21,9 %)	7 (21,9 %)		
HHF	3 (9,4 %)	3 (9,4 %)		
Pneumothorax	2 (6,3 %)	-		
Acidosis metabolic	1 (3,1 %)	-		
DKA	1 (3,1 %)	-		
COPD	1 (3,1 %)	-		
Asthma Attack Emergency	1 (3,1 %)	-		
Observation Ileus	-	2 (6,3 %)		
Stroke Hemorrhagic	-	1 (3,1 %)		
GEA	-	1 (3,1 %)		
Hyperglycemic	-	1 (3,1 %)		
RASS				
+1	1 (3,1 %)	3 (9,4 %)	2,06	0,039
0	16 (50,0 %)	19 (59,4 %)		
-1	-	4 (12,5 %)		
-2	10 (31,3 %)	6 (18,8 %)		
-3	4 (12,5 %)	-		
-4	1 (3,1 %)	-		
Sedation				
Yes	23 (71,9 %)	22 (68,8 %)	3,07	0,002
No	9 (28,1 %)	10 (28,1 %)		

Notes: [a]: Chi Square test, [b]: Mann- Whitney test; Z: Z count (Z table: 1,96); CHD: Coronary Heart Disease; UA: unstable angina; OMI: old myocardia infarct; HHF: Hypertension heart failure; DKA: diabetic ketoacidosis; COPD: chronic obstructive Pulmonary Disease; GEA: gastroenteritis acute.

Subjects in the intervention group were more than 50% male, while in the control group had a balanced amount between male and female sex. The subjects of the study in both groups had an average adult age to early elderly. Research subjects in the intervention group of less than 50% had diagnoses of OMI CHD while in the control group were CHD. Research subjects in the intervention group and control group found that more than 50% had a calm and alert awareness level (RASS 0). Majority subjects received sedation in the intervention group (71.9%) and control group (64%).

Table 2. Physical Cognitive Therapy Effect on Physical Function of Intervention Group and Critical Patient Control Group (n = 64)

Group	Negative Ranks		Positive Ranks		Z	ρ value
	Mean rank	Sum of Ranks	Mean rank	Sum of Ranks		
Intervention	0,00	0,00	32	528,00	-4,971	0,000
Control	4,50	36,00	0,00	0,00	-2,640	0,008

Description: ρ value: Wilcoxon test, significant (0,05), Z table = 1,96

The result of negative ranks test control group in mean rank is 4,50 and intervention group is 0,00, with each sum rank is 36 and 0,00. The result of positive ranks test result control group in mean rank is 0,00 and intervention group is 32, with each sum rank is 0,00 and 528.

Table 3. Differences of Physical Function Test Results between Intervention Group and Critical Patient Control Group (n = 64)

Group	Mean Rank	Sum Rank	U	ρ value
Physical Function			13,00	0,000
Intervention	48,09	1539,00		
Control	16,91	541,00		

Description: ρ value: Mann Whitney test, significant (0.05); U table: 105.

The result of the test result is the mean rank of the control group is 16,91 and the intervention group is 48,09, with each sum rank is 1539,00 and 541,00. The mean rank result is known that the physical function in the intervention group is better than the control group. The value of U arithmetic is (13 <105) with the significance of ρ value (0,000), which means that there is a significant influence difference between the physical function in the intervention group and the control group.

Table 4. Results of Differences in Cognitive Function Assessment of Intervention Groups and Critical Patient Control Groups (n = 64)

Group	Mean Rank	Sum Rank	U	ρ value
Cognitive Function			89,00	0,000
Intervention	45,72	1463,00		
Control	19,28	617,00		

Description: ρ value: Mann Whitney test, significant (0.05); U table: 105.

The result of test result is control group mean rank is 19,28 and intervention group is 45,72, with each sum rank is 1463,00 and 612,00. The mean rank result is known that cognitive function in the intervention group is better than the control group. The value of U arithmetic is (13 <105) with significance ρ value (0,000), which means that there is a significant effect difference between the cognitive function in the intervention group and the control group.

DISCUSSION

Physical Cognitive Therapy significantly affects physical function in critically ill patients in the ICU. The subjects of the study intervention group increased physical function after intervention with a mean difference of the increase in the intervention group of 3.2, whereas in the control group ecreased physical function with a mean of 0.2. The intervention group increased physical function because of physical exercise that is done properly and regularly. Physical exercise at each joint can increase the activity of mechanisms neuromuscular critical patients during bed rest. Physical activity done regularly prevents apoptosis activity. The control group decreased physical function due to a decrease in neuromuscular muscle debilitating up until the occurrence of cell apoptosis. Improved physical function occurs along with increased functionality and functional use of aid mobilization, step, shoulder strength, and the strength of the

knee. Physical-cognitive therapy is expected to be physiologically capable of activating mechanical neuromuscular patients, it is supported by the theory that in principle, the physical exercises to stimulate muscle nerves to recognize that when the patient bed rest does not happen mechanical unloading and decreased neuromuscular activity. The results of research supported by the theories Margaret that moment activity neuromuscular becomes better, it will inhibit the complex adaptation response (protein synthesis), protein degradation and apoptosis of muscle cells.[7–14] Mechanisms that occur are the main contributor muscle atrophy, loss of muscle strength in critically ill patients during bed rest. Physical-cognitive therapy is expected to increase muscle metabolism which further increases the formation of protein to energy solution for patient immobilization or bed rest. Physical-Cognitive therapy can improve physical function declined over the patient in the ICU, it was supported by the results of research Thomsen stated that ambulation and early mobilization in critically ill patients in the ICU were able to improve the patient's physical function and also decrease the use of sedation. [21] Critical patients in ICU should be done as soon as possible physical mobility exercises to improve muscle metabolism and does not activate a response or apoptosis mechanism. The results of research supported by Elliott in the prevention of damage to physical function after discharge from the ICU who stated that early mobility can mitigate the negative impact of critical illness and improved its physical function.[22]

Physical-cognitive therapy significantly impact on improving the cognitive functions of critical patients in ICU. These results correspond with the results of a study that critically ill patients in the ICU can experience mental health disorders such as anxiety and them have cognitive impairment and poor sleep quality.[5] Improved cognitive function was not affected characteristics of the study subjects from the intervention group. The decline in cognitive function is influenced also by gender in accordance with the statistical results and strengthened by the results of cognitive function decline Wreksoatmojo are motivated by a variety of risk factors that cannot be avoided such as age and gender, as well as some physical conditions and diseases.[23] The decline in cognitive function can slow recovery in patients. The research subjects in the control group was restless anxiety and pain scale settled on the first day to the third day.

The results also showed increased cognitive function occurred in all sub domains variable orientation, regression, attention-calculation, recall and language. Research shows that physical-cognitive therapy can improve the function of any existing variables. The results of research supported by the results of studies that suggest that cognitive therapy is able to change the perception of self in patients with heart problems.24 Research subjects most heart problems with a variety of conditions and consciousness and care in the ICU. The subjects of the study intervention group experienced an increase in all indicators of cognitive function. Cognitive function has several major functions which work is recertify function, memory function, the function of thinking and repressive function. This repressive function involves the ability to make the selection process, clarify and integrate the information provided. Researchers on the provision of physical-cognitive therapy provide the stimuli of orientation, registration, attention-calculation, recall until the language with the hope of the study subjects were able to do the selection process to integrate more complex information. The research subjects control group decreased cognitive function have a significant relationship to independence in accordance with the results of research conducted by Balquis.25 Subjects have been unable to carry out compliance activities of daily needs, and also experience pain in mild to moderate level. It also can affect the patient's condition, especially the condition of his illness. Based on the results that research subjects have the most control group cognitive impairment in moderate time in the ICU.

The research subjects in the control group with a decline in cognitive function may occur and demonstrate emotional response after discharge from the ICU such as anxiety, depression, fatigue, reflection and solitude in accordance expressed by Strahan.21 cognitive decline will worsen and weaken the function of other organs if not prevented in treatment in the ICU. 26 These results are also supported by another theory which states that the impact of decline in cognitive function for patients in ICU that increase the treatment time, a decline in cognitive function, physical function (organs, muscle contractility, functional capacity and

pain, vitality, fatigue), and worsening mental health (anxiety), emotional responses, depression, reflection, loneliness, inability to perform the activity and the use of instruments in everyday life. Condition of patients with worsening cognitive function for patients in ICU should be prevented to maintain the patient's quality of life and function as whole human beings with various functions in carrying out daily activities. Approach to symptom management theory indicated expected any problems can be overcome by a specific patient. Specific nursing interventions applied and overcome specific problems as well. The results also were able to study the possible factors that need to be improved in the provision of interventions, so as to provide maximum benefit to patients on the signs and symptoms of health problems in critically ill patients in the ICU.

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The Effectiveness of *Merremia mammosa (Lour.)* Extract Fractions as Diabetic Wound Healers on Diabetic Rat Model

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ABSTRACT

Introduction: Prevalence of diabetic ulcers in Indonesia ranges from 17.3 to 32.9% of hospitalized diabetes patients. Approximately 14-24% of them cannot be healed and require amputation. Treatment of diabetic ulcers is quite difficult, because of the failure of blood vessels and bacterial infection. *Merremia mammosa (Mm) (Lour.)* that contains flavonoids are thought to have potential antioxidant that helps the wound healing process. This study aimed to determine the effect of *Mm (Lour.)* extract fractions in wound healing process of diabetic rat model and searching the most potent fraction in 25 mg effective dose. The dose was proven effective in other plant fraction and smaller dose in our preliminary study was ineffective. **Method:** This experimental study used twenty-five male Wistar rats that were made diabetic by intraperitoneal injection of 40 mg/kg body weight streptozotocin. Rats divided into five groups, which consist of positive control (gentamicin 0.1%), negative control (aquadest) and *Mm (Lour.)* dose 25 mg each of n-hexane, ethyl acetate and water fraction. Wound was made by Morton method and treatment applied on the wound every other day for 10 days. Wound healing process were observed by calculating the percentage of reduction in wound size. Data were described and analyzed further using appropriate statistic tools. **Results :** The percentage of reduction in wound size comparison at day 11 of the excision showed n-hexane fraction group has 90,1% , ethyl acetate fraction group has 88,5% water fraction group has 93,4%, positive control group has 92,2% and negative control grup has 81,8%. It showed significant different in every fraction when compared with negative control and no significant different when compared with positive control. This study showed that among the three factions, water fraction showed the fastest healing rate (93.4 %). **Conclusion:** *Mm (Lour.)* extract fractions significantly accelerated the process of wound healing in diabetic rat model and the most effective fraction was water fraction. Therefore, it is potential to be developed further as a topical drug.

*Keywords**: Merremia mammosa (Lour), wound healing, diabetic ulcers***

Diabetic ulcer and gangrene diabetic is one of the diabetes mellitus complication. Prevalence of diabetic ulcers in Indonesia was 12 % of hospitalized diabetes patients and diabetes mellitus risk factors was 55.4%. Approximately 14-24% of them cannot be healed and require amputation. Treatment of diabetic ulcers is quite difficult, because of ineffectiveness inflammatory responses, endothel dysfunction, the failure of blood vessels and bacterial infection thus interfering wound healing process.14,17

Treating diabetic wound need comprehensive management which are blood glucose control, reduce offloading, keep the wound moisture and treat infection and also debridement of the wound. Highly cost, amputation requirement and the difficulty of diabetic wound care encouraging us to seek alternative treatment.16 One of the Indonesian medicinal plants have potential use for diabetic wound is Merremia mammosa (Mm) (Lour).11 Mm (Lour) has four chemicals component that needed in wound healing. There

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are four new resin-glycosides named merremosides A, B, H1 and H2 were isolated from the tuber of Mm (Convolvulaceae) has anti-inflammatory activity through COX1 and COX2 inhibitory.4 Mm (Lour.) has antidiabetic activity through dipeptidyl peptidase-IV (DPP-IV) inhibitory activity.11 Recent study show that ethanol extract of Mm (Lour) can decreasing blood glucose and accelerate wound healing of hyperglycemic rats by increasing fibroblast.2

Ethanolic extract of Mm (Lour) has some limitation because its contains all component of tuber bulb that gives wound healer effect or not. The amount of extract required is large (200mg) because the active ingredients are still mixed with other component that are not active. Fractionation or stratified purification of Mm (Lour) should be performed to increase the yield of the active ingredient and decrease side effect from the amount of non-active material. Fractionation can decrease the amount of non-active material contaminant in the extract to facilitate the formulation phase to improve the acceptability. The purpose of this study was determining the healing wound activity of fraction n hexane, ethyl acetate and water Mm (Lour) and standardize the most potent fraction of Mm (Lour) extract.

MATERIALS AND METHODS

Ethanolic Extraction method

The material used is the Mm (Lour) that has been identified in herbarium Jemberiense University of Jember. Two kg Mm (Lour) dried by airflow to get the simplicial, then mix and sieved to obtain simplicial powder. Simplicial powder extracted with ultrasonicator with 96% ethanol solvent for 1 hour. The extract produced was filtered with a Buchner funnel to obtain the filtrate and macerated three times. Filtrate concentrated with a rotary evaporator until a viscous extract is obtained.

Fractionation Method

Viscous extract is fractionated by partition using 3 different solvents of polarity i.e. n-hexane, ethyl acetate and water. 50 grams viscous extract added with 100 mL of water and stirred until homogeneous. This water fraction on the partition with n-hexane, ethyl acetate with comparison 2:3 three times. The partition evaporated with rotavapor to get n-hexane and ethyl acetate fraction. The water fraction concentrated with freeze dryer until viscous fraction is obtained7.

Diabetic rat model

This experimental study used twenty-five male Wistar rats aged 2 months. Rats were treated based on Helsinki convention. An ethical approval was obtained from the Ethical Committee of Facuty of Medicine University of Jember (No. 1.134/H25.1.11/KE/2017. After adapted in 7 days, rats were made diabetic by intraperitoneal injection of 40 mg/kg body weight streptozotocyn (STZ) dissolved in 0,05 mol/L buffer sitrat (pH 4,5)and given dextrose 10% in one night after injection of STZ to prevent hypoglycemic sudden death. Rat has positive diabetes if serum blood glucose were more than 200mg/dL on the fifth day after STZ injection. Blood glucose were measured once a week9.

Wound Excision

The diabetic wound was made by Morton modified method. Wound exicion made on the day after diabetic condition confirmed . Rats anesthetized with a combination of ketamine HCl dose of 50 mg / kg body weight and Xylazine dose 10 mg / kg body weight intramuscular. After anesthetic condition, excision was made until subcutaneous with an area of 2,5x2,5cm.6 The rats with non-infected wounds treated after one day of wounding. If there is pus or infection animal will be excluded. Treatment applied on the wound every other day for 10 days. Rats divided into five groups, which consist of positive control (gentamicin 0.1%), negative control (aquadest) and Mm (Lour.) dose 25 mg each of n-hexane, ethyl acetate and water fraction. After the completion of 11 days of topical application, the animals were subjected to wound examination and the percentage of reduction in the size of the wound was calculated5.

Measurement of percent wound healing

Wound margin was traced after wound creation by using transparent paper and the area was measured by graph paper. Wound contraction was measured every two days interval throughout the experimental period. Data were described and analyzed further using independent t-test statistic tools. Percent wound healing were observed by calculating the percentage of reduction in wound size.

Percent wound healing =

(initial wound size – specific day wound size) X 100% Initial wound size

Fraction standardization

Preparation of Mm (Lour.) extract using ethanol 70% with ultrasonication technique then continued fractionation phase with three different types of solvents ie water, ethyl, and n-hexane.

Fraction standardization has several parameters i.e. organoleptic examination, drying shrinkage, chromatogram profile and total flavonoid content. An organoleptic examination needs to be done to provide the identity of the fraction. Changes to this identity can be used to determine whether or not physical changes are extracted during the storage process. The chromatogram profile of the water content of upara bidara was carried out by TLC using eluent butanol, ethyl acetate and water ratio (6: 4: 1). The chromatogram profile is made by making payments to the elemation TLC plate at wavelength 254 using the TLC Scanner tool.

RESULTS

Induction of diabetes

The mean of body weight and non-fasting blood glucose level before and after diabetes induction were shown in figure 1. Mean of blood glucose before induction were 82,4mg/dL (negative control), 119,2 mg/dL (positive control), 92,4 mg/dL (dose 25 mg of ethyl acetate), 77,4 mg/dL (dose 25 mg of n-hexana), and 82,4mg/dL (dose 25 mg of water fraction). This result indicates that the initial condition of rats was normal. After STZ induction, mean of blood glucose were 472mg/dL (negative control), 472,4 mg/dL (positive control), 447,6 mg/dL (dose 25 mg of ethyl acetate), 450,2 mg/dL (dose 25 mg of n-hexana), and 444,6 mg/dL (dose 25 mg of water fraction). These results indicate that there is an increase of blood glucose level after induction with streptozotocin and rat had diabetic condition. Blood glucose were measured once a week and this hyperglycemia condition is still appeared until two weeks. It means that the diabetic rat models still in diabetic condition until the end of experiment.

Wound healing process

The rate of wound healing size and percent wound healing of the positive control group (gentamicin 0.1%), negative control group (aquadest) and Mm (Lour.) dose 25 mg each of n-hexane group, ethyl acetate group and water fraction group is shown in Table 1 and Figure 2. Water fraction group has 93,4% wound healing process when compared with negative control grup (81,8%) at day 11. Water fraction group has the smallest wound diameter (54 mm) when compared with control negative group (114 mm) and other fraction group at day 11.

Water Faction Standardization

Standardization parameters of the determined Mm (Lour) extract water fraction are organoleptic examination, drying shrinkage, chromatogram profile and total flavonoid content. The organoleptic parameters of water fraction examined the thick form, greenish brown, no odor and bitter taste. The loss on drying result showed 2.06% ± 0.13% w / w. The chromatogram profile of the Mm (Lour) water fraction was carried out by TLC using eluent butanol, ethyl acetate and water ratio 6: 4: 1. The chromatogram profile is made by scanning the TLC plate at wavelength of 254 using the TLC Scanner tool. Results of TLC prior to scan and after scan can be seen in Figure 1. Based on the image, the stain that appeared under the UV lamp were 5 stains with Rf respectively in sequence 0.706; 0.588; 0.459; 0.294 and 0.188. The scan results obtained 10 peaks with a larger area of 1000 AU (Figure 3).

Determination of chromatogram profile is used to provide an overview of the compounds contained in the water fraction. Analysis of the flavonoid and terpenoid compounds was also done by using TLC and the ammonia and anisaldehyde sulphate vapor smears. The results of the content analysis showed that the water fraction of Mm (Lour) extract contains no terpenoid but contains flavonoids. Figure TLC water fraction of Mm (Lour) extract after stained with ammonia vapor and anisaldehid sulphate dye can be seen in Figure 3.

Table 1. Wound size and precent wound healing*

Days	C(-)		C(+)		FE		FN		FW	
	Wound size	Percent wound healing	Wound size	Percent wound healing	Wound size	Percent wound healing	Wound size	Percent wound healing	Wound size	Percent wound healing
1	452.4	27.6	434.8	30.4	493.3	21.1	411.5	34.2	434.0	30.6
3	347.4	44.4	309.6	50.4	326.6	47.7	318.8	48.9	353.5	43.0
5	208.5	66.6	208.0	66.7	293.5	53.0	244.3	60.9	195.4	68.1
7	197.8	68.3	121.6	80.5	173.8	72.2	135.4	78.3	116.8	81.0
9	164.0	73.6	92.4	85.2	110.6	82.3	109.0	82.3	62.0	90.0
11	114.0	81.8	49.2	92.2[b]	71.8	88.5[a]	61.6	90.1[a]	54.0	93.0[b]

*All data are expressed as mean in millimeter for wound size and mean in percent for percent wound healing. C(+): Gentamycin 0.1%, C(-): Aquadest, and FE, FN, FW are ethyl acetate, n-hexane, water fraction of *Merremia mammosa (Lour).* [a]$p<0.05$ or [b]$p<0.001$ v.s. negative control (aquadest) at day 11 using unpaired Student T test.

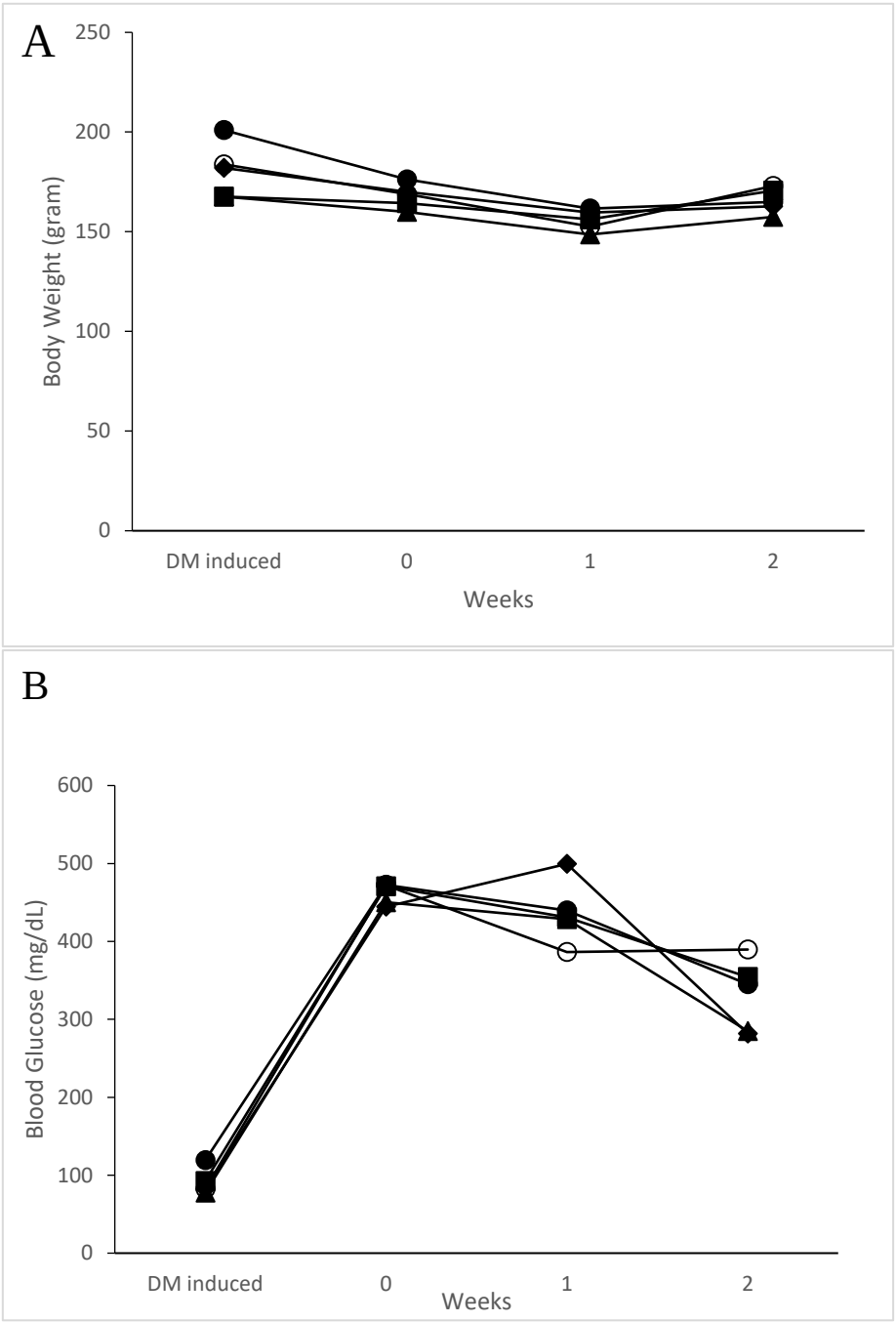

Figure 1. Body weight and non-fasting blood glucose level during the experimental period. Mean values of body weight (A), non-fasting blood glucose (B) and percent wound healing (C) are shown. Opened and closed circles represent negative and positive control. Closed square, triangle and diamond represent groups of *Merremia mamossa (Lour)* extract fraction dose 25 mg of ethyl acetate, n-hexane and water fraction in a sequent.

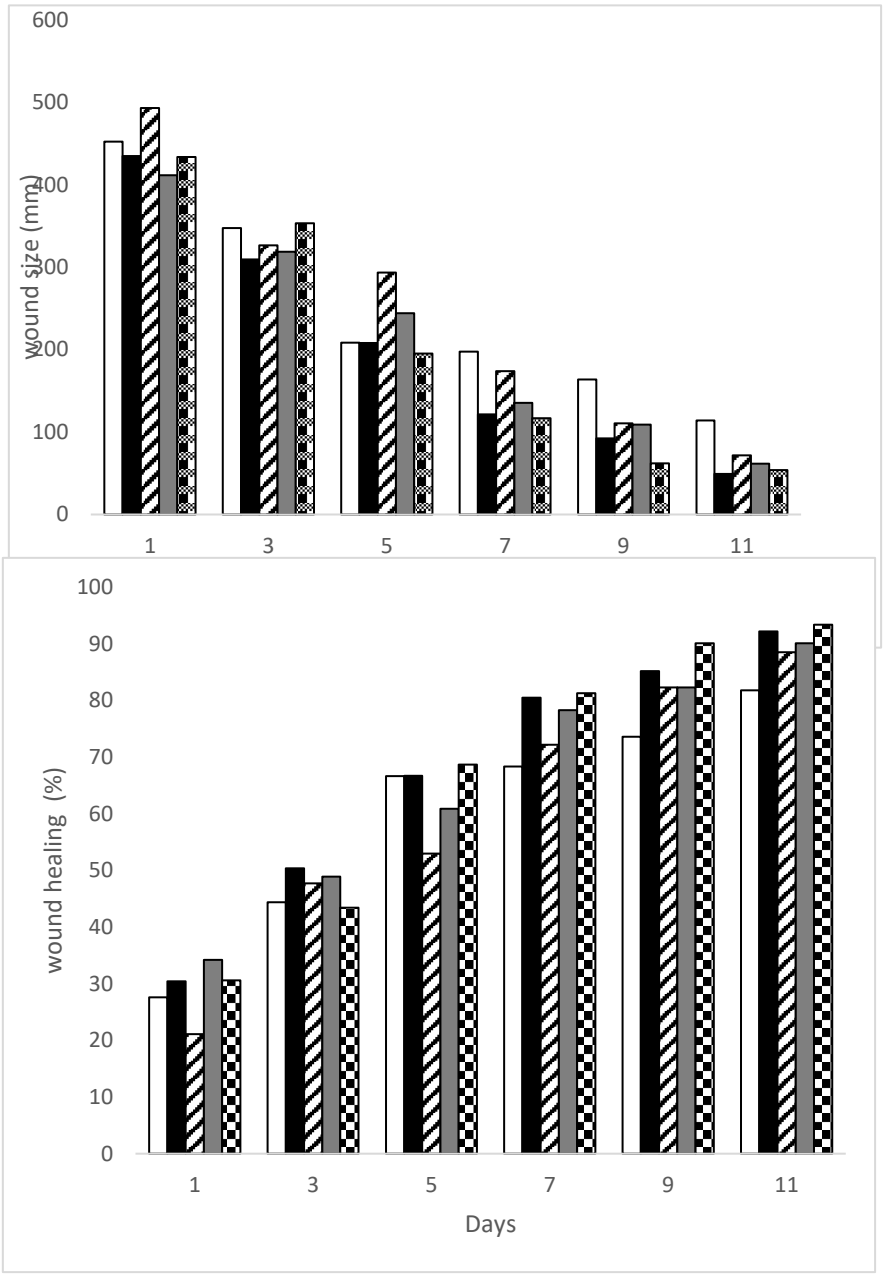

Figure 2. Wound size and percent wound healing during the experimental period. All data are expressed as mean in millimeter for wound size and mean in percent for percent wound healing. *Opened column*, negative control; *closed column*, positive control; *hatched, gray* and *cubed column* are ethyl acetate, n-hexane and water fraction of *Merremia mammosa (Lour).*

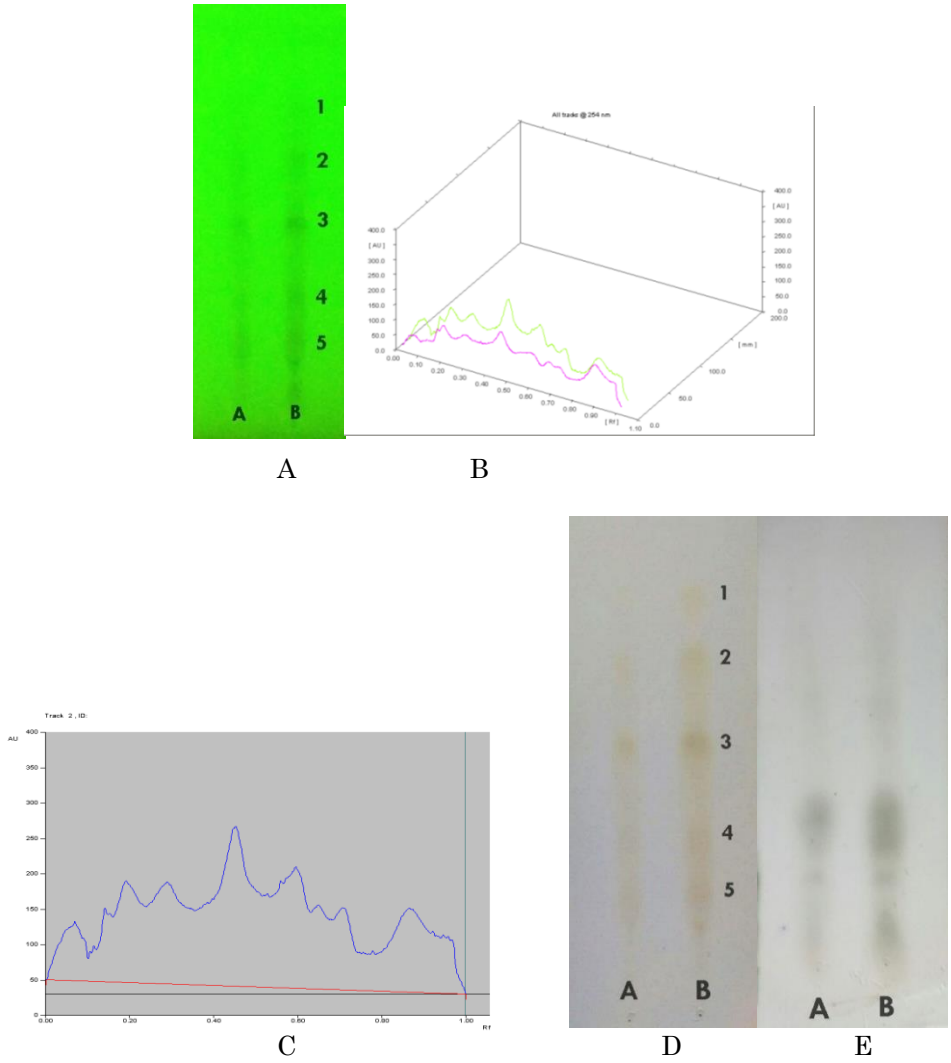

Figure 3. TLC chromatogram profile of Mm (Lour) water fraction using silica gel stationary phase, butanol mobile phase: acetic acid: water (6: 4: 1). (A,B,C); TLC results of water fraction of Mm (Lour) extract with butanol mobile phase: acetic acid: water (6: 4: 1), A. 2 uL Band; B. 4 uL Band. Ammonia vapor (1) and anisaldehyde sulfate (2) smear.

DISCUSSIONS

Diabetic ulcer is one of diabetes complication. In this study, we use 40 mg / kg body weight STZ to make a diabetic rat model. This dose has been successful in making the animal experience hyperglycemia and persisted until the end of this experiment in the second week. After STZ injection, body weight showed a slightly decreased in all group and then increased gradually, this is relevant with DM pathogenesis.6,9 STZ are diabetogenic agent that selectively beta cell pancreas toxicity. STZ will be uptakes the langerhans beta cell pancreas by GLUT2 and make beta cell pancreas damage so its failed to produce insulin resulting in increased blood glucose levels.1 Excisional wound is most suitable method in wound research due to the broader morphological changes during healing process. Wound size is an essential parameter to assess wound closure. Wound closure shows better re-epithelialization process during healing process.

The ethanol extract Mm (Lour) was partitioned using two solvents having different polarity levels i.e. n-hexane, ethyl acetate and water. It is aimed to separating the group of low-polymer compounds into n-hexane solvents, the group of compounds whose polymer is solvent of ethyl acetate and the high polarity to water. In this present study, treatment with water fraction resulted in faster wound healing process and smallest wound diameter compare to negative control and other fractions at day 11. This study showed that among the three factions, water fraction showed the most effective fraction for diabetic wound healing process. This is suggest that healing process occurs to be due to presence of glycoside flavonoid that have anti-

inflammatory effect more dissolved in the water fraction than with other fraction. It is because the flavonoid is a polar compound so it more easily soluble in the polar solvent fraction of water. This means that the solubility of an active material depends on polarity of solution, the active material of this compound dilutes mostly in water fraction..10,13Because of water fraction is most effective, to prove the existence of flavonoid compound in water fraction, standardization of water fraction of Mm (Lour) was done.

An organoleptic examination needs to be done to provide the identity of the fraction. Changes to this identity can be used to determine whether there were extract fraction physical changes or not during the storage process. Physical changes are generally followed by chemical changes that may affect the efficacy of the extract.13 Examination of loss on drying aims to determine the amounts of volatile compounds in the fraction. The loss on drying rate is determined using gravimetry. The loss on drying result showed 2.06% ± 0.13% w / w. This shows that from 100 grams of extract there are 2.06 grams of compounds lost during the heating process. The compound lost in the water fraction of this Mm (Lour) extract is probably water. Low water levels will prevent the growth of microorganisms and molds. Water content that exceeds 10% can cause the extract will easily overgrown with fungi.3

Determination of total flavonoid content of Mm (Lour) water fraction using colorimetric method of aluminum chloride with quercetin as comparison. Quercetin is a flavonoid of a flavonolol group that has a keto group on C-4 and a hydroxy group on C-3 or C-5 atoms. The maximum wavelength resulting from the measurement of quercetin is 438 nm. The quercetin regression line for this study was y = 0.0121x-0,0934 with r = 0.948. Flavonoid levels with AlCl3 method were 0.17 ± 0.009% w/w. Flavonoids are polyphenol compounds found in plants. Flavonoids have various activities including antioxidants and anti-inflammatory. Based on this result, it is indicated that the water fraction of Mm (Lour) contain flavonoids

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Positive Correlation between Ferritin and Activated Monocyte in Iron Overloaded Major β-thalassemia Patients

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ABSTRACT

Introduction: Regular blood transfusion for ß-thalassemia patients is a life-saving therapy, hence, it results in iron overload lead to immune dysregulation triggered by chronic activation of immune system. This fundamental notion contributes to their morbidity and mortality. Monocyte plays a critical role in regulating and bridging innate to adaptive immunity. Our pilot study analyzed the presence of activation markers, CD14 and CD69, on monocyte of major ß-thalassemia patients associated with their iron status**. Method:** Fifty pediatric ß-thalassemia patients routinely visited thalassemia clinic for clinical examination and blood transfusion were involved in this cross-sectional study. Flow cytometry applying antibody of CD14, HLA-DR, CD69 was used to dissect $CD14^+CD69^+$ monocytes from lysed-erythrocyte heparinized whole blood and defined as cell percentage also median fluorescent intensity (MFI) of CD69 of $CD14^+CD69^+$ monocytes. Iron status was indicated by ferritin and serum iron level. A correlation study was done. **Results :** We found 87.4% (76.1 – 91.4) CD14+CD69+ of dissected monocytes from iron overloaded pediatric ß-thalassemia patients (Ferritin level: 3118 µg/L, 1675 – 9718). Positive correlation was found between percentage of $CD14^+CD69^+$ monocytes and ferritin level ($r = 0.3$, $P = 0.04$). **Conclusion:** Considering the function of CD14 and CD69 on monocyte and the iron accumulation, our result may implicate that pediatric major ß-thalassemia patients have a tendency towards chronic inflammation. Future direction for research of our study aimed at discovering collateral activation of immune cells via monocyte to explain organ damage caused by iron overload is imperative.

Keywords: Monocyte, CD14, CD69, iron, β-thalassemia

INTRODUCTION

Beta (ß)-thalassemia is a blood genetic disease caused by the alteration of beta chains of hemoglobin protein synthesis distributed worldwide according to thalassemia belt where Indonesia was included [(1)]. The major type of this disease depended on regular blood transfusion to overcome the anemia caused by accelerated hemolysis and ineffective erythropoiesis [(1)]. Nowadays, major ß-thalassemia patients have a convenient access to the definite therapy, therefore increased their life expectancy. However this fact resulted into an inevitable iron overload condition which is related to persistent inflammation and increased risk for infection [(2, 3)].

Monocyte is a professional innate sentinel cell that participates in various inflammatory conditions through activation and recruitment in response to many cytokines. In addition, this cell bridges innate and adaptive immunities that also play a major role in iron regulation [(4)]. These integrated activities of monocytes can be studied by identifying and measuring their membrane protein. Previous study has showed that monocyte of thalassemia patients underwent hyperplasia and is hyperactive however demonstrate the low phagocytic activity in inflammation insult [(3)], however how monocyte was activated and therefore started the inflammation cascade in thalassemia patients need to be further elaborated.

CD69 is a C-type transmembrane lectin which is constitutively expressed in monocytes. Increase of which has been dubbed as an activation indicator of this cell [(5-7)]. All these studies lead us to think that there is an immune alteration caused by the constant state of iron overload. We hypothesize that monocytes are affected by the

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constant state of iron overload and activating the monocyte via this protein.

METHODS

Study Design, participants, and procedure

A study implementing cross sectional and analytic design involving 50 pediatric major ß-thalassemia patients that routinely visited the Thalassemia Clinic of Hasan Sadikin General Hospital, Bandung, West Java was done. Inclusion criteria were ß-thalassemia sufferers aged under 15 confirmed by genetic examination and had been transfused at least 2 years or more. Exclusion criteria were diabetes, autoimmune, cancer, tuberculosis, chronic infections such as HBV and HIV, and immunomodulatory treated patients.

Ethics

Ethics of this study was made in accordance to with Faculty of Medicine Universitas Padjadjaran and Hasan Sadikin General Hospital. The study was approved under the approval number of 74/UN6.C1.3.2/KEPK/PN/2016 and LB.02.01/C02/15691/XI/2016 for Faculty of Medicine and ethics committee of Dr. Hasan Sadikin Hospital respectively.

Laboratory Procedures

Characterization of CD69 monocytes

Blood was drawn from 50 patients through venipuncture and placed in vacutainer with lithium and sodium heparin. The blood was stored for 1 hour in room temperature before being analyzed. The tube that contains 200µL of heparinized blood was treated with 2000µL of PBA 0.5% and then vortexed/centrifuged for 5 minutes without break at 1500 rpm. The suspension is vortexed in FACS buffer with the antibodies, incubated for 20 minutes at 2-8^{o}C, covered by aluminum foil, given ten –time diluted red cell lysing buffer, incubated again for 12 minutes, and lastly washed with 2000µL PBA 0.5% and suspended with 200µL PBA 0.5%.

After preparations were done, the samples were put into flow cytometer. The antibodies used were HLA-DR, CD14, and CD69 antibodies (All from BioLegend, San Diego, CA, USA). Cells were read according to their phenotypic marker by BD Cell Quest Pro Software (Biosciences, San Jose, CA, USA) for 500,000 events, then the FCM output files were analyzed using FlowJo 10 (Tree star, USA). The monocytes were distinguished from granulocytes and lymphocytes with forward and side scatter, HLA-DR, and CD16 subsequently. The gated result was analyzed with CD14/CD69. The percentage that positively express CD69 and the mean fluorescent intensity (MFI) was recorded.

Hematology assessment: Complete Blood Count (CBC)

Vacutainer containing potassium EDTA (Becton Dickinson, Franklin Lakes, New Jersey, USA) was used for CBC. Automatic hematology analyzer (Sysmex Corp., Japan) was employed to measure hemoglobin (Hb), leukocyte, monocyte, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC).

Iron status measurement

Sera were extracted were analyzed for iron status. Serum iron and ferritin kit, The Elecsys ferritin immunoassay kit (Roche, Switzerland), was used to get ferritin concentration. All data was analyzed with a correlation study. Serum iron assay kit (Merck, Singapore) was used to measure serum iron.

Statistical analysis

Data with non-normally distribution are presented as median with interquartile range (IQR), while normally distributed data as mean with standard deviation (SD). Correlation between parameters was tested using Spearman correlation coefficient for non-normally distributed data, and Pearson correlation coefficient for normally distributed data. All analyses were performed with GraphPad PRISM version 7.0 (Graphpad Software, Inc., La Jolla, CA, USA). $P \leq 0.05$ is considered statistically significant.

RESULTS

Identification of CD69 monocytes

The $CD14^{+}CD69^{+}$ monocyte population result was depicted in figure 1. The selection of monocyte began by distinguishing the monocyte population according to size and granularity. Positive gating of the selected population employing HLA-DR and CD14 on monocyte membrane expression was used to identify true monocytes. Further, $HLA\text{-}DR^{+}CD14^{+}$ monocytes were selected. The final result, $CD69^{+}$ monocytes of true monocytes were counted also the MFI of CD69 expression was measured as showed in table 1.

Hematologic characteristic

Hematologic data acquired which depicted in Table 2 showed an excess of serum iron and ferritin content in these patients.

Correlation result

Results show ferritin is a significant positively correlated with $CD14^{+}CD69^{+}$ monocytes of total monocytes and $CD14^{+}HLA\text{-}DR^{+}$ monocytes. These p values are significant ($p<0.05$).

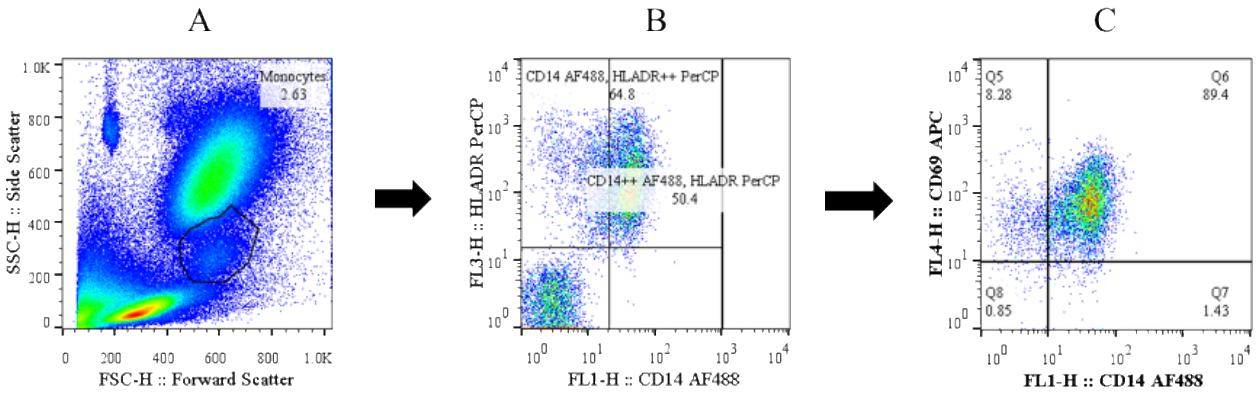

Figure 1. Identification of blood monocyte subset applying multicolor flow cytometry. Gating strategy for monocyte subsets identification presenting successive inclusion of monocytes population. (A.) Identification of monocyte subpopulation in blood. (B.) Selection for "true" monocytes by gating on CD14 positive and HLA-DR positive population. (C.) Remaining population was further discriminated on a CD14 vs. CD69 scatterplot to give CD14+CD69+ monocytes.

Table 1. Population of Monocytes

	Patients value
$CD69^{+}CD14^{+}$ monocyte (%) (Mean)	39.02
Median MFI of CD69 of $CD14^{+}CD69^{+}$ monocyte	80.2

Table 2. Correlation of monocyte characteristics in pediatric ß-thalassemia patients

Parameter		**Value**
Serum iron	CD14+/CD69+ of Total monocytes	r0.121
		p0.404
	CD14+/CD69+ of CD14+/HLA-DR+	r0.044
		p0.759
	Mean MFI for CD69+ of CD14+ /CD69+	r-0.004
		p0.978
	Median MFI for CD14+/CD69+	r-0.021
		p0.887
Ferritin	CD14+/CD69+ of Total monocytes	r0.278
		p0.050
	CD14+/CD69+ of CD14+/HLA-DR+	r0.291
		p0.040
	Mean MFI for CD69+ of CD14+ /CD69+	r0.012
		p0.934
	Median MFI for CD14+/CD69+	r0.015
		p0.916

Table 3. Clinical Characteristics

	Patients' value	Normal value
Gender		
Male, n (%)	25 (50)	Not Defined
Female, n (%)	25 (50)	Not Defined
Mean age (SD), year	8 (3.0)	Not Defined
Hematological assessment		> 29
Mean Hb (SD), g/dL	6.5 (1.2)	
Median leucocyte (IQR), /mm^3	6000 (4000-8100)	10.9-14.9
Median monocyte count (IQR), %	6 (5-7)	4500-14500
Median MCV (IQR), fl	75.6 (71.2-78.6)	
Mean MCH (SD), pg/cell	25.5 (2.7)	79-98
Mean MCHC (SD), g/dL	34.3 (1.6)	25-33
Iron status		32-36
Median ferritin (IQR), µg/L	3118 (1675-5009)	
Mean serum iron (SD), µg/dL	163.5 (60.4)	< 1000
		35-150

DISCUSSION

Monocyte has pivotal function in bridging the interplay between innate and adaptive immune cells in the progression of inflammation. Activated blood circulating monocytes are recognized by the expression HLA-DR protein on their cell membrane, while the pro-inflammatory ones are when they are expressing CD14 and CD69 [6, 8-10]. These facts suggested that in major ß-thalassemia patients burdened with iron overload complication the existence of $CD14^+CD69^+$ monocyte play important role in the pathogenesis of persistent inflammation happened in major ß-thalassemia.

The results acquired shows that there is a moderate positive correlation between ferritin level and the percentage of $CD14^+CD69^+$ monocyte population. Monocyte activation can be shown via the CD69 protein. This protein is encoded in chromosome 12p13. The cross-linking of this protein increases monocyte in producing prostaglandin, leukotriene B4, nitric oxide, and extracellular calcium influx [5-7, 11]. These functions implicate CD69 as a pro-inflammatory protein on monocyte. CD69 has shown increase in persistent inflammatory conditions, such as sarcoidosis and HIV. It is implicated in activation of monocytes, in which it is also tied to an induction of TNF-α release by macrophages [12]. The activation process or status in monocytes has also been related to more release of cytokines but less on phagocytic function. Studies showed a downgrading effect of monocyte activation making phagosomes less efficient [13, 14].

Iron may be significant to monocytes because the life cycle of erythrocytes ends in phagocytosis. Release of iron and storage of iron in the form of ferritin is exactly why monocytes can be affected. To manage iron content, cells such as monocytes express proteins such as DMT (Nramp2) to internalize iron. Free iron has to be controlled not just because of its dangers but also because of its benefits to microbes. In conditions of iron overload, free iron that is very toxic to the cells and tissues is formed and may continue to rise and catalyze the production of radical OH^- substances from peroxide molecules, known as the Fenton reaction. At the final phase, this condition endangered cell function by damaging their biomolecules and inflammation. This study shows excess of iron indicated by higher ferritin and serum iron level.

As iron levels rise in the blood, hepcidin need to create a waste disposal environment rather than keeping the balance. Thus, hepcidin functions to keep the internalizer, which is DMT, but turns off the externalizer, which is ferroportin. These findings in iron overloaded patients is why monocytes can become a reservoir for iron and hence became intoxicated by it. To prevent that, iron has to be contained in its more stable form which is incorporated into ferritin. However, this mechanism most likely to be imbalanced. May be this is why there is a correlation between ferritin content and the CD69 protein. But, since CD69 is indicated by many as an "activation" marker, and a part of the pro-inflammatory mechanism, how is it related to iron?

Our humble suspicion is that the main link between iron and activation in monocytes might be NFκB. A study in kupffer cells in the liver showed that iron in its ferrous form blocks IkBα, increased the number of p65 and p65/p50 binding to DNA, and it is a direct agonist to the IKK. These evidence may be the tying knot between iron and the activation of monocytes because it

shows a preference to the activation of NFκB canonical pathway, and CD69 may be a part of this pathway.

As many substances triggered by the canonical pathway is activated, an inflammatory state is created. The canonical pathway may affect the behavior of the monocytes because of the constant activation. High iron content, and hence the high ferritin content may just be the reason why the canonical pathway is more active and hindering the balance because the alternative pathway is constantly off. The imbalance in immune function may just be the reason why there is an increased susceptibility to infections and hence increased mortality rate in patients with iron overload.

CONCLUSION

Considering the function of CD14 and CD69 on monocyte and the iron accumulation, our result may implicate that pediatric major ß-thalassemia patients have a tendency towards chronic inflammation. Thus $CD14^{+}CD69^{+}$ monocytes are implicated as marker of chronic inflammation in patients suffering from iron overload and early identification by this study makes it even more pronounced. Many more studies are needed to increase our understanding of the mechanisms with which iron overload causes immune dysregulation. Future direction for research of our study aimed at discovering collateral activation of immune cells via monocyte to explain organ damage caused by iron overload is imperative.

Disclosure

M.Ghozali and M. Fariz Anggia are co-first authors

Competing Interests

Authors declare that they have no competing interests.

Author's Contributions

M. Ghozali, M. Fariz Anggia, Adi Imam Tjahjadi, Lelani Reniarti, Reni Ghrahani, MRAA. Syamsunarno, Budi Setiabudiawan, and Ramdan Panigoro conceived the study and participated in the design and data analyses. M. Ghozali and M. Fariz Anggia were involved in data acquisition. All authors contributed towards drafting and agree to be accountable for all respects of the work. M. Ghozali, Adi Imam Tjahjadi, Lelani Reniarti, Reni Ghrahani, MRAA. Syamsunarno, Budi Setiabudiawan, and Ramdan Panigoro critically reviewed the manuscript. All the authors read and approved the manuscript.

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The Sensitivity of Human Breast Cancer Stem Cells (ALDH+) Against Doxorubicin Treatment is Associated with PCNA and BIRC5 Gene Expressions

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ABSTRACT

Introduction: Breast cancer stem cells (BCSCs) are identified as side populations in breast cancer cells owing stem cell properties and tumorigenic characteristics. Previous studies revealed that breast cancer chemotherapy led to BCSC enrichment which contributed to therapy resistance. Our recent *in vivo* study using Next Generation Sequencing has demonstrated that PCNA - the proliferative gene - and BIRC5 – the anti-apoptosis gene – were under-expressed in human breast tumors after neoadjuvant chemotherapy. This study aimed to verify the role of PCNA and BIRC5 expression in doxorubicin-treated human BCSCs *in vitro* and its association with cell viability. **Method:** Human BCSCs (ALDH+) were treated with 0.25 uM of doxorubicin for 2, 4, 6, 8, 10, 12, 14 days respectively. Cell viability was measured using trypan blue exclusion assay and the expressions of PCNA and BIRC4 mRNA were determined using qRT-PCR. **Results:** This study demonstrated that the viability of ALDH+ BCSCs decreased after 2 days and increased again after 8 days of doxorubicin treatment, indicating the decrease of doxorubicin sensitivity. Interestingly, PCNA and BIRC5 genes were modulated in line with the modulation of cell viability during doxorubicin treatment of human BCSCs. **Conclusion:** In conclusion, we suggest that the PCNA and BIRC5 expressions play an important role on the BCSCs viability which associated with the sensitivity of doxorubicin treatment.

Keywords: human breast cancer stem cells, cell viability, PCNA, BIRC5, doxorubicin

Breast cancer is the most death-causing cancer among women all over the world due to therapy resistance and disease recurrence. Nowadays, neoadjuvant therapy is established for larger primary and locally advanced breast cancer as a preoperative approach. The advantages of neoadjuvant therapy is to downstaging the tumor, reducing the extent surgery and also evaluating the efficacy of the drugs that will be administered.(1) Yet, classic breast cancer therapy has not considered the presence of side populations, known as breast cancer stem cells (BCSCs), that are characterized by self-renewal, differentiation, and tumorigenic capabilities, similar to normal stem cells.(2)

Current treatments for breast cancer frequently fail to eradicate tumor since they are inable to effectively target BCSC populations. BCSCs have been proved to exhibit significant resistance to conventional chemotherapy. Furthermore, breast cancer chemotherapy may lead to BCSC enrichment which contributed to therapy resistance. It has been demonstrated that BCSC markers (CD44+/CD24-) were expressed abundantly after neoadjuvant chemotherapy of primary breast cancer patients.(3) Some previous studies have also reported that BCSCs are responsible for therapy resistance, recurrence and metastasis in breast cancer.(4)·(5)·(6) The presence of BCSC populations has been correlated with poor prognosis in breast cancer patients.(7)

Very recently, we have applied Next Generation Sequencing to assess the alteration of p53-pathway gene expressions in breast cancer cells from patients underwent neoadjuvant chemotherapy. We found that seven gene expressions in p53-pathway were significantly down-regulated after neoadjuvant chemotherapy of breast cancer patients, namely ATM, BID, BIRC5, CASP8, CASP9, CDK1 and PCNA.(8) Proliferating cell nuclear antigen (PCNA) is

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known as a molecular marker for cell proliferation, thereby it has an important role in replication. The function of PCNA is indispensable for the maintenance of genomic integrity and propagation in actively growing cells.(9) BIRC5 or survivin is a member of the inhibitor of apoptosis (IAP) protein family that suppresses caspase-9 activity leading to apoptosis inhibition. This protein is highly expressed in most cancers and is associated with a poor clinical outcome. (10) Our previous study has demonstrated that BIRC5 was highly expressed in BCSCs after oxidative stress induction and led to high cell survival.(11)

The present study aimed to verify the role of PCNA and BIRC5 expressions on the sensitivity of human BCSC against chemotherapy. We analysed the decrease of BCSC sensitivity against doxorubicin by determining cell viability in association with PCNA and BIRC5 expressions in human BCSCs (ALDH+) after repeated exposure to doxorubicin treatment. The result of this study is consistent to our previous next generation sequencing analysis, suggesting that PCNA and BIRC5 could be used as markers for determining chemotherapy sensitivity, particularly in BCSCs.

MATERIALS AND METHODS

Cell culture

Human BCSCs (ALDH+) were grown in serum free DMEM F12 medium with 1% penicillin/streptomycin and 1% amphotericin B and incubated in 5% CO2 at 37˚C.

Cytotoxic Assay

MTS assay was performed to determine cytotoxicity of human BCSCs. About $1x10^3$ human BCSCs (ALDH+) were plated triplicate in each well of 96-well plate and grown in non-serum DMEM/F12 medium supplemented with 1% penicillin/streptomycin and 1% amphotericin at 37^0C and 5% CO_2. After 24-incubation, cells were treated with various concentrations of doxorubicin (0.1, 0.5, 1, 5, 10 and 20 µM) for 24 hours. As controls, cells were grown without doxorubicin under standard conditions for 24 hours. Afterwards, medium was replaced with 100 µl fresh DMEM/F12 medium containing 20 µl MTS/PMS (20:1) and incubated for 1 hour. After the brown colour has appeared, the absorbance was read at 490 nm using microplate reader (Varioskan®, Thermo Fisher Scientific USA). The absorbances of doxorubicin-treated cells were normalized to that of control cells to calculate the percentage of cell viability.

Doxorubicin treatment

Approximately $1x10^5$ cells were plated each well in12 well-plate and incubated overnight. Afterwards, medium was replaced with fresh DMEM/F12 medium containing doxorubicin of 0.25 µM for 2, 4, 6, 8, 10, 12, and 14 days respectively. As controls, cells were grown in DMEM/F12 without doxorubicin for 2, 4, 6, 8, 10, 12, and 14 days respectively. After incubation, cells were harvested and counted using trypan blue exclusion assay and automated cell counter (Luna®, Logos Biosystems Korea). The number of viable doxorubicin-treated cells was normalized to that of the respective control cells to calculate the percentage of cell viability. Total RNA and isolated from the cells using Tripure® isolation reagent kit (Roche) according to the manufacturer's instructions.

Quantitative RT-PCR

The concentration of total RNA was measured using spektrofluorometer (Varioskan, Thermo Fisher Scientific USA). One-step quantitative RT-PCR was carried out using ECO48® real time qPCR system (PCRmax UK). The RT PCR was performed in 20 µL volume with 100 ng total RNA. The primer sequence for quantitative RT-PCR are listed in table 1. PCR cycles are 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, 55□C for 30 seconds, 72□C for 30 seconds, and melting curve incubation.

Relative expression was relatively calculated using Livak formula(12) with 18S rRNA gene as a reference gene and normalized to its respective control.

RESULTS

Cytotoxic Assay

Cytotoxicity of doxorubicin treatment on human BCSCs (ALDH+) was determined using MTS assay. The 50% cytotoxicity concentration (CC50) was calculated using cytotoxicity curve generated by logarithmic regression equation ($y= -9.572\ln(x) + 62.291$). As shown in Figure 1, CC50 value of doxorubicin on human BCSCs was 3.63 µM. Based on this data, we used doxorubicin concentration lower than CC50 for all experiments.

Cell Viability

Percentage of cell viability was determined by comparing the number of viable doxorubicin-treated cells with that of control cells. Figure 2 demonstrates that BCSC viability after 2-day doxorubicin treatment decreased (66%), however

started to increase on day 8 (81%) until reached 92% viability on day 14.

Relative mRNA expression of PCNA and BIRC5 after doxorubicin treatment

To analyze the role of PCNA and BIRC5 on the sensitivity of BCSCs against doxorubicin, we examined the relative expression of PCNA and BIRC5 mRNA following 2-, 6-, 10-, and 14-day doxorubicin treatment. Compared to each respective controls, PCNA mRNA expression was significantly decreased after 2-day treatment (0.787-times; p=0.040), but slightly increased on day 10 (1.337-times; p=0.236) and day 14 day of treatment (1.437-times; p= 0.169) (Fig. 3). PCNA relative expressions in BCSCs treated with doxorubicin for 10 and 14 day was significantly higher compared to that with 2-day treatment (p=0.016 and p=0.011 respectively, One-way ANOVA test). Relative expression of BIRC5 mRNA in human BCSCs treated with doxorubicin was also modulated in a similar manner to PCNA expression. It was decreased after 2-day treatment of doxorubicin (0.846; p=0.089), but started to increase on day 10 (1.257; p= 0.140) and day 14 of treatment (1.530; p=0.094) compared to each respective controls (Fig. 4). Although there were no significant differences compared to controls, the increase of BIRC5 relative expression in BCSCs after 10- and 14-day doxorubicin treatment was significantly higher compared to 2-day treatment (p=0.027 and p=0.001, One-way ANOVA test).

Table and Figures

Table 1. Primer sequence of PCNA, Survivin and 18S rRNA

No.	Gene	Primer Sequence	Poduct
1.	PCNA	F: 5'- CTT CCC TTA CGC AAG TCT CAG -3'	189 bp
		R: 5'- TTG AGT GCC TCC AAC ACC TT -3'	
2.	Survivin	F: 5'- AGG ACC ACC GCA TCT CTA CA -3'	186 bp
		R: 5'- GTT CCT CTA TGG GGT CGT CA -3'	
3.	18S rRNA	F: 5'- AAA CGG CTA CCA CAT CCA AG -3'	155 bp
		R: 5'- CCT CCA ATG GAT CCT CGT TA -3'	

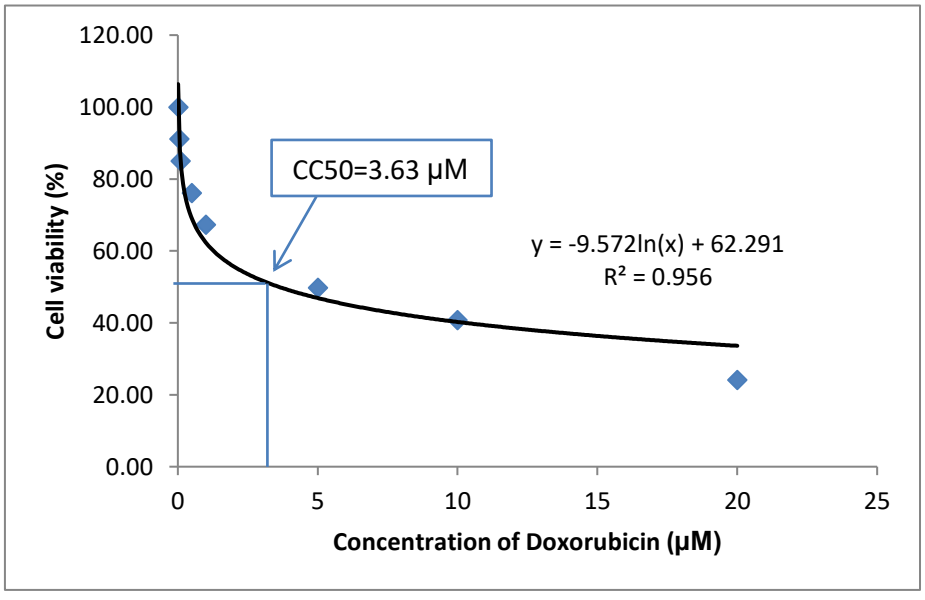

Figure 1. Cytotoxic assay of doxorubicin-treated BCSCs. MTS assay was performed to determined cytotoxicity of human BCSCs. About 1x10^3 human BCSCs (ALDH+) were plated triplicate in each well of 96-well plate and grown in non-serum DMEM/F12 medium supplemented with 1% penicillin/streptomycin and 1% amphotericin at 37^{0}C and 5% CO_2. After 24-incubation, cells were treated with various concentrations of doxorubicin (0.1, 0.5, 1, 5, 10 and 20 µM) for 24 hours. As controls, cells were grown without doxorubicin under standard conditions for 24

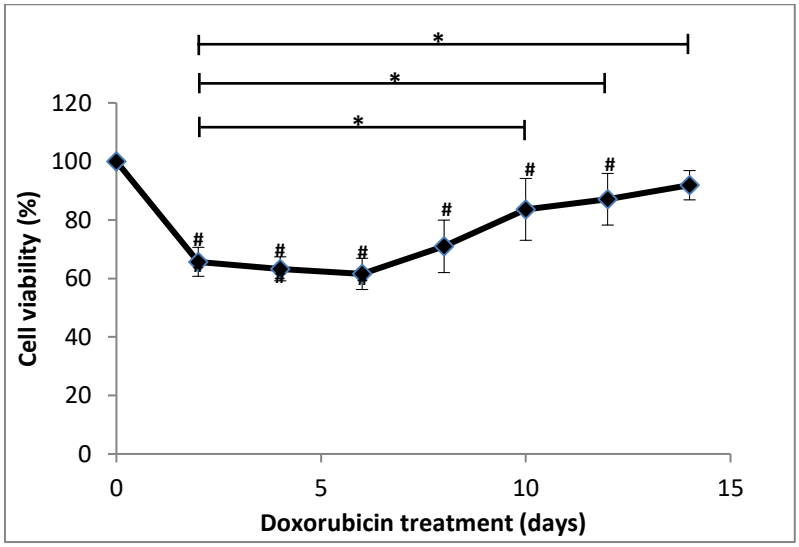

Figure 2. Viability **of doxorubicin-treated BCSCs.** Approximately 1x10^5 cells were plated in each well of 12 well-plate and incubated overnight. Afterwards, medium was replaced with fresh DMEM/F12 medium containing doxorubicin of 0.25 μM for 2, 4, 6, 8, 10, 12, and 14 days respectively. As controls, cells were grown in DMEM/F12 without doxorubicin for 2, 4, 6, 8, 10, 12, and 14 days respectively. After incubation, cells were harvested and counted using trypan blue exclusion assay and automated cell counter (Luna). The number of viable doxorubicin-treated cells was normalized to that of the respective control cells to calculate the percentage of cell viability. Significant differences are considered at *p<0.05 (One-way ANOVA test) compared to cell viability after 2-day doxorubicin treatment. Significant differences compared to each respective controls are considered at ##p<0.01 and #p<0.05 (Student's t-test).

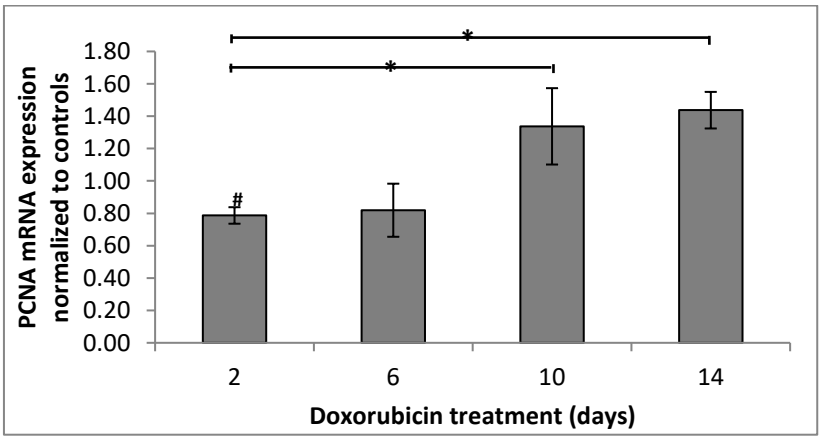

Figure 3. Relative expression of PCNA mRNA in doxorubicin-treated BCSCs. Human BCSCs (ALDH+) were treated with doxorubicin 0.25 μM and incubated for 2, 6, 10, and 14 days respectively. As controls, cells were grown without doxorubicin for 2, 6, 10, and 14 days respectively. After incubation, cells were harvested for total RNA isolation. Quantitative RT-PCR was performed using 100 ng total RNA to determine PCNA mRNA relative expression levels in human BCSCs, as described under Materials and Methods. The expression was relatively calculated using Livak formula with 18S rRNA gene as a reference gene and normalized to its respective control. All values are means ± SE, n = 6. Significant differences are considered at *p<0.05 (One-way ANOVA test) compared to 2-day doxorubicin treatment. Significant differences compared to each respective control are considered at #p<0.05 (t test).

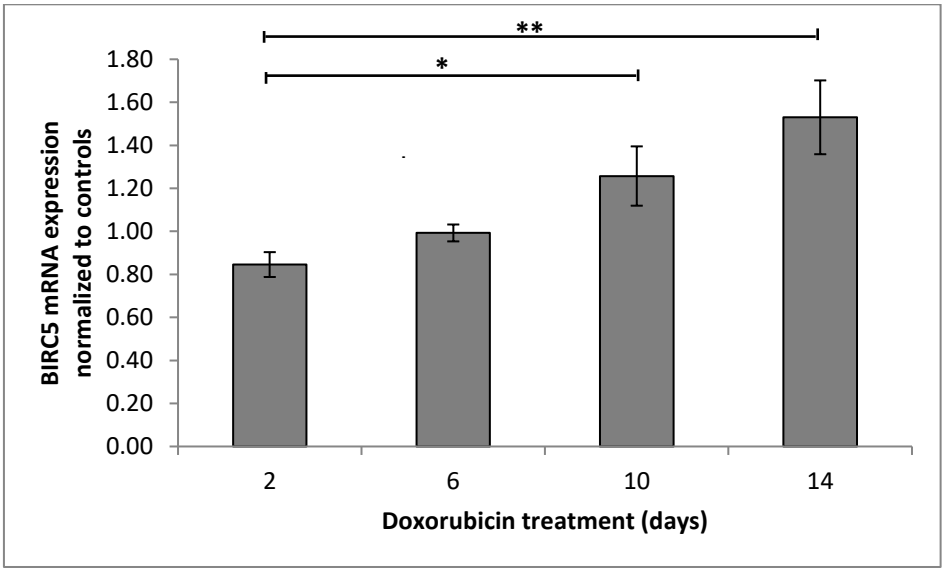

Figure 4. Relative expression of BIRC5 mRNA in doxorubicin-treated BCSCs. Human BCSCs (ALDH+) were treated with doxorubicin 0.25 μM and incubated for 2, 6, 10, and 14 days respectively. As controls, cells were grown without doxorubicin for 2, 6, 10, and 14 days respectively. After incubation, cells were harvested for total RNA isolation. Quantitative RT-PCR was performed using 100 ng total RNA to determine BIRC5 mRNA relative expression levels in human BCSCs, as described under Materials and Methods. The expression was relatively calculated using Livak formula with 18S rRNA gene as a reference gene and normalized to its respective control. All values are means ± SE, n = 6. Significant differences are considered at **p<0.01 and *p<0.05,(One-way ANOVA test) compared to 2-day doxorubicin treatment.

DISCUSSION

Breast cancer stem cells could be identified based on several surface antigen markers such as CD44□CD24□□or ALDH1+.(13)·(14) High expression of ALDH1 is correlated with poor prognosis. Some studies reported that high number of BCSC was shown to be capable of tumorigenesis, metastasis, and drug resistance.(6)·(15) This study was performed to analyse the role of PCNA and BIRC5 gene expression in response to repeated exposure of doxorubicin especially in ALDH1+ breast cancer stem cells.

Result of cell viability percentage shows the decreasing of the BCSC viability in the early of doxorubicin treatment (until day 6), suggesting that BCSCs were still sensitive to doxorubicin therapy. However, this study shows that the sensitivity of BCSC against doxorubicin began to decrease after 8-day doxorubicin treatment and reached 92% viability on day 14.

The present study indicated that the expressions of PCNA and BIRC5 were modulated in line with the modulation of human BCSC viability during doxorubicin treatment for 14 days. Both genes were expressed lower than control on day 2 and 6, but significantly higher on day 10 and 14 of doxorubicin treatment. Therefore, we confirm that PCNA and BIRC5 gene expressions involved in the sensitivity of BCSC against doxorubicin by regulating the cell viability, which is consistent to our previous study using next generation sequencing.[8]

PCNA is a responsible factor for DNA replication and repair and contribute to cell proliferation. Cell growth/proliferation is a requirement for cancer progression, so PCNA plays important role in cancer progression at both primary and metastatic sites. PCNA phosphorylation is stimulated by epidermal growth factor (EGF). When EGF binds the receptor (EGFR), it will lead the PCNA phosphorylation on tyrosine residue 211 (pY211). The phosphorylated-PCNA (pY211-PCNA) was found frequently in human breast cancer, and the levels of pY211-PCNA were correlated with poor overall survival.(16) On the other hand, PCNA also acts as an antiapoptotic activity through interaction with proteins of the Gadd45 family (Gadd45, MyD118, and CR6), which are contributed in growth control, apoptosis, and DNA repair. PCNA interaction in these protein will inhibit their activities.(17) PCNA has also been widely used as a tumor marker, and its expression is correlated poor prognosis.(18)

BIRC5 (Survivin) is an inhibitor of apoptosis protein and is expressed in a lot of malignancies,

including breast cancer.(19) Overexpression of BIRC5 gene in cancer may overcome cell cycle checkpoints to facilitate aberrant progression of transformed cells through mitosis, suggesting the involvement of this gene in tumor aggressiveness and therapy resistance.(20) Its expression levels correlate with more aggressive disease and poor clinical outcome. Khan et al reported higher expression of BIRC5 as a critical factor for radioresistance in head and neck squamous cell carcinoma (HNSCC) cell lines.(21) A study reported that reducing the mRNA levels of BIRC5 to approximately 40%, results in a concomitant reduction of OCT4 and NANOG mRNA. It suggesting that BIRC5 plays a role in pluripotency.(22)

PCNA and BIRC5 are genes that involved in p53 signalling pathway. The p53-binding sites are present in PCNA gene promoter.(23) Induction of PCNA transcription is regulated by p53 protein in response to DNA damage (radiation or other stresses) to stimulate DNA repair. Low and moderate levels of p53 positively stimulate transcription of PCNA, while high levels of p53 inhibit PCNA expression.(24) BIRC5 is a target of p53 protein for its action and downregulation. P53 protein may induce apoptosis by antagonizing the anti-apoptotic activity of BIRC5. The promoter of BIRC5 gene has a p53 binding element, thereby it may give the possibility of p53 binding to BIRC5 promoter either alone or in combination with other protein to repress BIRC5 expression.(25) Based on our results, we suggest that BCSCs modulated PCNA and BIRC5 gene expressions that regulate cell viability via proliferation and anti-apoptosis mechanism in response to prolonged exposure to doxorubicin .

In the presen study, we conclude that the PCNA and BIRC5 expressions play an important role on BCSCs viability which associated with the sensitivity of doxorubicin treatment. Hence, PCNA and BIRC5 expressions should be considered as markers for determining chemotherapy response and sensitivity in particular to breast cancer stem cells.

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Effects of Engineered Stimulation of Oxytocin on Hormonal Status of Postpartum Women

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ABSTRACT

Oxytocin in the postpartum women is very necessary to maintain breastmilk production. An engineered stimulation of oxytocin by using a tool in a previous study was evident to give effects on the production of breast milk. The purpose of this study was to analyze the effects of an engineered massage tool for stimulating oxytocin on the levels of oxytocin, prolactin and beta-endorphin in the postpartum women. Quasi-experimental study employed a post-test with control group and was conducted in two hospitals in Semarang. A consecutive sampling was used to recruit the samples, involving 32 postpartum women in the control group, 26 in the intervention group I (intervention once-daily) and 30 in the intervention group II (intervention twice-daily). After 9 hours 30 minutes since the labor, the blood samples from the postpartum mothers were taken and examined for the oxytocin, prolactin, and beta-endorphin. The results showed a mean of 353.58 ng/ml for oxytocin, 231.41 ng/ml for prolactin and 178.75 ng/ml for beta-endorphin. There was a significant relationship between the frequency of breastfeeding and the prolactin (p=0.004), and there was also a significant relationship between the breast milk secretion and the prolactin (p=0.005). Beta-endorphin had a significant association with the oxytocin (p=0.000). There was a difference in beta-endorphin levels between the groups given once-daily stimulation, twice-daily stimulation and the control group (p=0.041). The stimulation of oxytocin had an effect on the increase of beta-endorphin which indirectly affected the oxytocin.

***Keywords**: Engineered oxytocin, postpartum, oxytocin, prolactin, beta-endorphin*

A mother's ability to breastfeed is shown in the production and secretion of breast milk which is affected by the prolactin and oxytocin. The letdown reflex is a response of the nervous system that causes the breast milk producing cells contract so that the milk inside is squeezed out, flows along the milk duct, and comes out through the nipples. The letdown reflex will work if only given a command from the oxytocin. A high level of oxytocin maximizes the amount of breast milk in its reservoir [(1)].

A postpartum mother by nature has a duty to breastfeed her baby exclusively for six months, and this will continue until the age of two. Breastfeeding provides some benefits; one of which is increasing the secretion of oxytocin that plays a role in the uterine involution and breast milk secretion. In a mother with caesarean section, the involution occurs slower than that of normal labor since the uterine condition of C-section mom is not stimulated to immediately return to its normal form [(2)].

Oxytocin affects the affiliate behaviors such as trust, empathy, social memory, and interpretation of facial expressions. Oxytocin provides an enhanced effect of compassion quality such as wisdom, strength, and goodness [(3)]. The function of oxytocin is just opposite to the stress hormone since oxytocin makes someone feel calm and relaxed. Oxytocin leads someone to feel relaxed, avoid stress, have more open communication, feel connected, and get rid of isolated feelings. Oxytocin can make a person be in his/her best mood and develop a feeling of loving and being loved [(4)]. Also, oxytocin can induce anti-stress such as decreased blood pressure and cortisol levels. Repeated exposure to oxytocin causes a long-term effect which affects one's other activities.

In addition to affecting the breastfeeding mechanism, oxytocin also gives effects on the work of the other organs. In the uterine smooth muscles, oxytocin stimulates the uterine contractility. In the kidneys, antidiuretic hormones (ADH) and oxytocin have some similar

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effects since the chemical structure between the ADH and oxytocin is very identical. Furthermore, oxytocin in the blood vessels also works on the ADH receptors which cause a decrease in diastolic pressure [5].

The circulation of oxytocin will increase when there is a signal of trust, and vice versa, a high level of oxytocin will increase the trust. Trust is an emotional aspect. There are some factors which improve a mother's emotions, including a pleasant condition, maternal attraction, touch, and happy and warm feelings [6].

The stimulation of oxytocin is a necessary intervention for the postpartum women. In the practice of postpartum care, such intervention has not become a standard of care as it is only given to those who are in need. So far, the existing intervention has been in the form of a manually administered oxytocin massage. A person who is given a massage increases oxytocin and reduces adrenocorticotropin [7]. Postpartum women who were given the oxytocin massage produced colostrum of 5 cc more than those who did not receive any massage [8]. In addition, the provision of oxytocin massage also gave an effect on the involution of uterine [9]. A similar study also showed that oxytocin massage provided an effect on the level of oxytocin [10].

The stimulation of oxytocin can be performed using an engineered assisting tool. It is as suggested by Anggorowati who stated that the use of digital massager of oxytocin (DMO) gave effects on the increased volume of breast milk production. In this study, the stimulation can be independently practiced so that it is suitable for the purpose of nursing, that is to develop one's self-reliance. Furthermore, this self-administered intervention is also practical for the postpartum women under any circumstances as long as their condition is conscious. Also, the operation of the tool is also simple. However, a previous study by Anggorowati has not been able to determine the effective dose of DMO use for oxytocin stimulation [11].

The present study aimed to determine the effects of engineered stimulation of oxytocin on the hormonal status of postpartum women. The hormonal parameters in this study include the oxytocin, prolactin, and beta-endorphin.

METHODS

This study employed a post-test quasi-experimental design with a control group and was conducted at public hospitals in Semarang and Semarang regency particularly at the maternity room. A consecutive sampling was used to recruit the samples of postpartum women after 6 hours of labor who met the criteria of compost mentis awareness and willingness to participate.

The first sample group was taken as a control group from the public hospital in Semarang municipality and Semarang regency (n=32). The other two sample groups were also recruited simultaneously from both hospitals and were assigned to the intervention group I and intervention group II. The number of each sample group was similar with that in the control group. However, during the process of data collection, some women dropped out and refused to participate. Therefore, the number of samples included in the analysis was 32 in the control group, 26 in the intervention group I and 30 in the intervention group II. Before blood sampling in treatment group 1 was given massage stimulation with tool once and group 2 treatment was given 2 times massage stimulation with interval of 3 hours.

After 9 hours and 30 minutes since the labor, the blood samples from the women in the control group and intervention group were taken; each was for 3 ml. The blood samples were then centrifuged in the hospital laboratory and stored in a cooling cabinet. Furthermore, the samples were then sent to GAKY laboratory at Diponegoro University for further analysis. By using the ELISA technique, the centrifuged blood samples were then examined to identify the status of oxytocin, prolactin, and beta-endorphin.

Data collection is done after obtaining ethical approval from the Ethics Committee of Faculty of Medicine Diponegoro University - Kariadi Hospital with number 712/EC/FK-RSDK/2016. Respondents were given an explanation of the purpose of research and involvement in research as well as approval in the study proved to provide a signature.

RESULTS

The results describe the characteristics of respondents, including the demographic data and

other characteristics of the post-partum mothers in table 1 In addition, they also explain the hormonal condition in table 2 and the relationships among the hormones of postpartum women in table 3.

Table 1. Characteristics of postpartum women at hospitals in Semarang

Variables	Control (n=32)	Group I (n=26)	Group II (n=30)	p value
Age	27,46 (15-43)	28,73 (18-47)	26,03 (17-39)	0,907**
Education				0,000*
Elementary school	4 (12,5%)	2 (7,7%)	2 (6,7%)	
Junior high	12 (37,5%)	5 (19,2%)	9 (30%)	
Senior high	15 (46,9%)	16 (61,5%)	17 (56,7%)	
University	1 (3,1%)	3 (11,5%)	2 (6,7%)	
Employment status				0,000*
Employed	9 (28,1%)	11 (42,3%)	6 (20%)	
Unemployed	23 (71,9%)	15 (57,7%)	24 (80%	
Type of labor				
Vaginam				
Section caesarean	21 (65,6%)	14 (53,8%)	19 (63,3%)	0,033*
	11 (34,4%)	12 (46,2%)	11 (36,7%)	
Number of birth	1,75 (1-4)	1,61 (1-4)	1,63 (1-4)	0,525**
Birth weight	3129,7 (2300-3900)	3121 (1500-4200)	2963,3 (1600-3500)	0,363**
Early initiation of breastfeeding				0,522*
Yes	16 (50%)	17 (65,4%)	14 (46,7%)	
No	16 (50%)	9 (34,6%)	16 (53,3%)	
Frequency of breastfeeding every day	1 (0-5)	1,26 (0-4)	1,73 (0-5)	0,135**
Secretion of breastmilk (hour after birth)	42,56 (0-72)	28,86 (0-72)	27,63 (0-72)	0,063**

*chi-square ** annova

Table 2. Hormonal differences in postpartum women after the stimulation of oxytocin massage using DMO in Semarang in 2016 (n=88)

Group	Hormones		
	Oxytocin	Prolactin	Beta-endorphin
Control	346,05 (46,74-1045,20)	245,36 (76,37-488,49)	190,41 (23,13-418,57)
Group I	341,11 (36,61-1019,10)	238,22 (63,66-425,40)	211,84 (78,22-458,80)
Group II	372,42 (132,64-1027,60)	210,64 (21,85-474,21)	137,64 (28,94-357,56)
Difference Control-Group I	0.491*	0.845*	0.839*
Difference Control-Group II	0.678*	0.139*	0.068*
Difference Group I & II	0,643***	0,392***	0,013***
Difference Control-GroupI-GroupII	0.583**	0.358**	0.041**

*= Mann Whitney ** = Kruskal Wallis ***independent t test

Table 3. Relationship among the hormones of postpartum women in Semarang in 2016 (n=88)

N	R	P
Oxytocin and prolactin	-0.308	0.004
Beta-endorphin and oxytocin	0.536	0.000
Beta endorphin and prolactin	-0.034	0.754

The mean of oxytocin in the postpartum women on the first day was 353.58 ng/ml, indicating a normal level of oxytocin in breastfeeding mothers. The results of present study showed that the mean of oxytocin level among the women was within a normal category. However, some were found to have less than that. The lowest level was 36.61 ng/ml, and this was far beyond the normal value (75ng/ml).

The level of prolactin in the postpartum women on the first day showed a mean of 231.41 ng/ml. It was within a normal range for breastfeeding mothers. The lowest level was 21.55 ng/ml, indicating an extreme difference from the normal one (90ng/ml). Some mothers would be at risk of inadequate production of breast milk if not controlled by any stimulation of the breast milk production.

Beta-endorphin on the first day showed a mean of 178.75 pg/ml, which belonged to the normal value. Nevertheless, the lowest score was 23.13 pg/ml. Beta-endorphin represents a stressful condition of breastfeeding mothers.

DISCUSSION

Prolactin level in the other study in two weeks is 124ng/dl, eight weeks is 68 ng/dl [12]. From this study prolactin level more than study, its 231ng/ml. The potential for breastfeeding was better in control group with higher prolactin levels after intervention in accordance with other studies that oxytocin and prolactin levels were related to the mother's ability to breastfeed [13]. Based on the level of prolactin in the intervention group, this study could not conclude that the intervention did not have the potential to increase breast milk because of unknown prolactin levels in all groups prior to intervention.

There was a relationship between the oxytocin and prolactin as shown in a correlation value of -0.308 and a p-value of 0.004. Likewise, there was also a strong relationship between beta-endorphin and oxytocin with a correlation value of 0.536 and a p-value of 0.000. This is in line with other studies stating that beta-endorphin is closely related to oxytocin (14). However, there was no significant relationship between beta-endorphin and prolactin (p = 0.754).

Endorphin is an opioid of the body, produced by the hypothalamus which provides pleasing and relaxing effects [15]. A hormonal level in a normal limit provides a relaxed response, and on the contrary, a lower endorphin level will provide the opposite response.

The results showed that there was a difference in the level of beta-endorphin among the groups with one-time stimulation, two-times stimulation and the control group (p = 0.041). The massage stimulation using DMO had an effect on the changes in the level of beta-endorphin. Increased beta-endorphin was more clearly seen in the postpartum women with one-time massage stimulation. Beta-endorphin mutually synergizes with oxytocin. These hormones facilitate or inhibit the effects of pain and stress, maternal and bonding relationships [16]. Beta endorphin also improves one's self-defense ability against disease progression [17].

The provision of massage provides effects on the decrease of stress. The stressful condition is represented by the low level of beta-endorphin (14). High level of beta-endorphin will induce oxytocin, and thereby gives an indirect effect on the oxytocin [7].

In a previous study, the provision of massage for three consecutive days provided significant changes in the oxytocin. Repeated massages would have effects on the oxytocin changes [18]. In addition, another study also revealed that administering DMO to the mothers for three consecutive days could increase the volume of breastmilk of 23 ml [19]. The results of this study reinforce that the giving of a positive effect that is given once a day for a certain period.

Oxytocin is associated with beta endorphin. Giving once daily has an effect on beta endorphin

but has no effect on oxytocin. This suggests that DMO stimulation needs to be given over a longer period. Oxytocin has an effect on lactation mediating let down reflex along with prolactin stimulation resulting in milk production. Oxytocin improves adaptation behavior of maternal and behavior immediately after delivery [14].

The weakness of this study is that between the control and intervention groups there are different types of labor. The type of labor distinguishes the standard procedure of hormone oxytocin that affects stress. The effects of anesthesia on cesarean section affect the stimulation of oxytocin and prolactin, this is different from normal labor. Differences in these characteristics are related to the results of the study that DMO stimulation did not show any effect on oxytocin and prolactin. In a cesarean section, administering a three-day oxytocin massage may increase prolactin [20]. Massage of postpartum mother with normal labor shows the effect of increased milk production [21]. Stimulation of breastfeeding in normal and cesarean section differs as in the initiation of early breastfeeding [22].

CONCLUSION

The hormonal status of the postpartum women on the first day showed level of oxytocin, prolactin and beta-endorphin on normal range. The engineered stimulation oxytocin affect to beta-endorphin level. Stimulation of oxytocin with once-daily dosage its difference effect from twist-daily dosage. There was a strong relationship between beta-endorphin and oxytocin.

For the breastfeeding mothers, it is suggested that they breastfeed their baby frequently in accordance with the results of some studies which stated there was a relationship between the frequency breastfeeding and the prolactin. In addition, the postpartum women are recommended to immediately breastfeed their baby after birth.

For the health workers managing the postpartum mothers, it is suggested that they implement the early initiation of breastfeeding and encourage postpartum mothers to increasingly breastfeed their babies. In addition, they can also give some interventions to stimulate the oxytocin.

Further studies on the use of oxytocin stimulation using DMO with a once-daily dosage for at least three days are necessary. The stimulation of oxytocin in breastfeeding mothers can be measured through the mother's independence parameters such as the smooth secretion and sufficiency of breast milk.

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The Correlation between Serum Vascular Endothelial Growth Factor (VEGF) Levels and Size of Colorectal Cancer Tumors

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ABSTRACT

Background: Angiogenesis plays an important role in progression of colorectal carcinoma (CRC). Vascular endothelial growth factor (VEGF) is the predominant angiogenic factor in CRC and plays important role in cell mitosis, change in cell shape and increases vascular permeability. Vascular endothelial growth factor is expressed in approximately 50% of CRCs, and considered an important angiogenic factor in growth and development of CRC. In this study, we examined VEGF serum levels to asses corelation between serum VEGF levels and size of CRC tumors. **Patients and Methods:** This cross sectional study involved 17 CRC patients with stage I, II, III. who had undergone large bowel resection at Karyadi Hospital and had not chemotherapy. Size of tumors grading system according to TNM system based on abdominal CT, whether serum levels of VEGF was assessed by ELISA. **Results:** In this study, we found the grading tumor size T1, T2 and T3 was 23,5%, 29,4 % and 47,1 % respectively. Significant statistical correlation (p=0.001) was found between serum VEGF levels and size tumors of CRC with strong relationship (rho>0,7). **Conclusion:** This study showed a correlation between serum VEGF levels and size of colorectal cancer tumors. Whether VEGF levels is affected by surgical procedure, we need further study to evaluate serum VEGF levels before surgical and tumor size according to treatment response.

Keywords: *Angiogenesis, Colorectal Cancer, Tumor size, VEGF*

Colorectal cancer is the second most common cancer in the world. Colorectal cancer incidence rates is more prevalent in developed countries such as Australia, New Zealand, Canada and USA. In the United States, colorectal cancer is the second leading cause of death among those developed countries. In 2014, an estimated 136.830 people were diagnosed with colorectal cancer and 50.310 died. It is estimated that approximately 95270 new cases of large bowel cancer and 39220 new cases of colorectal cancer are diagnosed in 2016 [1-4].

Colorectal cancer is the abnormal growth of cells originates in the colon and the rectum. Colorectal cancer cells growth and development need vascularization to support food supply (angiogenesis) [5, 6].

Angiogenesis process is the formation of new blood vessels from the preexisting vasculature. Angiogenesis is a vital process in tumor growth and development and Vascular Endothelial Growth Factor (VEGF) is a potent stimulator in the angiogenic response [7].

VEGF contributes to tumor growth due to its capacity to induce blood vessels permeability (6).VEGF is the target of anticancer therapy because of its fundamental role in tumor growth [8]. Although tumor size remains a major prognostic value in many other solid organs cancer, its value in colon cancer may have long been neglected. In additional to our knowledge, the aim of this study is to determine the association of the serum Vascular Endothelial Growth Factor (VEGF) levels with tumor size in colorectal cancer.

METHODS

Sampels

Blood samples were taken from 17 colorectal cancer patients who had undergone large bowel resection at Karyadi Hospital. No patients had had previous malignant disease nor had received chemotherapy or radiotherapy. Blood samples were taken, at the time about 2 weeks after surgery.

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Five cc blood was taken into a into serum separator tubes (SST) for serum analysis, and shaken to homogeneous. Wait for 30 -45 minutes until clothing. Samples were centrifuged at 3000 rpm for 15 minutes. The samples were stored at −20°C until assay by ELISA (Quantine R&D America). Using calibrator diluent RD5K, the minimum detectable dose of VEFG by ELISA is typically less than 5.0 pg/ml. All of the samples will sent with dryice to Prodia central laboratory in Jakarta.

All specimens were systematically reevaluated by expert pathologist in Karyadi hospital. Tumour staging was performed according to the TNM stage classification American Joint Committee on Cancer system 7th (2010) based on contrast abdominal CT.

Study Design

This was a cross-sectional study conducted over a period of 3 months at RSUP Dr. Kariadi Semarang, 17 patients were included. Correlation test was used to assess the association between the serum VEGF levels and tumor size in colorectal cancer.

Statistical Analysis

All collected data were tabulated and calculated using SPSS v.16, presented in numerical data, median and its variations.Spearman's rank test was used for the correlation analysis. A p-value <0.05 was considered statistically significant.

RESULTS

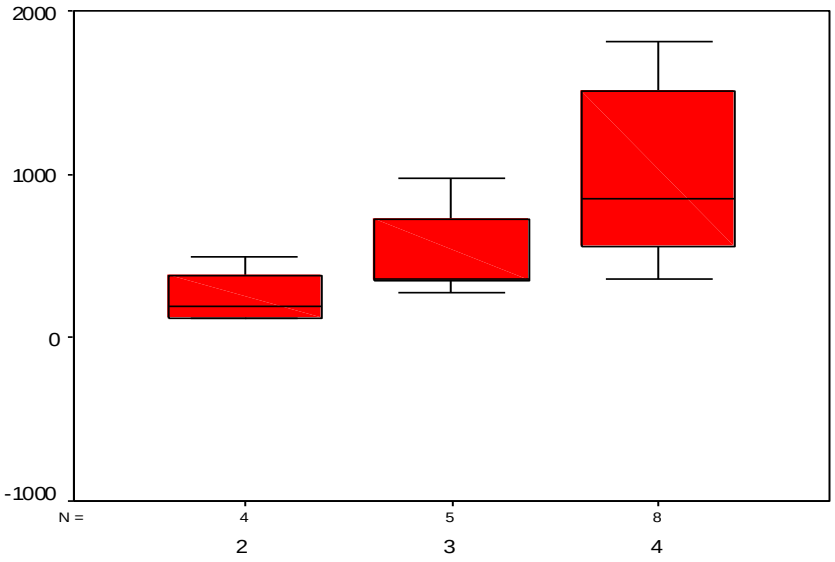
This study was conducted over a period of 3 months, a total of 17 samples were studied, consisted of 10 males (58,8%) and 7 females (41,2%). The age range of the subjects varied between 31 years and 81 years. According to age category, the subjects were divided into ≤ 50 years; 51-60 years; 61-70 years; 71-80 years; and >80 years with a count of 7(41,2%); 4(23,5%); 4(23,5%); 1 (5,9%), and 1(5,9%) respectively.

In this study we found tumor size of T4 in 8 subjects (47,1%), T3 in 5 subjects (29,41%), and T2 in 4 subjects (23,52%). Clinicopathologicalof samples are adenocarcinoma. Colorectal cancer stages of the subjects consisted of stages I to III who underwent surgery and never received chemotherapy All pertinent clinical and histopathological data of the patients are summarised in Table I.

Table1. Characteristics of the study population

Variables	N(%)	Mean ±SD	Median (Minimum-Maximum)
Gender			
Male	10 (58,8%)		
Female	7 (41,2%)		
Age (years)			
≤ 50 years	7 (41,2%)	53,71 ±13,959	
51-60 years	4 (23,5%)		
61-70 years	4 (23,5%)		
71-80 years	1 (5,9%)		
>80 years	1 (5,9%)		
Tumor size			
T2	4 (23,5%)		193 (120-494)
T3	5 (29,4%)		356 (276-973)
T4	8 (47,1%)		855 (357-1809)
Cancer stage			
I	2 (11,8%)	308 ± 263	
IIA	1 (5,9%)	729 ± 0	
IIC	1 (5,9%)	517 ± 0	
IIIA	2 (11,8%)	192 ± 101	
IIIB	10 (58,8%)	762 ± 479	
IIIC	1 (5,9%)	1809 ± 0	

This study found significant association between serum VEGF levels with tumor size in colorectal cancer P= 0,001 (P<0,05) with strong correlation (rho >0,7).



Tumor size

DISCUSSION

This study involved 17 colorectal cancer patients stages I, II, and III who underwent surgery and never received chemotherapy. Several studies have shown that colorectal cancer increases with age. Colorectal cancer is rare before the age of 40. The risk of developing colorectal cancer increases at the age of ≥50 years and becomes twice as large as in each subsequent decade. This study also found the that colorectal cancer rates washigher among the subjects who were older than 40 years compared to younger subjects, with the youngest participant of 31 years, and most of the study subjects were from the age of 40 - 60 years.

Vascular Endhotelial Growth Factor (VEGF) is endothelial cell-specific mitogen. VEGF is a mediator of angiogenesis that plays an important role in cell mitosis, cell shape alteration and increases vascular permeability.VEGF is involved in the growth and development of colorectal cancer. VEGF is correlated with the invasion of colorectal cancer cells, tumor vascular density, metastasis, tumor recurrence and poor prognosis, furthermore there is a strong association between colorectal cancer with apoptosis and angiogenesis [(9)]. Serum VEGF levels in the subjects of this study (mean 686,82 ± 516,729 pg/ml).

Large tumors may have independent prognostic significance as a result of several factors. Larger tumor size may also be a reflection of biologically more aggressive tumor. Although tumor size remains a major prognostic value in many other solid organs cancer, its value in colon cancer may have long been neglected [(10)]. In this study we found that tumor size of T4 in 8 subjects (47,1%), T3 in 5 subjects (29,41%), and T2 in 4 subjects (23,52%).

VEGF has an effect on the progressivity of colorectal cancer. According to several studies, increased levels of VEGF is associated with increased size of colorectal cancer tumors [(10-12)].

This study found significant association between serum VEGF levels with tumor size in colorectal cancer P= 0,001 (P<0,05) with strong correlation (rho >0,7). Increased VEGF tumor may be used as an independent prognostic parameters in the management of colorectal patients stages II and III [(13-15)]. Thisfinding is consistent with some previous studies. FerroniP, et al in 2005 reported that serum VEGF was associated with a large tumor (p = 0.005); another study by KemikOzgur et al, in 2011 found an association between serum VEGF levels and a large tumor with p = 0.0001, elevated serum VEGF levels was associated with the development of colorectal cancer. This finding is similar to the study conducted by Kwon et al's in 2010 with p = 0.012; Sahasukamal et al study in 2014 also revealed the correlation of high serum VEGF levels with increased tumor size, high serum VEGF levels was associated with poor prognosis

and larger tumor size decreased patient survival rate by 5 years [10, 11, 13, 15].

CONCLUSION

The present results demonstrate in This study has demonstratethat serum VEGF levels is significantly difference inassociated with tumor size in colorectal cancerinpreviously untreated colorectal cancer patients who never received chemotherapy. High level VEGF serum associate with the larger the tumor. Surgical procedures may affect VEGF levels, so further studies are may needed to assess the effect of surgery to VEGF alterations. VEGF seems to be an indicator of poor prognosis, as well was correlated with tumour size, further studies needed to see association between VEGF levels with less favourable long-term survival.

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TNF - α Gene Polymorphism is Likely to be a Risk Factor for NASH in Indonesia

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ABSTRACT

Introduction : Non alcoholic steatohepatitis (NASH) is a subset spectrum of NAFLD that can progress toward cirrhosis. Tumor necrosis factor- α (TNF- α) polymorphism play a significant role in liver inflammation and Insulin resistance its pathophysiological hallmark. The association between TNF- α (-238 and -308) polymorphism) and the severity of NAFLD was evaluated. **Method:** A total of 155 subjects (80 NAFLD cases and 75 controls) were included. Liver biopsy was performed for evaluated severity of NAFLD based on NAFLD Activity Score-CRN in all cases. Controls subjects defined was not any liver disease by ultrasound. Plasma TNF– α was measured in all subjects. Polymorphism of` TNF- α promoter gene -308 and -238 were identified using PCR-RFLP confirmed with direct sequencing. **Results:** Liver biopsy established the diagnosis of NASH in 29 cases. There was no association between the incidence of NAFLD with TNF- α polymorphism at the TNF– α -308 or the -238 ($p>0.05$). The prevalence ratio of TNF– α polymorphism -238 was significantly higher for subject with NASH ($p< 0.02$). There was no different prevalence ratio for TNF– α polymorphism -308 ($p>0,05$). We found novel polymorphism of TNF- α -245 T/C in a subject with possible NASH, high plasma TNF- α level (20.27 pg/cc) and very high value of Homeostasis Model of Assessment - Insulin Resistance HOMA-IR (22.73). **Conclusion :** Polymorphism TNF- α -238 is likely to be a risk factor for NASH in Indonesia. The identification of new possible polymorphism of TNF- α -245 requires further study in more samples.

***Keywords**: Polymorphism TNF- α, risk factor, NASH*

INTRODUCTION

Non Alcoholic Steatohepatitis (NASH) is the most severe form of Non Alcoholic Fatty Liver Disease (NAFLD). NASH is clinically a manifestation of chronic liver disease frequently occur in industrial countries and its rate has been increasing in developing countries [1]. Recent epidemiology study (2010) in Asia, Europe, Middle East, North and South America stated the prevalence of NAFLD ranging from 6-35% with median 20%[2]. Hasan Irsan reported that NAFLD was found in 30% of the population in Jakarta based on abdominal ultrasound [3].

Tumor Necrosis Factor (TNF)-α polymorphism has been reported to have effect on the susceptibility of several liver diseases including NASH and more scientific proof showed the involvement of TNF-α in the pathogenesis and progression of liver disease caused by many etiologies [4, 5]. Data from several clinical and experimental studies revealed TNF-α played roles not only in early stage of fatty liver disease but also in transition process to more advanced liver damage. The role of TNF-α in NAFLD is a crossroad of pathogenic pathway. Major difference between patients with simple steatosis and NASH is that serum level of TNF-α is found higher in NASH even though the result did not always reach statistical significance [6].

Two polymorphisms in TNF-α gene promoter have been identified: at position 308 (TNF2 allele)

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and at position 238 (allele TNFA). Studies on the allele TNF-α proved the increased formation and expression of these cytokines compared to the wild type (TNF-1), although the reported data of TNF- α allele was conflicting, but many researchers believed that TNF- α allele increases the release of this cytokine [7].

Luca Valenti (2002) in his research revealed genes that influence the effect of TNF-α , that is polymorphism in gene promoter -238 that correlates well with alcoholic steatohepatitis or NASH(4). Katsutoshi T (2007) reported that serum level of TNF-α was higher in NASH patients than that of steatosis and normal patients. There was no significant deviation in the analysis of TNF-α gene polymorphism between all groups. The number of gene promoter -1031C and -863A in patients with NASH was higher than that of simple steatosis group. Multivariate analysis has proven that polymorphism is an independent factor which differentiate NASH from from simple steatosis. It was concluded that TNF-α polymorphism influences TNF-α production and correlates with the progression of NAFLD [8]. However, data from several studies were conflicting that further study is needed to confirm its correlation.

MATERIAL AND METHODS

A case-control study held in outpatient clinic of dr. Kariadi General Hospital Semarang on January 2009 – December 2011.

Inclusion criteria for case group includes: aged > 14 years old having metabolic syndrome components and proven to have NAFLD based on abdominal ultrasound. Subjects will be excluded if known to have hepatitis A, B, C virus infection (AST and ALT > 5 times higher than normal with positive IgM anti HAV or positive HBsAg or positive anti HCV); autoimmune hepatitis (positive ANA test); alcoholic hepatitis; history of alcohol consumption (> 30 gr/ day for male and > 20 gr/day fpr female); history of taking drugs causing fatty liver (glucocorticoid, estrogen, tamoxifen, amiodarone, metothrexate, valproate, diltiazem).

Inclusion criteria for control group includes no history, and clinical symptoms of liver disease (with normal AST and ALT, negative HbsAg, negative anti HCV) and no liver abnormality found on abdominal ultrasound. Subjects will excluded if known to have liver disease by anamnesis, physical examination and laboratory findings; hepatitis virus infection (A,B,C); autoimmune hepatitits, alcoholic hepatitis; history of alcohol consumption; and history of taking drugs causing fatty liver.

All subjects underwent these following physical and laboratory examinations: blood pressure, body weight, height, body mass index, waist circumference, liver enzymes (AST, ALT), lipid profile (HDL, total cholesterole, triglyceride) and fasting blood glucose. Serum level of TNF-α was tested in all subjects of case and control groups. Liver biopsy was performed only in NAFLD subjects for classification of severity by Kleiner et al; NASH Clinical research network scoring system (NAFLD activity score =NAS). NASH was defined if NAS > 5, Possible NASH if NAS 3-4 and Simple steatosis if NAS < 3 consecutively. TNF-α polymorphism in gene promoter -238 and -308 was tested using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) and confirmed by direct sequencing.

RESULTS

Basic Characteristics

During study period, 150 subjects were enrolled and divided into 2 groups. Case group (n=75) consisted of NAFLD patients and control group (n=75) consisted of healthy subejcts without fatty liver. The clinical characteristics of all subjects in both groups are shown in table 1.

Table 1. Clinical characteristics, serum level of TNF-α and gene promoter polymorphism of subjects in case and control group

Characteristic	Group		p
	Case (n=75)	Control (n=75)	
Sex α			
Male	45 (55,6%)	36 (44,4%)	
Female	30 (43,5%)	39 (56,5%)	< 0,100*
Age (year)£	44,7 ±10,28 (23,0 - 74,0)	44,3 ± 9,98 (20,0 - 72,0)	< 0,800§
Male age (year)£	42,6 ±10,36 (25,0 - 74,0)	42,0 ± 11,04 (20,0 - 72,0)	< 0,800§
Female age (year)£	48,0 ± 9,42 (25,0 74,0)	46,5 ± 8,47 (26,0 - 63,0)	< 0,500§
Body weight (kg)$	77,0 (54,0 - 115,0)	56,0 (41,0 - 74,0)	< 0,001¶
Height (cm)£	162,1±8,37 (140 – 178)	161,1±6,31 (147,0 - 179,0)	< 0,400§
Body mass index $	29,0 (22,0 - 47,4)	22,5 (17,3 - 27,5)	< 0,001¶
Waist circumference (cm)$	99,0 (77,0 - 134,0)	80,0 (64,0 - 98,0)	< 0,001¶
TNF-α (pg/ml) $	7,98 (0,00-332,56)	2,24 (0,04-77,65)	<0,001¶
TNF-α (pg/ml) >3,645	58 (76,3%)	18 (23,7%)	
□3,645	17 (23,0%)	57 (77,0%)	<0,001*
TNF-α gene promoter polymorphism			
Region-238			
G/G	72(96,0%)	73 (97,3%)	
A/G	3 (4,0%)	2 (2,7%)	
A/A	0 (0,0%)	0 (0,0%)	
Frequency alel A (%)	2,0	1,3	0,700□
Region-308			
G/G	74(98,7%)	72(96,0%)	
A/G	1(1,3%)	3(4,0%)	0,600□
A/A	0(0,0)	0(0,0)	
Frequency alele A(%)	0,7	2	

£ Mean ± standard deviation (minimum-maximum); $ Median (minimum – maximum)
*x^2 test; §Independent t-test; ¶Mann-Whitney test, zFisher-exact test, Frequency of alele A=(frequency of alele A/total alele) x 100%

Serum level of TNF-α

Serum level of TNF-α of subjects in case and control groups is shown in table 1. The result shows that mean serum level of TNF- α in case group was significantly higher than that of control group (p<0,001).

Cut-off level of TNF- α to diagnose NAFLD

The result of receiver operating characteristics (ROC) analysis for TNF- α to determine the incidence of NAFLD. Area under the ROC curve of serum level of TNF- α to determine the incidence of NAFLD was 0,80 (95% CI=0,73 s/d 0,87) and it was statistically significant (p<0,001). ROC analysis also demonstrated cutoff point for serum level of TNF- α to determine the incidence of NAFLD was 3,645 ng/ml. Based on the cutoff point, further analysis was done. The subjects with serum level of TNF- α > 3,645 pg/ml were in the case group (76,3%), and on the opposite, subjects with TNF- α ≤ 3,645 pg/ml were mostly in the control group (77%). Statistical analysis confirmed that the significant correlation between serum level of TNF- α and the incidence of NAFLD.

TNF- α gene promoter 238 dan -308 polymorphism in NAFLD

The results of PCR-RFLP investigating TNF-α gene promoter -308 G/A and -238 G/A polymorphism are visulized in figure 1a and Ib

Results of liver biopsy.

Liver biopsy is a gold standard for the diagnosis of NAFLD severity. Most of the subjects in this case group of severity were included in the Possible NASH in 38 subjects (50.67%), then NASH in 29 subjects (38.67%) and at least the Simple Steatosis in 8 subjects (10.67 %).

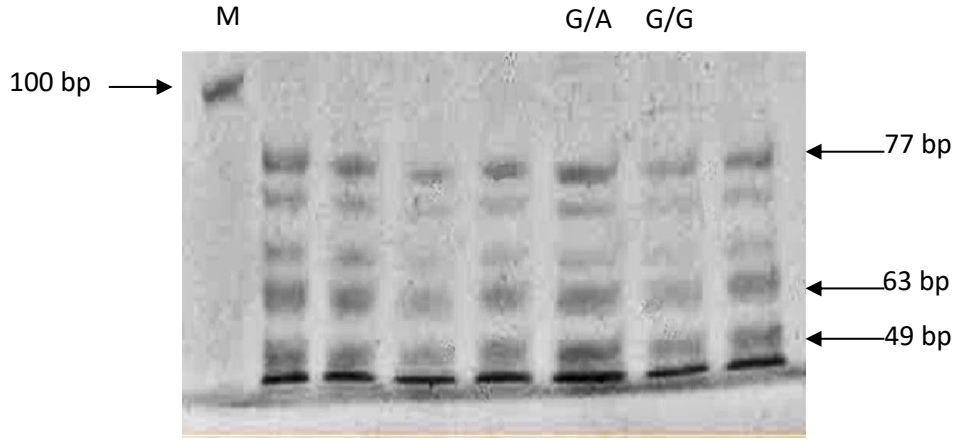

Figure 1b . Agarose gel visualization for TNF-α gene promoter -238 (PCR-RFLP) polymorphism

Showing agarose gel as the result of PCR-RFLP TNF-α regio promoter -238 polymorphism. Size of product after RFLwas 77 bp, 63 bp, 49 bp and 21 bp for -238 G/A polymorphism. Size of *wildtype* -238 G/G was 77, 70, 63, dan 49 bp. Product sized 70 bp and 21 bp were not visualized on agarose gel. No allele 2 (-238 A/A) was found

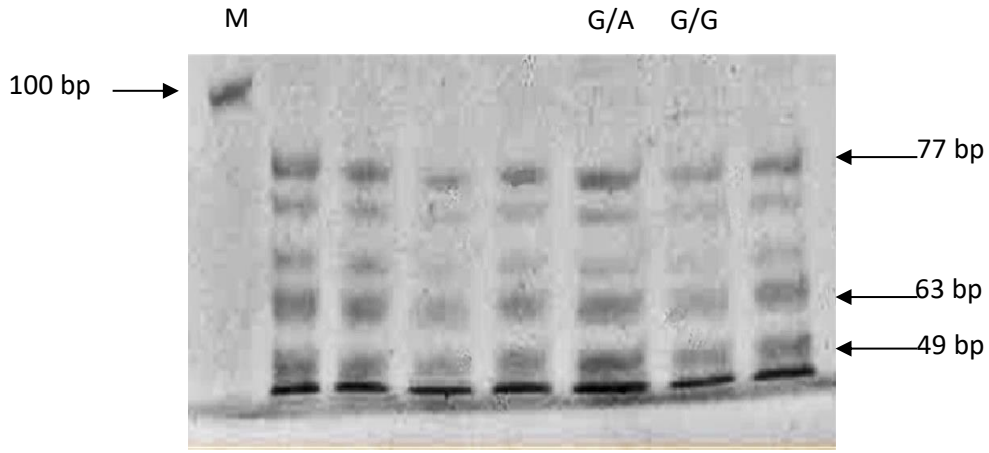

Figure 1b . Agarose gel visualization for TNF-α gene promoter -238 (PCR-RFLP) polymorphism

Showing agarose gel as the result of PCR-RFLP TNF-α regio promoter -238 polymorphism. Size of product after RFLwas 77 bp, 63 bp, 49 bp and 21 bp for -238 G/A polymorphism. Size of *wildtype* -238 G/G was 77, 70, 63, dan 49 bp. Product sized 70 bp and 21 bp were not visualized on agarose gel. No allele 2 (-238 A/A) was found

TNF- α gene promoter -238 and -308 polymorphisms

TNF- α polymorphism test using PCR-RFLP method was confirmed by direct sequencing due to very little polymorphism found by the PCR-RFLP in Indonesian population, or there was possibly other kind of univestigated polymorphism. Distribution of TNF- α polymorphism located in gene promoter -238 and -308 is listed in table 1. In table 1, the genotype of TNF- α promoter -238 mostly seen in both case and control group was G/G. TNF- α -gene 238A/G polymorphism was found in 5 subjects (3 in case group and 2 in control group). TNF- α -gene 238A/A polymorphism was not found. The frequency of allele A TNF- α -gene 238 polymorphism in case group was higher than that of control group. Statistical analysis shows no significant difference in the incidence of TNF- α -gene 238A/G polymorphism between case and control group (p=0,7).

In table1, the genotype of TNF- α promoter -308 mostly seen in both case and control groups was G/G. TNF- α -gene 308A/G polymorphism was found in 4 subjects (1 in case group and 3 in control group). Uncommon allel in control group was found more frequent in control group compared to case group (typical for Javanese-Indonesian population). TNF- α -gene 308A/A polymorphism was not found. The frequency of allele A TNF- α -gene 308 polymorphism in control group was higher than that of case group. Statistical analysis shows no significant difference in the incidence of TNF- α -gene 308A/G polymorphism between case and control group (p=0,6).

Serum level of TNF- α based on TNF- α - gene 238 and -308 polymorphisms

Table 2 below shows serum level of TNF- α based on TNF- α - gene 238 and -308 polymorphisms.

Table 2. Serum level TNF- α based on genotype of TNF- α -238 and 308

Promoter region	Allel TNF- α		p¶
	GG	AG	
- TNF- α -238	3,56 (0,00 - 332,56)	22,42 (3,56 - 107,87)	0,020
- TNF- α -308	3,89 (0,00 - 332,56)	2,36 (1,51 - 31,93)	0,600

§ Median (minimum – maximum)

¶ Mann-Whitney test

Data on table 2 shows that serum level of TNF-α in subjects with TNF- α gene -238A/G polymorphism (n=5) has higher level of TNF- α than that of without polymorphism (n=145) with significant difference (p=0,02). Serum level of TNF- α in subjects without TNF- α gene -308 polymorphism (n=146) has higher level of TNF- α than that of with TNF- α -308A/G polymorphism (n=4) but the difference was not statistically significant (p=0,6).

Distribution of TNF- α gene promoter -238 and -308 polymorphisms

TNF- α -gene 238 polymorphisms was found in 5 subjects (3 NASH and 2 central obesity (-)). There was no difference in TNF- α -gene 238 polymorphisms between groups (p = 0,1). TNF- α -gene 308 polymorphisms was found in 4 subjects (1 NASH, 2 central obesity (+), 1 central obesity (-)). There was no difference in TNF-α -gene 308 polymorphisms between groups (p = 0,1). DNA sequencing of TNF- α gene -308 G/A, -238 G/A, -245 T/C and wild type is picturized in figure 2.

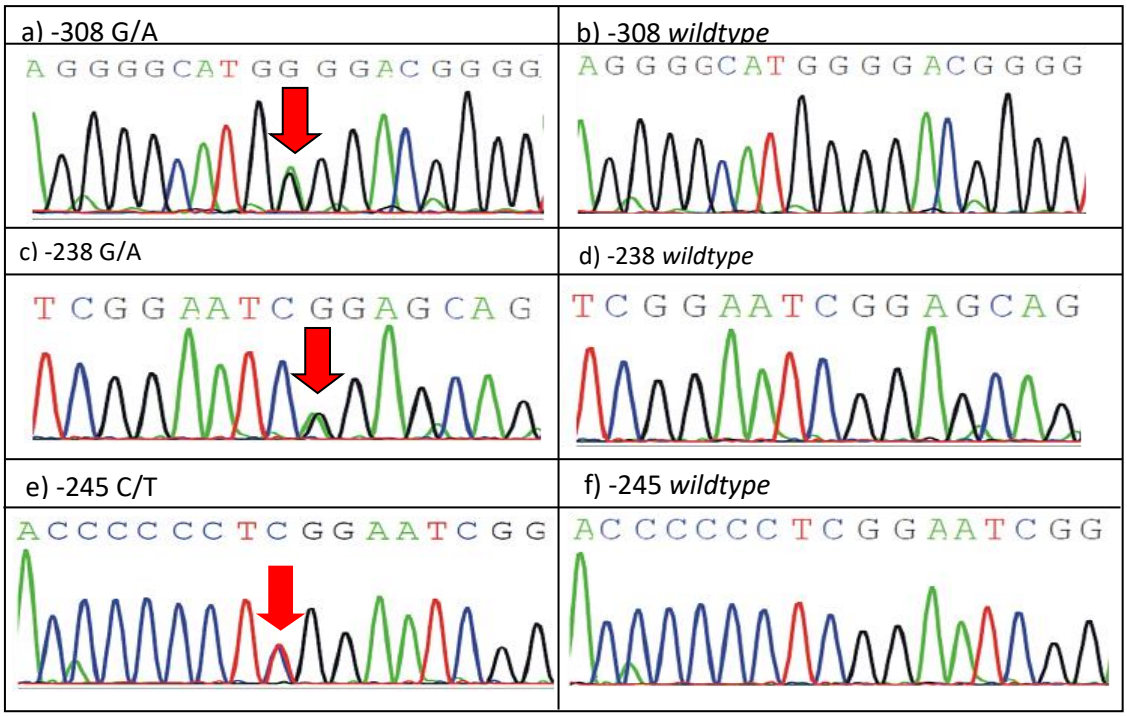

Figure 2. DNA sequencing chromatogram of gene polymorphism and wild type of TNF-α -308, -238, and -245

As seen in figure 2, sequencing chromatogram shows the presence of polymorphism (red band) (overlapping band/ heterozygote) in TNF- α –gene promoter -308 G/A (a), TNF- α -gene promoter -238 G/A (c), and TNF- α -gene promoter -245 C/T (e). Figure b,d and f consecutively are the wild type of TNF- α -gene promoter -308, -238 dan -245.

Table 3. Risk factor of NAFLD

Risk factor	Group		Odd Ratio (95% confidence interval)	p*
	Case n=75 n (%)	Control n=75 n (%)		
TNF- α (pg/mL)				
> 3,645	58 (76,3%)	18 (23,7%)	10,8 (5,1 s/d 23,0)	< 0,001
α 3,645	17 (23,0%)	57 (77,0%)		
TNF- α -gene 238A/G Polymorphism				
Present	3 (60%)	2 (40,0%)	1,5 (0,2 s/d 9,4)	0,700
Absent	72 (49,7%)	73 (50,3%)		
TNF- α -gene 308A/G Polymorphism				
Present	1 (25,0%)	3 (75,0%)	0,3 (0,03 s/d 3,2)	0,600
Absent	74 (50,7%)	72 (49,3%)		

* α ^{2} test

TNF-α and TNF-α gene polymorphism as risk factor of NAFLD

Analysis of TNF-α level TNF-α gene polymorphism as risk factor of NAFLD can be seen in table 3 Subjects with serum level of TNF-α > 3,645 pg/mL are 10,8 times more likely to develop NAFLD compared to those whose level of TNF- α □ 3,645 pg/mL. As listed in table 3, TNF-α > 3,645 pg/mL is a risk factor for developing NAFLD (p < 0,001). On the other hand, TNF- α gene polymorphism was not proven to be risk factor of NAFLD.

TNF-α and TNF-α gene polymorphism as risk factor of NASH

Analysis for risk factor of NASH is listed on table 4. Non NASH group consists of combination of subjects with suspected NASH, simple steatosis, central obesity (+) and central obesity (-).

Table 4 . Analysis for risk factor of severity of NAFLD

Risk factor	Group		Prevalence ratio (95% confidence interval)	p
	NASH n=29 n (%)	Non NASH n=126 n (%)		
TNF- α (pg/mL)				
> 9,41	18 (39,1%)	28 (60,9%)	3,9 (2,0 s/d 7,5)	< 0,001*
α 9,41	11 (10,1%)	98 (89,9%)		
TNF- α -gene 238A/G Polymorphism				
Present	3 (60,0%)	2 (40,0%)	3,5 (1,6 s/d 7,7)	0,020¥
Absent	26 (17,3%)	124 (82,7%)		
TNF- α -gene 308A/G Polymorphism				
Present	1 (25,0%)	3 (75,0%)	1,3 (0,2 s/d 7,6)	0,700¥
Absent	28 (18,5%)	123 (81,5%)		

* α ² test n=75 healthy control by ultrasound, n=80 cases by liver biopsy (Nafld activity score)
¥ Fisher-exact test

TNF- α -gene 238A/G polymorphism has prevalence ratio 3,5 (1,6 s/d 7,7) which means subjects having TNF-α -gene 238A/G polymorphism are 3,5 times more likely to develop NASH compared to those without TNF-α -gene 238A/G polymorphism. Based on 95% confidence interval, TNF- α -gene 238A/G polymorphism was concluded as risk factor of NASH. TNF- α -gene 308A/G polymorphism was not considered as risk factor of NASH based on the analysis despite the prevalence ratio (4, 9)

DISCUSSION

This study was first performed in Indonesia nonalcoholic fatty liver disease patients linked to the level TNF- α, severity and polymorphism gene.

Tumor necrosis factor- α is well-known as major cytokine that plays important role in the occurance of liver damage, including fatty and inflammatory liver disease (steatohepatitis) [10, 11]. Studies investigating about TNF- α gene polymorphism in NAFLD have been showing conflicting results. Several studies reporting the correlation of TNF- α polymorphism and susceptibility of NASH[11-14] yet there were also other studies not supporting the theory [8, 15]. This study focused on 2 polymorphisms located in region -308 dan -238

The findings in our study were inconsistent with previous studies by Valenti et al (2002) and Murillo et al (2011) in a population of Mexican and Italian that reported gene polymorphism [9, 12, 13, 16]. This study reported TNF-α -238 as risk factor of NASH /severity of NAFLD, whereas TNF-α gene polymorphism-308 was not a risk factor of NAFLD. In contrast to previous research results by Aller et al (2010) in Spanish population TNF-α gene polymorphism -308 was found to be associated with histopathologic changes of the liver [13]. Tokushige (2007) reported in the Japanese population polymorphism associated with TNF-α increased blood levels of TNF-α [8]. Additionally Tokushige also reported TNF-α gene polymorphism positions -1031, -863 instead of the -857, -308 and -238 associated with progression of NAFLD (incidence of NASH)[12]. The results of the meta-analysis by Wang et al (2012) in 7 case-control study TNF-α gene polymorphisms and 8 case-control studies concluded; TNF-α gene polymorphism -238 was a risk factor NAFLD, whereas gene polymorphism TNF-α-308 was not a risk factors NAFLD [17]. Murillo at al (2011) also reported gene polymorphism TNF-α -238 associated with the onset of sensitivity NASH [12].

In accordance to the previous studies, we found that serum level of TNF-α is significantly higher in subjects with TNF-α gene -238 polymorphism compared to those without TNF-α gene -238 polymorphism. There was no difference in serum level of TNF-α between subjects with and without TNF-α gene -308 polymorphism. TNF-α gene -238 polymorphism is not only related to the increase of serum level of TNF-α, but also to the occurence of insulin resistance [8]. TNF-α has been reported to cause trauma and apoptosis of liver tissue, play role in netrophil chemotactic, activation of stellate cells of the liver and related to insulin resistance locally in liver tissue or systemically [18]. Crespo et all (2001) reported significant increase of TNF-α expression and receptors in liver and increase of TNF-α expression in adipose tissue in NAFLD patients with obesity [19].

Ethnical variety determines the different roles of metabolic and sosio-demography risk factors in NASH [20], therefore further study involving different ethnicity is needed to confirm the pathogenesis. This study has shown that TNF-α polymorphism in Indonesian population (specifically Javanese-dominant) is different from Kaukasian population. It is suggested to conduct a larger study (multicenter) to prove that TNF-α gene -238 A/G polymorphism is a risk factor of severity of NAFLD (risk factor of incidence of NASH) considering the significantly high level of TNF-α was found in subjects with TNF-α gene -238 polymorphism.

This study also found one subject with 2 (doubles) polymorphisms (haplotypes) that have the highest severity of NAFLD (NAS score = 7). In addition, it was also found in this study 1 subject with novel polymorphism, that is TNF- α - 245 T/C with clinical characteristics showing high level of insulin resistance and very low level of adiponectin (1401 ng/cc). Further study with a larger sample is suggested to explain better the consistency of the relationship.

The limitation of this study were tools for determining the presence of fatty liver in case group and the healthy control were not the same, liver biopsies were performed only in the case group, while the healthy control group with ultrasonography, this potentially arising the measurement bias.

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The Effect of Stress on IL-17 Levels in an OVA-immunized Mice Allergic Model

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ABSTRACT

Objective: Evaluate the effect of stress on eosinophil count, levels of cortisol and levels of IL-17 in an OVA-immunized Mice Allergic model. **Method:** Eight teen female BALB/c mice divided into 3 groups. Negative control in group 1, Allergy control in group 2 group received ovalbumin sensitization only and stress treatment in Group 3 received ovalbumin sensitization and stress using water-immersion stress test on day 24th, 26th and 30th. All mice are terminated on 31st day, lungs and blood sample are collected and measured for eosinophil count microscopically, level of cortisol and level of IL-17 using ELISA. **Results:** Mean eosinophil count for group 1 is 0.48 per high power field (hpf), group 2 is 2,13 hpf, group 3 0.8 hpf. Mean cortisol level for group 1 is 9.13 ng/mL, group 2 is 10.85 ng/mL, group 3 25.47 ng/mL. Mean IL-17 level for group 1 66.71 pg/mL, group 2 is 22.36 pg/mL, group 3 is 26.38 pg/mL. **Conclusion:** The number of eosinophils in the allergy control in group 2 (2.13/hpf) was significantly higher than the negative control in group 1 (0.48/hpf) (p = 0.004). Levels of cortisol in the stress treatment in group 3 (25.47 ng/mL) were not significantly different from the allergy control in group 2 (10.85 ng / mL) (p = 0.180). Levels of IL-17 in the stress treatment in group 3 (26.38 pg / mL) did not differ significantly from the allergy control in group 2 (22.36) (p = 0.394).

Keywords: Allergic Rhinitis, Ovalbumin, Stress, Cortisol, IL-17

Prevalence of allergic diseases continue to increased up to 30-40% of the world population, with 10-30% of which suffer from allergic rhinitis with annual cost about 1.6 4.9 billion USD in the United States of America.(1)(2) Stress can worsen allergic symptoms by activation of *Hypothalamic-Pituitary-Adrenal* (HPA) axis and increased the cortisol levels. Cortisol influenced the immune system into a dominant T helper 2 (Th2) cell condition with increased in Th2 cytokines such as Interleukin 4 (IL-4), IL-5 and IL-13 that result in increased eosinophils infiltration and levels of Immunoglobulin E (IgE) thus lowering the success rate of immunotherapy, although the exact mechanism is still unknown.(3)(4)(5)

Recent pblication on allergy has reported that other factors come into play in allergy mechanism that is the activity of *regulatory* T cells [T*reg* CD4 + CD25 +] that produce IL-10, TGF-ß1 (*Transforming Growth Factor Beta-1)* and Th17 cells that produce IL-17 which is a pro-inflammatory mediator that can cause local inflammatory processes.(6) Research on relationship between stress and allergy has been reported that stress can increase levels of IL-4, IL-5, IL-10, IL-13, and worsen allergy symptoms and lower the success rate of allergic rhinitis treatment causing disturbance in daily activity that can lead to frustration, low self-esteem and even depression.(7)(8)(9)(10)(11)(12)(13)

Stress in allergy also affect HPA axis and increase cortisol levels. Cortisol then can reduce the activity of Th1 thus creating a more Th2 dominant condition in immune system that can lead to worse allergic rhinitis manifestation.
(14)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25)(26)(27)(28)(29)

Recent research on allergy now focusing in IL-7, a pro-inflammatory cytokine that can reduce neutrophil infiltration and increase eosinophil infiltration that lead to local inflammation in the upper airway.(30)(31)(32)(33)(34) Local inflammation of the upper airway has been related to the increased IL-17 levels in the lungs tissue(35)(36)(37)(38)(39)(40) and also blood serum of allergic patient was related to worsening of symptoms, more drugs usage,

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elevated eosinophil count and worse immunotherapy result.

Animals allergic models used in interventional allergy research can help explain the mechanisms of various process that affected allergy. Stress simulation method such as electrical shock, immobilization, or water stress test can increased levels of the hormone cortisol in the blood[5]. Species that most often used as allergic models BALB/c mice with sensitization using ovalbumin given intraperitoneal and inhalation. Caution should always be used in conclusion before the result can be applied to human.(41)(42)(43)(44)

Hypothesis of this study are i) the number of eosinophils count in allergy control in group 1 is higher than in mice without allergy group, ii) cortisol level in stress treatent in group 3 is higher than in allergy control in group 2, and iii) IL-17 level in stress treatment in group 3 is higher than in allergy control in group 2.

The purpose of this study is to provide evidence of elevated eosinophil count in the allergy control in group 2 compared to the negative control in group 2, and also to provide evidence of elevated cortisol and IL-17 levels in the stress treatment in group 3 compared to allergy control in group 2.

This study is required to established evidence for effective methods of sensitization to create an allergic mice models, evaluate the effectiveness of the water stress test protocol in creating stress in mice and to explore the relationship between stress and IL-17 levels that both seem to influence the worsening of allergic symptoms.

MATERIALS AND METHODS

This is an experimental posttest only control group design with laboratory animals using BALB/c mice. This study is done in Biology Laboratory of Faculty of Science in State University of Semarang and ELISA examination done in GAKI laboratory of Medical Faculty of Diponegoro University Semarang in June 2016 for 30 days. Number of sample are 18 BALB/c mice according WHO criteria in which minimum of 5 mice per group is considered sufficient. Inclusion criteria are female BALB/c, 6-8 weeks of age, weight 22-25 grams, with exclusion criteria of sick mice, and drop out criteria of death mice.

All samples are then divided according to simple random sampling into 3 groups, in which negative control in group 1 in which mice are not sensitized and not given stress treatment. Allergy control in group 2 which mice are sensitized but not given stress treatment. And Stress treatment in group 3 mice are sensitized and given stress treatment.

Sensitization were given by injecting intraperitoneally a mixture of ovalbumin 10µ and 2 mg of *$AL(OH)_3$ in 0,2 mL normal saline* on day 1^{st} , 7^{th} and 14^{th} followed with inhalation of 1% ovalbumin on day 19^{th} – 22^{nd} for 30 minutes daily. (7)(19)(20)(45)(46) Stress treatment were given using Water-immersion stress test, done by immersing individual mice in a glass jar with the size of 25cm x 12cm x 25 cm filled with 15 cm deep of room temperature water (22±3°C) for 6 – 10 minutes on day 24^{th}, 26^{th} and 30^{th} .(47)

Samples are obtained on day 31^{st} with taking lungs samples to check for the eosinophil count microscopically, blood samples obtained from retrobulbar to evaluate the cortisol levels using Cortisol (mouse/rat) ELISA kit from Biotech and IL-17 levels using Mouse IL-17 Quantikine ELISA kit from R&D Systems in GAKI laboratory of Medical Faculty of Diponegoro University Semarang, Central Java, Indonesia.

Statistical analysis

Data obtained in this study are primary data of eosinophil count, cortisol levels and IL-17 levels. Results are expressed as the mean ± standard deviation (SD). Data were tested for normality with Saphiro-Wilk test and found to have abnormal distribution. Difference between means were analyzed with Kruskal-Wallis and Mann-Whitney test with significance of $p \leq 0,05$ and 95% confidence interval, and 80% power, using SPSS system.

Ethics

This study used animal as research subject and treated according to animal ethics, in which the animal kept at cage with sufficient airflow, food and drinks during research. Analgesia are used when blood samples are obtained and before termination. Ethical clearance had been obtained from Ethical Review Board of Diponegoro University with document number: 531/EC/FK-RSDK/2016.

RESULTS

Effects of Ovalbumin Sensitization on Eosinophil count

To evaluate the sensitization process, the eosinophil count was performed from the lung tissue of the mice in all group. The statistical analysis done to compare means from allergy control in group 2 and negative control in group 1, as shown in table 1. The eosinophil count in allergy control in group 2 (2.13/hpf) is higher than negative control in group 1, with p=0.004.

Effect of Stress on Cortisol Levels

To evaluate the result of water stress test, cortisol levels was measured from blood serum of the mice in all group using Cortisol (mouse/rat) ELISA kit from Biotech. The statistical analysis done to compare means from stress treatment in group 3 and allergy control in group 2, as shown in table 2. The cortisol levels in stress treatment in group 3 (25.47 ng/mL) is higher than allergy control in group 2, with p=0.180.

Effect of Stress on IL-17 Levels

To evaluate the result of water stress test, cortisol levels was measured from blood serum of the mice in all group using Mouse IL-17 Quantikine ELISA kit from R&D Systems. The statistical analysis done to compare means from stress treatment in group 3 and allergy control in group 2, as shown in table 3. The IL-17 levels in stress treatment in group 3 (26.38 pg/mL) is higher than allergy control in group 2, with p=0.394.

DISCUSSION

Effects of Ovalbumin Sensitization on Eosinophil count

Results from table 1 shows there was a significant higher means of eosinophil count in allergy control in group 2 compared to negative control in group 1. This suggests that the sensitization process in this study managed to increased the means of eosinophils count found in lungs of the allergy control in group 2. This is consistent with several studies of allergy mice models that also use BALB / c mice with intraperitoneal and inhaled ovalbumin sensitivity.(7)(19)(20)(45)(46)

The increased means of eosinophil count in the allergy control in group 2 occurred due to ovalbumin sensitization given from intraperitoneal injection and ovalbumin inhalation, causing eosinophilic inflammation in the peri bronchial region causing the airway hyperresponsivity state.(19)(20) Thus, the sensitization technique done in this study can be used in subsequent research that requires model of allergic mice.

The means of eosinophils count in the Stress treatment in group 3 were higher compared with the negative control in group 1, but lower than the allergy control in group 2. This is because cortisol is a steroid hormone in the glucocorticoids group that can decreased eosinophils count by inhibiting eosinophil survival, increasing eosinophil apoptosis but inhibiting neutrophil apoptosis. Therefore, in a conditions of airway inflammation and increased glucocorticoids in certain levels, will lead to decreased of eosinophils count but at the same time increased the of neutrophil count.(48)

Increased of neutrophils count in the upper respiratory tract has been shown to play a role in worsening asthma symptoms through airway obstruction mechanisms due to epithelial damage and tissue remodeling. The neutrophils count in the airway could still be elevated despite treatment with high doses of corticosteroid, and it has been proven in laboratory setting that corticosteroids can improve neutrophil survival by reducing neutrophil apoptosis.(49)

This is consistent with findings in this study, where eosinophils count in stress treatment in group 3 were lower than the allergy control in group 2, however in this study the neutrophils count is not measured.

Effect of Stress on Cortisol Levels

Results from table 2 shows that the cortisol levels in stress treatment in group 3 is higher than in allergy control in group 2 but the difference is not significant with p=0.180. This suggests that the Water-immersion Stress Test protocol as described by Gupta can increased cortisol levels in stress treatment in group 3[47].(47) However, the difference was not statistically significant could be due to time of stress exposure is not enough. This suggests that the Water-immersion Stress Test protocol if it were going to be used in subsequent research to produce stress should be further investigated, by modification of different time duration of stress exposure.

Interestingly, cortisol level of the allergy control in group 2 were higher than the negative control in group 1. This finding was

contradicting with the results of a Bakkeheim study in which cortisol levels in asthma and allergic rhinitis were lower than those of healthy subject[51].(50)

Effect of Stress on IL-17 Levels

Results from table 3 shows that the IL-17 levels in stress treatment in group 3 is higher than in allergy control in group 2 but the difference is not significant with p=0.394. This suggests a tendency to increase levels of IL-17 in allergic conditions that experience stress. This is in accordance with the results of Miyasaka and Viswanathan research where in patients with asthma who experienced psychological stress there is an increase in IL-17 which results in a decrease in response to glucocorticoid therapy and result in worsening of symptoms as a result of airway inflammation associated with increased neutrophil[52.53].(51)(52) Limitations This study did not measure the number of neutrophils, and other confounding factors that could affect IL-17 levels such as IL-4 and IL-6.

CONCLUSION

The number of eosinophils in the allergy control in group 2 (2.13/hpf) was significanly higher than the negative control in group 1 (0.48/hpf) (p = 0.004), thus the first hypothesis was proven. The cortisol levels in the stress treatment in group 3 (25.47 ng/mL) were not significantly higher than the allergy control in group 2 (10.85 ng/mL) (p=0.180), thus the second hypothesis was not proven.

IL-17 levels in the stress treatment in group 3 (26.38 pg / mL) were not significantly higher than the allergy control in group 2 (22.36) (p = 0.394), thus the third hypothesis were not proven.

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Correlation between HPV Vaccination and Cervical Cancer Incidence in Southeast Asian Population

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ABSTRACT

Human Papillomavirus (HPV) is the most common sexually transmitted disease of genital tract that may cause cervical cancer, the second most frequent type of cancer in South East Asia. By far, HPV vaccination is widely used for risk reduction; however, the rate of developing cervical cancer post-vaccination is still not well-studied. The aim of this study is to evaluate the association between HPV vaccination and development of cervical cancer in Southeast Asia. Analysis of data on HPV vaccination in Southeast Asia was performed, based on literature from 2010 to 2016 accessible in PubMed, Google Scholar, and ScienceDirect. Vaccination coverage rates and changes in cervical cancer incidence in particular countries were subjected to comparative analysis using Pearson's correlation coefficient. The statistical analysis showed HPV vaccination coverage and cervical cancer incidence has negative correlation but not significant ($r=-0.04$, $p>0.05$). This might due to HPV vaccination introduction is still at early stage (<10 years of implementation). In addition, 5 out of 9 countries are running the vaccination program as pilot project rather than nationwide program. Other factors may also influence the incidence of cervical cancer such as: genetics and lifestyle factor, socioeconomic status as well as having many children. Nevertheless, follow up study is needed to assess effect of HPV vaccination introduction and coverage to cervical cancer incidences in Southeast Asian countries.

Key words: *Cervical Cancer, HPV, Southeast Asia, Vaccine*

Human Papillomavirus (HPV) is the most common sexually transmitted disease of genital tract that has high risk cause cervical cancer.(1) Most of HPV infected do not have any symptoms/asymptomatic. However, the persistence of HPV infection may result in the development of cervical cancer.(2)

Current estimates of cervical cancer in the world in 2012 indicate 527.624 diagnosed annually and 265.672 are died from this disease. That makes cervical cancer as the fourth ranks cause of cancer death in the world.(3) While in Southeast Asia, cervical cancer become the second most frequent type of cancer, and become one of the highest incidence rates of cervical cancer in the world with 50.566 incidences and 23.989 number of deaths annually in 2012.(1)(4) In fact, data from World Health Organization (WHO) said that cervical cancer in Southeast Asia is contributing to nearly 35% of the global burden of disease which become major public health program.(5)

Human Papillomavirus (HPV) associated with cervical cancer is considered as carcinogenic to human that based on the strong, consistent, and persistent between infections with the diseases.(6)(7) HPV vaccination is proved significantly reduces the HPV related disease; cervical cancer and provide high protection against it.(5)(2)

HPV vaccine works by stimulating antibody-mediated immunity, that will make neutralizing antibody before HPV virus infects the host cells by recognizing and inactivating it.(8) The vaccines that are available in the market: Cervarix, Gardasil, and Gardasil 9. Cervarix, only works for HPV types 16 and 18. Gardasil vaccine have the ability to high level of protection from types

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6, 11, 16, and 18. While Gardasil 9 gives additionally protection against HPV types 6, 11, 31, 33, 45, 52, and 58.(1)(8) Both types of vaccines are approved in around 100 countries globally and have a known duration of protection of at least 5 years. However, since Gardasil 9 is still newer vaccine, it still has not been acceptable by WHO and approved in Asian countries yet.(1)

WHO recommended HPV vaccine should be implemented as national immunization programs and strategy by government in order to prevent cervical cancer. The strategy itself including training the health workers, give education, sexual, and reproductive health information to the people specially females about diagnosis, screening, and treatment of HPV related with cervical cancer, introduction to HPV vaccination as cervical cancer prevention. Especially, increased access to screening and treatment services.(9)(2)

Besides cervical cancer, HPV vaccine is found out to be effective in prevent other cancers from anogenital tract such as anus, vagina, penis, vulva, and oropharynx.(10)(8)

Therefore, HPV vaccination is offer simple and effective strategy widely to reduce the risk of cervical cancer. However, the rate of developing cervical cancer post-vaccination is still not well-studied. The objective of this review is to evaluate the association between HPV vaccination and development of cervical cancer in Southeast Asia.

METHODS

Data Collection

Analysis of data on HPV vaccination in Southeast Asia was performed, based on literature from 2010 to 2016 accessible in PubMed, Google Scholar, and ScienceDirect, using the following keywords: "HPV vaccination"," Cervical Cancer", and "Southeast Asia".

For each included study, the following key information was extracted: country, vaccine coverage, vaccine status, year of introduction, and incidence of HPV (see appendix A1).

Statistical analysis

Vaccination coverage rates and change in cervical cancer incidence in particular countries were subjected to comparative analysis using Pearson's correlation coefficient with R software.

Table A1. Results of data collection

Country	Vaccine_Coverage	Vaccine_Status	Vac_Year	B_Inc	A_Inc	delta_inc
Brunei Darussalam	93	Y	2012-2015	20	16.9	-3.1
Malaysia	94.33	Y	2010	16	15.6	-0.4
Vietnam	96	Pilot	Pilot	20.5	10.6	-9.9
Cambodia	77.8	Pilot	2015	38	23.8	-14.2
Indonesia	76.6	Pilot	2012	16	17.3	1.3
Lao PDR	76.9	Pilot	Pilot	17	12.5	-4.5
Philippines	70	Y	2015	21	16	-5
Singapore	8.8	Y	2010	13	8.1	-4.9
Thailand	60	Pilot	2014	19.5	17.8	-1.7

Vaccine_Status: Y = National Program; Vac_Year : Year of vaccine introduced; B_Inc : Incidence rate before Vaccine introduced; A_Inc: After vaccine intro; delt_inc : differences

RESULTS AND DISCUSSION

In this study, HPV vaccination coverage and incidence of cervical cancer of 9 Southeast Asia countries were collected (Brunei, Cambodia, Indonesia, Lao PDR, Malaysia, Philippines, Singapore, Thailand, and Vietnam). There are only nine out of eleven countries in Southeast Asia were subjected. Since there are no available data for Myanmar and Timor Leste and the HPV vaccine in those two countries have not being introduced yet even become their national program.(4)

Five countries in Southeast Asia still in pilot program for HPV vaccine; Cambodia, Indonesia, Lao PDR, Thailand, Vietnam. Those countries that are still in pilot program may influence the data quality and may affect to the result. While the other four countries; Brunei, Malaysia, Philippines, and

Singapore already have their national program. In 2010, Malaysia and Singapore are being first countries that introduced HPV vaccination programs in ASEAN.(1)(11)

In the end, the result from statistical analysis showed HPV vaccination coverage and cervical cancer incidence in Southeast Asian population has negative correlation but not significant (r=-0.04, p>0.05). It might be due to limited data since there are two countries that are being eliminated; no data available from Myanmar and Timor Leste, five out of nine countries (Cambodia, Indonesia, Lao PDR, Thailand, Vietnam) are running the vaccination program as pilot project rather than nationwide program, while only four countries (Brunei, Malaysia, Philippines, and Singapore) have their nationwide program for HPV vaccination, M alaysia and Singapore the first countries that introduced it in Southeast Asia.

In addition, several factors may also influences the result; first the HPV vaccine in developing country, the database system that record medical activity is not well established and other factors like genetics and lifestyle factor, socioeconomic status as well as having many children. Such factors might produce less statistically powerful result. Moreover, to achieve more powerful result, establishment of a system to record medical activity is required. In order to analyze in more reliable and valid data to evaluate association between HPV vaccination and cervical cancer more deeply

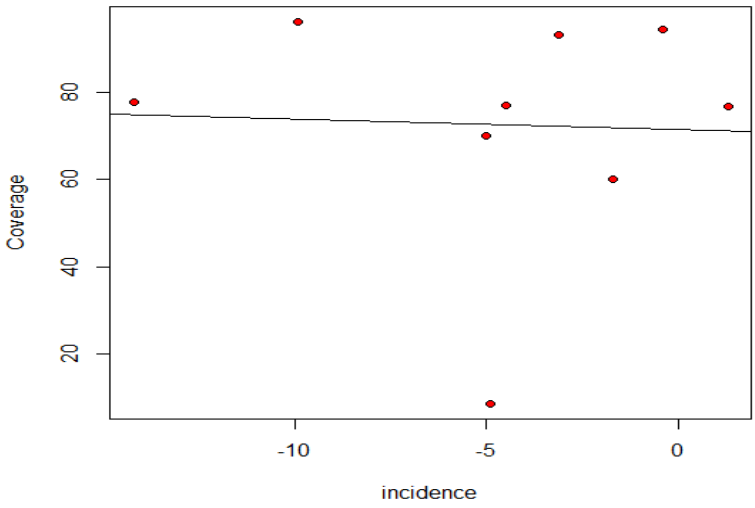

Figure A2. Scatter plot of HPV vaccination against cervical cancer

CONCLUSION

Overall, the analysis showed it is not really significant result. Since HPV vaccination introduction is still at early stage (<10 years of implementation). Nevertheless, follow up study is needed to assess effect of HPV vaccination introduction and coverage to cervical cancer incidences in Southeast Asian countries

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Polymorphism in 4'-UTR Region of *PITX2* in Vertical Mandibular Symmetry

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ABSTRACT

Study on specific genetic pathways of condylar phenotype variation related to vertical mandibular asymmetry remain rare. *PITX2*, a gene active in the Nodal Pathway that determines the left-right symmetry during embryogenesis, has been reported in expression and regulation of skeletal-muscle development as well as differentiation of satellite cells in adult muscle. The aim of this study is to analyze the phenotypes of expressed *PITX2* and its polymorphisms in vertical mandibular asymmetry based on skeletal and dental analysis. Pre-treatment panoramic radiographs from selected 62 orthodontics patients (20.7 ± 3.2 year old) were analyzed using Kjellberg symmetry Index. Subdivision of malocclusions that are limited to Angle's classification was recorded. DNA material was obtained using buccal swabs, followed by Polymerase Chain Reaction (PCR) and Sanger sequencing with ChromasPro 2.13 software (Technelysium, Queensland, Australia) and then compared to archival data from gene bank number ENSG00000164093 (www.ensembl.org).Genotype analysis showed 3 polymorphisms (rs72554076, rs761511445, rs372257787) in 4'-UTR of 16 subjects (25.8%) with various vertical mandibular asymmetry causing a C>A change at 47-105 in 13 patients, G>A change at 47-9 in 1 patient and G>T change at 46+100 in 2 patients, respectively. The characteristics of vertical mandibular asymmetry and canine subdivision dominated in these subjects. Our findings suggest that complex polygenic trait of vertical mandibular asymmetry should consider *PITX2* polymorphisms that related to muscular disorder.

***Keywords**: PITX2, vertical mandibular asymmetry, subdivision malocclusion*

Mandibular asymmetry is a common craniofacial deformity related to asymmetrical muscular function of jaw movement which results from asymmetric growth of mandibular condyle or certain diseases affecting the facial growth.(1)(2) There are vertical and rotational growth patterns in mandibular asymmetry related to mandibular undergrowth or overgrowth. The deformity often worsens with time as the imbalance between affected and unaffected sides is progressing.(3) As the primary center of mandibular growth, the condyle undergoes a remodeling process, responding to continuous stimuli during jaw movements.

There were some documentations about

Abbreviations: PITX2, Paired-like homeodomain transcription factor2; NSP, Nodal Signalling Pathway

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specific growth factors or other signaling molecules expressions in the regulation of mandibular morphogenesis related to condylar growth. However, asymmetrical jaw function could alter the intra-articular mechanical dynamics which could persist or renew the muscular activities in one or both of the condyles.(4) The major focus of skeletal muscle regeneration research will help the clinician to understand the developmental of muscle program at the genetic, cellular, and molecular levels.(6)

The muscular compensatory mechanism might be responsible for asymmetrical ramus height found on both sides of the subjects with malocclusions. The expression of *PITX2* as a mandibular compensatory mechanism to the asymmetry of the middle third of the face in subjects with posterior asymmetry in the vertical dimension of facial growth was studied.(7) There was suggestive association ($p<0.05$) with *PITX2* identification in 3-dimensional dentoalveolar phenotypes in subjects with malocclusion analysis.(8) The Angle classification of class II division 1 has significantly higher condylar asymmetry index compared to class II division 2, class III, and normal occlusion group. The malocclusions could be a predisposing factor of asymmetric condyles if left untreated.(9)(2)

Genetic factor, as well as environmental factors, plays an important role in the etiology of skeletal anomalies. A better understanding of the genetic variables contributing to the skeletal anomaly should be developed as a new preventive strategy in personal orthodontics treatment.(10) Based on twin studies conducted to show the genetic effects in dentofacial skeletal characteristics, the heritability of skeletal characteristics appears stronger than dental characteristics. Genetic components have stronger influence on variability in vertical dimension compared to sagittal dimension.(11)(12)

The development of molecular biology can help the clinician to recognize various genes contributing to the shape, size, and acceleration of mandibular growth. Determination of candidate genes in particular skeletal variability is related to the polygenic nature of craniofacial traits, which is involved in the formation of any malocclusion with mandibular asymmetry.(3)(11)(13) Single nucleotide polymorphisms are valuable genetic markers to reveal the evolutionary history, while common genetic polymorphisms could explain the heritable risk of common disease or anomaly,such as mandibular asymmetry.(14)

Panoramic radiograph is a routine radiograph for diagnosing mandibular asymmetry and is relatively more reliable in vertical measurements.(15)(5) The phenotype of variation in skeletal asymmetry was obtained by Kjellberg's method as it was easier to perform in terms of identifying and measuring points in vertical mandibular asymmetry.(16)(17) The extended period of mandibular growth and its rigid attachment to the maxillary located in cartilage of the condyle-fossa relationship are responsive in any biophysical environmental changes, including orthodontics treatment.

Previous study reported that differences in the NSP of *PITX2* promoted the development of left-right patterning of mesoderm and endoderm during embryogenesis and remained in masseter muscle in adults.(18)(7) Therefore, phenotype-genotype correlation studies of vertical mandibular asymmetry are greatly needed as the fundamental concept to understand the mechanisms responsible for malocclusions and craniofacial anomalies. This research was conducted to analyze the phenotypes of expressed *PITX2* and its polymorphisms in vertical mandibular symmetry based on skeletal and dental analysis.

MATERIALS AND METHODS

This is a cross-sectional study conducted on a selected 62 orthodontics patients (20.7 ± 3.2 years old) of Dental Hospital Faculty of Dentistry, Universitas Sumatera Utara from July 2016 to March 2017. The study protocol was reviewed and approved by the ethics committee of Medical Faculty, Universitas Sumatera Utara (100/DATE/KEPK FK USU-RSUP HAM/2017). All pre-treatment panoramic radiographs taken by the same X-ray machine (AUGE series, Asahi Roentgen, Japan) were measured by using an x-ray reviewer. The following contours (mandibular line, angle, ramus and notch, and condylar process) were marked on transparent paper on the x-ray film with a pencil and measured according to the Kjellberg's symmetry index.(15) PCR analysis was done in the Integrated

Laboratorium of Medical Faculty, Universitas Sumatera Utara and sequencing was performed by the 1st base Laboratories, Selangor-Malaysia.

Experimental inclusion

The subjects with poor quality panoramic radiographs and dental casts were excluded. Based on medical records, the exclusion criteria includes previous orthodontic treatment or prosthodontics history, congenital history, facial trauma, incomplete denture except third molar, and oral bad habits history. The 62 subjects, regardless of temporomandibular joint status, were recalled for genotyping analysis. Samples of DNA material were collected by buccal swab with wooden stick (Nesco®).

Problem formulation

Selection of samples for vertical mandibular symmetry was based on Kjellberg's symmetry index that less than 93% difference categorized as asymmetry (Figure 1). The symmetry between the right and left condylar was estimated using the following formula:

$$\text{Kjellberg Symmetry Index (SI)} = \frac{\left(\frac{CH}{RH_A}\right)}{\left(\frac{CH}{RH_B}\right)} \times 100$$

Molar and Canine classification was verified by asymmetric malocclusions in sagital based on Angle's subdivision classification on pre-treatment dental cast analyze (Figure 2). To assure validity and reliability of intra-rater digitized panoramic radiograph measurements, *Cohen's Kappa* was performed. There is no requirement to assure consistency in scoring of the occlusal parameters since dental cast was used as indicator.

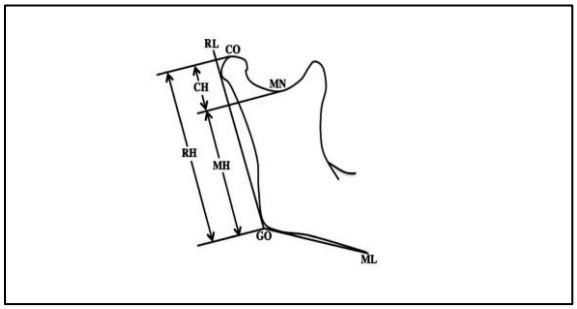

Fig. 1. Measurement of vertical mandibular symmetry with Kjellberg's technique

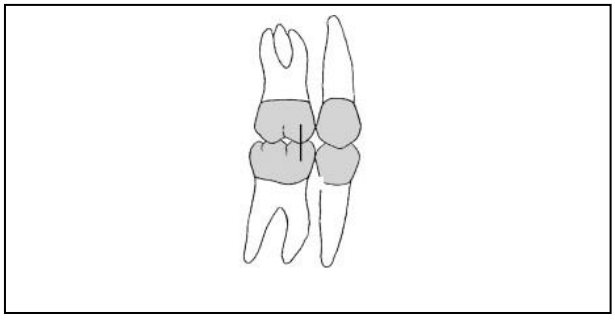

Fig. 2. Classification of Angle's Molar and Canine subdivision

Genomic analysis to obtain Patients' DNA using Presto TM (Geneaid, Taiwan) with buccal swab method. The following primers were used: Region A forward 5'-AATCTCTGCTGACGTCACGT and reverse CCAGACTCGCATTATCTCAC-3'; Region B forward 5'-TAGTCTCATCTGAGCCCTGC and reverse TTCTTGCGCTTTCGCCCGA-3'; Region C forward 5'-CTTGACACTTCTCTGTCAGG and reverse AAGCGGGAATGTCTGCAGG-3'; Region D forward 5'-CAGCTCTTCCACGGCTTCT and reverse TTCTCTCCTGGTCTACTTGG-3'; Region E forward 5'-GTAATCTGCACTGTGGCATC and reverse AGTCTTTCAAGGGCGGAGTT-3'. PCR was performed at 55°C annealing temperature. Amplified products were separated on

agarose gels, reamplified, separated on 3% agarose gels, purified with KAPATaq™ Extra Hotstart readymix with dye (Kapa Biosystem) and sequenced directly with CLUSTAL 2.1 multiple sequence alignment. Chromatograms were visualized with Chromas Pro 2.13 software (Technelysium, Queensland, Australia) and compared to archival data from gene bank number ENSG00000164093 (www.ensembl.org).(19)

RESULTS

In this study, the author sequenced the *PITX2* coding region and intron–exon boundaries of 62 selected multi-ethnic patients who came for orthodontics treatment and received no prior orthodontics treatment. The finding showed 16 subjects with PITX2 polymorphisms. The study identified 3 polymorphisms (rs72554076, rs761511445, rs372257787) in 4'-UTR with various vertical mandibular asymmetry, both near (in close proximity of the) splice junctions. The SNPs rs72554076 caused a C>A change at 47-105 in 13 patients (Figure 4). The SNPs rs761511445 caused a G>A change at 47-9 in 1 patient (Table 1) and SNPs rs372257787 caused a G>T change at 46+100 in 2 patients (Table 1). The clinical data of phenotype including vertical mandibular asymmetry and subdivision of molar and canine classification can be observed on Table 1. The higher score of phenotype characteristics were found in subjects with compound mutation and new rs number that have never been reported before.

Table 1. Clinical data of SNPs of *PITX2* and phenotype vertical mandibular symmetry

Subject	Base	SNPs	Sequence	Age	Vertical mandibular symmetry (0=Symmetry; 1=Asymmetry)	Molar Symmetry (0=Symmetry; 1=Asymmetry)	Canine Symmetry (0=Symmetry; 1=Asymmetry)
3	46+100	G>T	CAGCGAGGGGCGCTTCCC	22	1	0	1
10	47-105	C>A	CCTCTTTCTCCTCCGGCC	23	1	0	0
15	47-105	C>A	CCTCTTTCTCCTCCGGCC	15	1	0	1
20	47-105	C>A	CCTCTTTCTCCTCCGGCC	24	0	1	1
24	47-105	C>A	CCTCTTTCTCCTCCGGCC	22	1	1	1
29	47-105	C>A	CCTCTTTCTCCTCCGGCC	11	1	0	0
30	47-9	G>A	CCTCTTTCTCCTCCGGCC	25	1	0	1
51	47-105	C>A	CCTCTTTCTCCTCCGGCC	24	1	0	0
59	47-105	C>A	CCTCTTTCTCCTCCGGCC	16	1	0	0
62	46+100	G>T	CAGCGAGGGGCGCTTCCC	24	1	0	0
85	47-105	C>A	CCTCTTTCTCCTCCGGCC	23	1	0	1
105	47-105 184+11 184+23	C>A G>A G>A	CCTCTTTCTCCTCCGGCC TAAGGCCGGGAGGGAAGC GGAAGCGCAGGCCGCGCG	21	1	1	1
112	47-105	C>A	CCTCTTTCTCCTCCGGCC	19	0	0	1
122	47-105 46+80	C>A C>A	CCTCTTTCTCCTCCGGCC CTGGCCCTGCGGCGAGGC	23	1	1	1
126	47-105	C>A	CCTCTTTCTCCTCCGGCC	18	1	1	1
142	47-105	C>A	CCTCTTTCTCCTCCGGCC	16	1	1	0
TOTAL					**14**	**6**	**10**

DISCUSSION

The complexity of mandibular asymmetry explains, in part, the reason of why most treatment approaches for malocclusion are directed to the symptoms rather than to its etiology. In our study, we hypothesized that an asymmetric malocclusion may be the result of dental arch asymmetry development due to skeletal asymmetry in the maxillofacial skeletal complex. Remodelling the condyle and the glenoid fossa in mandibular deviation suggests that the asymmetry may be an adaptive response to functional demands. However, the study of genetics is fundamental to understand the underlying biology of craniofacial growth and dental relations.(11)(20)

Bio-molecular explanation of the original DNA coding (A, T, C or G) for certain locus in a genome showed that vertical mandibular variances had specific genotype locations. There are natural variations in DNA sequence, which means a specific gene on a

locus can vary even within homologous chromosomes in the same individual. The mode of inheritance describes how the genetic information is passed down through "allele" which forms homozygotes or heterozygotes from one generation to another. Inspite of these specialized cell types, most cells within an individual's differentiate into muscle, nerve, skin cell, etc, or become an organ based on the pattern of genes that are turned "on" or "off" within each cell. The activation process of the gene is referred as "gene expression" which mostly lead to production of protein or a set of related proteins. (21)(10)

Previous studies have shown that malocclusions have a remarkable effect on condyle morphology in mandibular asymmetry and vice versa. The high prevalence of moderate and severe asymmetries were reported in 327 Caucasian subjects aging between 8 to 12 years old when comparing condylar and gonial angle on both sides of the mandible based on panoramic radiograph.(22) A clinically significant prevalence of molar and canine subdivision relationships was reported in Kuwait and hungarians adolescents.(23)(13)

Based on previous studies regarding vertical mandibular asymmetry, the measurement of asymmetry were various and no specific 'gold standard' for the threshold norm value in each population was found. This research was a basic novel research combining skeletal and dental phenotype with wide genome analysis of vertical symmetrical zone that has never been conducted on Indonesian population with its diverse demographic background with similarity in seeking orthodontics treatment.

The clinical data of SNPs (rs72554076, rs761511445, rs372257787) of *PITX2* and phenotype reported that vertical mandibular asymmetry asymmetry and canine asymmetry dominated in vertically. Asymmetries between both sides of the mandible may be due to an adaptive response of the mandible to deviations during functions, which may cause modelling of the condyle and glenoid fossa, as well as remodeling and modelling of the mandibular bone. The process would then lead to dimensional differences of size or shape between the right and left sides of the mandible.(4) The *PITX2* is a master regulator gene that regulates the production of a protein that binds to specific region of DNA and regulates other genes expressed in the eyes, teeth, heart, and abdominal organs.(12)

In this study, the asymmetry of condylar height was dominated by subjects with polymorphism, except in polymorphism SNPs rs372257787 which caused a G>T change at 46+100 in 2 patients. There was no Molar asymmetry in subjects with G>T change, except in C>A. Moreover, the presence of gene-gene and/or gene-environment interaction in mechanisms of the trait's etiology, partial or incomplete phenotypic approaches and the combined effect of genetic variants in non-coding regulatory regions could also result in missing heritability.(20) In this study, a new mechanism of the regulation of *PITX2* transcriptional activation through the action of *PITX2* isoform was found. This can explain the craniofacial abnormality in non-syndromic patients related to *PITX2* gene expression in muscle function, especially masseter muscle which might influence the development of malocclusion in asymmetrical dental relationship. These new *PITX2* functions on satellite-cell biology might be considered in developing therapeutic strategies for muscular disorders in mandibular asymmetry.(8)

Since the subjects consists of multiethnic patients, further studies regarding ethnicity are required. Determining the effect of different diets on individuals with and without *PITX2* polymorphisms would be of interest as a means to reveal the interaction of genetic and environmental factors. According to previous studies of genetic basis and its relation to orthodontics, orthodontists should be aware of the etiological factors of vertical mandibular asymmetries to determine the best treatment plan for each patient in the era of truly personalized orthodontics.(10)(11)

This data provided by the human genome project have made it feasible to map inherited vertical mandibular asymmetry related to Polygenic "complex" trait since this *PITX2* with its multiple proteins. Identification of *PITX2* polymorphisms might influence dental compensation, especially in the symmetrical zone. Our findings suggest that complex polygenic trait of vertical mandibular asymmetry should consider *PITX2* polymorphisms that are related to

muscular disorder in order to develop interceptive orthodontics.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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Effect of Chronic Organophosphate Poisoning on Attention Deficit and Memory Impairment

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ABSTRACT

Introduction: Organophosphate pesticide poisoning is a common health problem in Indonesia. By inhibiting acetylcholinesterase mechanism, chronic organophosphate poisoning may lead some cognitive consequences included attention deficit and memory impairment. This study investigates the effect of chronic organophosphate exposure in attention deficit and memory impairment. **Method**: A cross-sectional study was conducted in 2 different groups, each consisting of 33 male farmers in Banjarnegara, aged 18-59 years old who are occupationally exposed to organophosphate pesticide for at least 2 years. First group was carried on memory examination using memory impairment screen (MIS) instrument while in second group, attention examination was measured by attention network test (ANT). Vein blood samples analyzed semiquantitatively using Tintometer to know the level of blood acetylcholinesterase (AChE) activity. Data were analyzed using Chi square and Spearman. **Result**: In the first group, 15 (45.5%) samples were found mildly poisioned, while 18 (54.5%) samples were found normal. Among 33 samples, 11 (33.3%) samples have memory impairment. Overall, memory impairment prevalence was higher in sample with mild poisoning, with prevalence ratio of 1,78 (p=0.026). In second group, 54.5% (n=18) farmers were found mildly poisioned while 45.5% (n=15) others had normal AChE activity. Lower AChE activity were significantly correlated with poorer performance in total attention score (r = -0.539; p< 0.00), especially alerting (r= -0.653; p< 0.00) and orienting function (r = -0.632; p< 0.00). **Conclusion.** Chronic organophosphate poisioning seems to have deleterious effects on memory and attention.

Key Words: Organophosphate pesticide, chronic, memory, attention, acetylcholinesterase

Organophosphate pesticide (OP) has toxic property in order to be effective in controlling pest. As its toxic effect, organophosphate has hazardous potential to human and environment.(1) In Indonesia, OP is increasingly used for improving agriculture product quality and quantity.(2) However, this condition is not being followed by proper using of OP.(3) Therefore, Indonesia has high pesticide poisoning rate.

A study had found acute and chronic toxicity in farmers whose exposed by OP. Clinical manifestation of OP pesticide acute poisoning has been well explained and caused by acetylcholinesterase enzyme inbitory. However, study about the correlation of low dose OP chronic expose toward neurobehaviour disruption still inconsistent. Many epidemiological studies said that there is a correlation between OP chronic expose with neurobehaviour disruption.(4–7) As in contrast, other study did not find any correlation between OP chronic expose with neurobehaviour disruption.(8) The aim of this study is to assess the correlation between neurobehaviour disruption, especialy memory impairment and attention deficit in farmers whose exposed by chronic OP pesticide.

MATERIALS AND METHOD

Time and setting of the study

This study was an analytic observational study with cross sectional design. The study was held in Kepakisan village, Banjarnegara in May 2017. Subjects of this study were potato farmers with chronic expose OP pesticide. The test held in the morning in nearby regency office. It was

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choosen for the quiet and comfortable situation of the place so that it will minimize distraction.

Participants

A study was conducted in 2 different groups, each consisting of 33 male farmers in Banjarnegara, who are occupationally exposed to organophosphate pesticide for at least 2 years. An official permission letter had been acquired from nearby village office for cooperation guarantee. Subjects were potato farmers with OP pesticide chronic exposure which had stayed for two years or more in Kepakisan village and had sprayed using OP pesticide within two year or more. Subjects should be male, aged between 18-59 years old, haemoglobin level ≥ 12.5 gr/dl, Body Mass Index (BMI) ≥ 18.5 and at least elementary school graduated or equal. Exclusion criterias was ensured that they had no history or symptoms of any significant disease or conditions including neurologic, kidney and liver; not consuming alcohol habitually; no prescription medication that could inhibit or trigger cholinesterase; and did not agreed to participate in this study.

Tests administered

Subjects that agreed to participate for the research had been explained and asked to sign written informed consent. Furthermore, subjects were asked to fill the questioner about their personal information, job, and medical history. Measuring height and body weight to assess body mass index (BMI). Haemoglobin level evaluation was measured by Haemoglobin Meter. Organophosphate pesticide poisoning rate was assessed by measuring acetylcholinesterase enzyme blood level with Tintometer. Further, subjects were divided into two groups, in group A subject memory was examined using memory impairment screen (MIS), while in group B subject attention level was measured using attention network test (ANT).

RESULT

Table 1. Demographics Status of the Subjects

Characteristics	n (%)		Mean ± SD / median (minimum-maximum)	
	Group A	Group B	Group A	Group B
Age (years)			38.82 ± 8.93 (23-53)	37.73± 10.16 (19 - 57)
Working periode (years)			15 (3-30)	11 (3 - 37)
BMI			23.3 (18-33.6)	24.05 ± 3.26 (18.5 – 32.9)
Hb			14.6 (13.4-17.1)	14.71 ± 0.84 (13.4 – 17.1)
Education				
Junior high school	18 (54.5%)	22 (66.7%)		
Senior high school	13 (39.4%)	10 (30.3%)		
Bachelor	2 (6.1%)	1 (3%)		
Self Protection				
Fully equipped	28 (84.85%)	30 (90.91%)		
Not fully equipped	5 (15.15%)	3 (9.09%)		

Group A

There were 33 subjects in this study which suited the inclusion and exclusion criterias. Potato farmers age mean was 38.82 ± 8.93 with the youngest age was 23 years old and the oldest 53 years old. No subjects had body mass index and haemoglobin level less than normal. Beside that, farmers work time median was 15 years with the least time was 3 years and 30 years the longest. From 33 subjects, there were only 5 subjects used personal general precaution properly.

Group B

In group B, there were also 33 subjects which suited inclusion and exclusion criterias. Subject age mean was 37.73 ± 10.16 with the youngest age

was 19 years old and the oldest was 57 years old. No subjects had body mass index and haemoglobin level less than normal. While, work time variable which had abnormal distribution resulted in median value between 11 years with the least work time 3 years and the longest was 37 years. From 33 subjects, there were only 3 subjects used personal general precaution.

Table 2. Acetylcholinesterase enzyme level examination

Acetylcholinesterase activity	n (%)	
	Group A	Group B
Normal	18 (54.5%)	14 (57.6%)
Low poisoning rate	15 (45.5%)	19 (42.4%)

Group A

Poisoning rate characteristic was assessed with acetylcholinesterase enzyme blood level activity. Normal cut off for acetylcholisnterase enzyme blood level was ≥ 75%, low poisoning 75%-50%, moderate poisoning 50-25%, and severe 25%-0%. There were 15 subjects had low poisoning rate, while other 18 subjects was normal.

Group B

From 33 subjects, 57.6% or 19 people had low poisoning rate, while other 42.4% or 14 people were normal.

Memory and attention examination

Group A

Memory function characteristic measured by memory impairment screen (MIS) score with the scoring was >7.5 (memory impairment) and <7.5 (no memory impairment).(10) There were found 11 subjects had memory impairment (33.3%) and 22 subjects (66.7%) had no memory impairment.

Group B

Attention level characteristic data had abnormal distribution with alerting function median value was 33 which the lowest was -36 and the highest was 141, orienting function median value was 40 which the lowest was -15 and the highest was 138, while median value of executive control was 143 with 48 as the lowest and 442 as the highest. Total attention level median value was 195 with minimal score 27 and maximal score 721.

Correlation between acetylcholinesterase with memory function and attention level

From 15 subjects that had low poisoning rate, 8 subjects encountered memory impairment, while 18 sample were clear from poisoning, only 3 subjects had memory impairment. Hypothesis test used in this study was *Chi- Squared* test, with p value 0.026. There was a significant correlation between acetylcholinesterase enzyme activity category with memory function which the prevalence ratio was 1.78.

Table 3. The attention examination using Attention Network Test (ANT)

Characteristic	Median (minimum-maximum)
Alerting	33 (-36 – 141)
Orienting	40 (-15 – 138)
Executive control	143 (48 – 442)
Total attention	195 (27 – 721)

Table 4. Correlation between acetylcholinesterase with memory function

Acetychilinesterase activity	**Memory catagory**		**p***
	No memory impairment	**Memory impairment**	
Normal	15	3	0.026
Low poisoning rate	7	8	

*RP: 3.18

Table 5. Correlation between acetylcholinesterase with attention level

	Acetylcholinesterase activity	
	Correlation coefficient	P value
Alerting	-0.65	< 0.01
Orienting	-0.56	< 0.01
Executive	-0.20	0.27
Total Attention	-0.47	0.01

There was a negative correlation between acetylcholinesterase blood activity and total attention level, alerting function and also orienting function which the corelation rate consecutively was -0.47 (p = 0.01), -0.65 (p < 0.01) and -0.56 (p= < 0.01). Thus, acetylcholinesterase blood activity had moderate negative correlation rate toward total attention level and orienting function, also high negative correlation rate toward alerting function. Beside that, statistically *executive control* function showed unsignificant low negative correlation rate (r = -0.20; p = 0.27).

DISCUSSION

Blood acetylcholinesterase activity was one of the organophosphate toxication markers which was easy to be measured. The low activity of blood acetylcholinesterase indicated a magnitude of organophosphate effect in inhibiting acetylcholinesterase enzyme.

The result showed that there was a correlation between chronic exposure of organophosphate pesticide to memory and attention function. Based on statistic test, farmers in group A who suffered from low poisoning rate of organophosphate pesticide had 3,38 times chance more often to have memory impairment. It assessed memory function of subject especially recall ability. While in the group 2, the result showed significant negative correlation between acetylcholinesterase and attention especially in alerting and orienting function after tested using Spearman test. This study was in accordance with some previous studies which state that chronic exposure of organophosphate pesticide would cause memory and attention impairment. (4–7)

Malekirad et al in 2013 found decreasing memory, attention, spatial function, psychomotor speed together with inceasing incidence of anxiety, depression and insomnia in chronic-organophosphate-exposured farmers compared to the control. Those neuropsychiatric symptoms called by C*hronic organophosphate-induced neuropsychiatric disorder* (COPIND) as a result of low dose chronic organophosphate exposure without being started with cholinergic syndrome.(10)

The primary mechanism of organophosphate toxicity was the inhibition of acetylcholinesterase enzyme. Aboudonia stated a hypothesis about organophosphorus ester-induced chronic neurotoxicity (OPICN) which caused necrosis and apoptosis of the brain cells through the accumulation of acetylcholin in the central nervous system. The oversimulated acetylcholin receptor (mAChR) would produced excitotoxin which activate NMDA (N-methyl-D-aspartate) subtype of glutamate receptors. It lead to a massive influx of calsium ions to the postsynaptic cell and disturb its homeostasis. Then free radical was produced and the intracelular component was degraded, resulting neuronal degradation.(11) This early lesion then would produce secondary lesion through inflammation and oxidative stress cascade which induced apotosis of the other neuron and escalate the damage.(10) Organophosphate toxicity also having direct effect on increasing oxidant and decreasing antioxidant, leading to neuronal death and gene expression alteration.(12)

Nevertheless, chronic poisoning mechanism was not limited only in that inhibition process, some studies had proven that there were other mechanisms took in charge. As in result, memory and attention function impairment mechanism caused by chronic exposure of organophosphate pesticide was not only limited by acetylcholinesterase enzyme inhibition.(13)

Blood Brain Barier Impairment(14)

In healthy nervous system, blood brain barrier consist of endothelial cells forming a tight junction to detain big molecules. Brain traumatic injury, hipoxia, and other chemical substances, such as organophosphate pesticide would increase this permeability of blood brain barrier.

Cytotoxicity(10,14,15)

Low dose of OP chronic exposure would cause gradually cell death from the effect of free radical formation or *reactive oxygen species* (ROS). Organophosphate pesticide induced mithocondria damage as of morphology disruption and decrease in mithocondria cell amount. This would cause adenosine triphosphate (ATP) depletion and ROS formation enhancement leading to oxidative stress. ROS induced a fatal ATP mithocondria depletion, activated proteolytic enzyme, and DNA fragmentation, at the end leading to cell apoptosis.

Axonal Transport Impairment(15)

Organophosphate pesticide might disrupt fundamental neuron process, axonal transporting. Axonal transporting was responsible in lipid mobilization, mithocondria, synaptic vesicle, mRNA, enzyme, protein receptor, *growth factor,* and some macromolecules transporting from neuronal cell body to cytoplasm troughout axon and otherwise. Axonal transport disruption process was identified in some neurologic diseases such as, *amyotropic lateral sclerosis, Alzheimer's disease, Huntington's disease, and Pick's disease*.

Neuroinflamation(14)

Organophosphate pesticide would provoke gliosis process. Gliosis is an inflammation respond from the brain as a result of neuron damage. Those inflammation respond usually exhibit a hipertrophy and proliferation of glial cell, especially astrocyte and microglia. This microglia activation accompanied by proinflammatory cytokine enhancement.

Yet this result also found an insignificant correlation between blood acetylcholinesterase and executive control. Executive control consist of planning and decision making, assosiated with dopamin activating process in cyngulus anterior cortex, basal ganglia and lateral prefrontal cortex.(16)(17) Those result was different with an experimental study in rats exposed by organophosphate, which the result showed there was a decline in dopamin level inside basal ganglia.(18)

Many factors that could affect this study result were diet, smoking and technical factor, as executive control measurement test had the highest difficulty level task, wherefore it was possible to have understanding biased among the subjects. Beside that, subjects heterogenity in terms of computer operating skill caused wide data variation in which the subject who able to operate computer fluently had shorter measurement time, as in contrast, subject who unable to operate computer had longer measurement time result.

A spesific study that assessed organophosphate effect to each components of attention function still had not been found so that the underlying mechanism was still unclear. The hypothesis still attributed to inhibitory of acetylcholinesterase inhibition and other mechanisms as described before.

Limitation

The limitation in this research was the design of the study, considered to be frail showing correlation between the variables. Cross sectional design was choosen as for the time and distance impediment. Inability to monitor any potential factor that might affect the result optimally, such as computer operating skill, wherefore attention level data had wide variations. Another inadequency was the instrument used in assessing acetylcholinesterase was tintometer and attention measurement instrument had long duration and repetitively.

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Factors That Influence the Quality of Doctor's Services in Children's Diarrhea Cases in Indonesia

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ABSTRACT

Diarrhea is one of the diseases that can increase child mortality rate. More than 2.3 billion cases and 1.5 million children under five years die from diarrhea. Good quality doctors can have positive influence on the quality of service. Outcome of good service is the increased level of health, including reduced child mortality caused by diarrhea. Analysis of the quality of doctors in Indonesia needs to be done to improve the quality of health workers. **Methods:** This study was a quantitative study using secondary data analysis Indonesian Family Life Survey (IFLS) 2007 using cross-sectional design. IFLS 2007 data were collected from 13 provinces. Number of doctors as respondents were 786. Analysis of the quality of doctor was assessed from vignette questions on the health centers block and private practice block by scoring each question that can be answered. Data was analyzed by bivariate test with independen T test using STATA. **Results:** The average correct answer of doctor was 22.25 ± 7.9 out of 56 questions. Doctors who worked for < 6 years had a score of 2.43 points higher than who worked for > 15 years and 2.18 points higher than who worked for 6-15 years. The quality of doctors who received diarrhea training were 2.18 points higher than those who did not. Doctors who worked in eastern Indonesia had an average quality of 3.42 points higher than who worked in Sumatra and 1.84 points higher than who worked in Java-Bali. The work place was not influenced on doctor quality. **Conclusion:** The quality of doctors in handling child diarrhea cases in Indonesia needs improvement. Doctors who have a working period of less than six years, attend diarrhea training and work in eastern Indonesia have a better quality than the other.

Keywords*: diarrhea, quality of doctors, IFLS*

Diarrhea is one of the diseases that can increase child mortality rate. More than 2.3 billion cases and 1.5 million children under five die from diarrhea. According to National Survey 2007, there were 17.2% mortality due to diarrhea in children under 5 years old.[17)] Survey in 2012 data also stated that 65% of children with diarrhea are treated in primary health facilities.[18)]

The quality of human resources for health (HRH) affects the health status in Indonesia, including diarrhea in children.[10)] The low quality of health workers leads to inadequate health services. The outcome of the condition is the declining health status of the community.[4,19)] In Papua New Guinea it was mentioned that 69% of health workers examined only 2 of 4 criteria for the diagnosis of pneumonia, and only 24% of health workers were able to diagnose malaria appropriately. Only 56% of health workers in Pakistan are able to diagnose diarrhea caused by virus, and only 35% can provide standard therapy. These conditions lead to a decrease quality of public health.[15)]

Doctor is one of the important human resources for health. Sang O Rhee et al states that factors that affect the quality of doctor's performance in handling the patient is the participation of doctors in training. The length of a person in participating practice also influences the doctor's performance. Sang O rhee also mentioned that doctors who practice for 6-15 years have better performance compared with doctors who practice less than 6 years or more than 15 years. The location of a doctor's practice also affects the doctor's performance in the provision of health services.[16)]

This study attempts to analyze the various factors that affect the quality of doctor's services, especially in children diarrhea cases in Indonesia. The analysis covers various aspects related to factors influencing the attitude of doctors in Indonesia.

METHODS

It was a quantitative study with cross sectional design and using large-scale secondary data from the 2007 Indonesian Family Life Survey (IFLS) collecting through Survey Meter website. Data were collecting in 13 provinces in Indonesia, consisting of North Sumatra, West Sumatra, South Sumatra, Lampung, DKI Jakarta, West

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Java, Central Java, Yogyakarta, East Java, Bali, West Nusa Tenggara, South Kalimantan and South Sulawesi. The number of respondents were 786 doctors. The independent variables were period of work, participation in diarrhea training, region area (Java-Bali, Sumatra, Eastern Indonesia), workplaces in community health centre (CHC) and private practice, location (underdeveloped - developed area). This study used IFLS 2007 questionnaire data on CHC and private health facility section H (vignette of child health care facility). This data was available on the Survey Meter website in STATA format. In the data there were vignettes that can be used to score the quality of doctors.

We focused on assessments of technical quality for child curative care using clinical case scenarios. A scenario was read aloud to one service provider per facility. Then, the subject was asked with a series of questions about history taking, physical examination, diagnostics, and therapy. The interviewer evaluated the subject's answer based on the clinical guidelines. The scenarios used in the IFLS were pilot-tested before implementation with direct observation to ensure clarity and minimal measurement error. The case scenario methodology has been validated in other settings. The raw scores were expressed as the sum of the criteria spontaneously mentioned as a proportion of the total. The scores aimed to capture knowledge about evidence-based procedures for child curative care.[2] We used all of the item in the scenario.

Data were analysed by using STATA program version 12.1. Bivariate analysis was conducted to explain the relationship between dependent and independent variable.

The study has received ethical approval from the Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine, Gadjah Mada University with Ref: KE/FK/710/EC/2015 dated June 17, 2015. All data processed collected from Survey Meter by first doing registration of personal data on the RAND website.

RESULTS

The study were analysed 786 doctors, most of them (41.6%) have worked for 6-15 years and 60.8 % have received diarrhea training. Most doctors (74.1 %) come from Java-Bali province, 57 % respondents work in private practice and 14.1% work in underdeveloped areas (Table 1).

Table 1. ***Characteristics of respondents (doctor)***

No	Variable	n	%
1	**Working period**		
	6-15 years	327	41.6
	>15 years	298	37.9
	≤ 5 years	161	20.5
2	**Participation in diarrhea training**		
	Yes	478	60.8
	No	308	39.2
3	**Region Area**		
	Java-Bali	582	74.1
	Sumatra	115	14.6
	Eastern Indonesia	89	11.3
4	**Workplaces**		
	Community health center	338	43.0
	Private practice	448	57.0
5	**Location**		
	Underdeveloped area	111	14.1
	developed area	675	85.9

Table 2 showed the average ability of doctors to answer the right questions of anamnesis, physical and laboratory examination questions, therapy, and the ability to answer total questions in vignette. From table 2 it can be seen that the average ability to answer the right questions of anamnesis was 42.3% (11 of 26 questions), about physical and laboratory examination questions was 41.1% (7.62 of 19 questions), about therapy was 32.9% (3.62 of 11 questions), and the ability to answer the total question was 39.7% (22.25 of 56).

Table 2. Average score of doctor's ability in answering question of IFLS vignette

Question's Group (Number of question)	Mean Score of Correct Answer	%
Anamnesis (26)	11 ± 4.1	42.3
Physical and laboratory examination (19)	7.62 ± 3.1	41.1
Therapy (11)	3.62 ± 1.8	32.9
Total (56)	22.25 ± 7.9	39.7

Table 3 showed that there was a significant difference between doctor quality and working period (p <0.05). Doctors who worked < 6 years have a score of 2.43 points higher than who worked more than 15 years and 2.18 higher poin than who worked 6-15 years.

Participation in diarrhea training also affects doctor quality. Doctors who received diarrhea training 2.18 points higher than those who did not follow diarrhea training. Doctors who work in eastern Indonesia have an average quality of 3.42 points higher than who work in Sumatra and 1.84 points higher than who work in Java-Bali. The work place was not influenced on doctor quality.

Table 3. Factors that influence the quality of doctor

Variable	Mean	Mean Different (CI)
Working periode		
– 6-15 years	21.90±7.6	-2.18{-3.67-(-0.68)}**
– >15 years	21.65±7.9	-2.43{-3.96-(-0.91)}**
– < 6 years	24.08±8.3	
Participation in diarrhea training		
– Yes	23.11±7.9	2.18{1.04-3.31}**
– No	20.93±7.8	
Regional		
– Java-Bali	22.28±8,2	-1.84{-3.61-(-0.06)}
– Sumatra	20.69±7.2	-3.43{-5.62–(-1.23)}
– Eastern Indonesia	24.12±6.8	
Workplaces		
– Community health center	21.98±7.9	-0.64{-0.49 - 1.77}
– Private practice	22.62±7.9	
Location		
– Underdeveloped area	22.75±7.0	-0.58{-2.18-1.03}
– Developed area	22.17±8.1	

**: p-value < 0.05

DISCUSSION

The quality of health workers especially doctor is crucial in providing quality health services. Nevertheless, assessing the quality of health personnel is not an easy things to do, especially in Indonesia that has various diversity. Vignette can be an alternative to assessing the quality of health workers.[9,15] In

the IFLS 2007 data there is a vignette of child health that more specifically assesses the quality of health personnel in the delivery of diarrhea health services in children. Questions on the vignette are in accordance with the guidelines and have been in the previous validation.[1,2)]

Sang O Rhee stated that health workers with a working period of 6-15 years have better quality than working period more than 15 years and less than 6 years. While the working period more than 15 years is better than the working period less than 6 years.[16)] Unlike the study, in this study found that doctors have higher score in working period less than 6 years. The results are consistant with John W Peabody's research which said that young doctors (less than 35 years), female doctors working in tertiary services, and specialist perform better than others.[14,15)] Another study by Payne mentioned that doctors with a working period of less than 10 years had better performance compared with doctors with working period of 10-19 years and more than 20 years.[12)]

Doctors who attend the training have better quality than those who did not attend the training. This is in accordance with previous study which states that training influences the improvement of knowledge.[5)] Health workers who have attended the training are able to work more effectively in addition to the latest skills and the competence of health workers can be improved.[7)] Conferences, workshops can not replace the knowledge gained by handling directly in patients. It is therefore important to carry out a combination of training including in practice.[3)]

The regional complexity in Indonesia also influences the research results. Doctors who working in eastern Indonesia have higher score compared to doctors who working in Sumatra, as well as those working in Java-Bali. This condition may be caused by the large number of exposure cases of diarrhea so it can improve the ability of doctors in dealing with diarrhea problems. Yasuko et al mentioned that the more cases handled correlated with the higher ability of doctors in providing health services.[6)] Eastern Region of Indonesia that participating in IFLS 2007 is West Nusa Tenggara, South Kalimantan, and South Sulawesi. The three provinces, especially South Kalimantan and South Sulawesi, have relatively developed area characteristics in an infrastructure that enables better quality of doctors. While the Eastern Region of Indonesia such as Papua, NTT or Maluku which has relatively less conditions in terms of infrastructure facilities did not participate in IFLS 2007.

In this study, there was no difference between physicians working in the public sector compared to health workers that working in private practice. This is in accordance with previous study which states that the quality of public and private health personnel is no different.[11)] But this study is in contrast with the previous study by R Bojalil who stating that health workers in the public sector have better quality than the private sector.[3)]

From the results of this study we found that there was no difference between the quality of health workers in underdeveloped areas compared to health workers in developed areas. A previous study by Ihsan Husain stating that health workers in villages with inadequate infrastructure condition have worse quality compared to health workers in the city.[8)] This is in accordance with the limited facilities and infrastructure, easy access to information and so on.

CONCLUSION

The quality of doctors in handling child diarrhea cases in Indonesia needs improvement. Doctors who have a working period of less than six years, attend diarrhea training and work in eastern Indonesia have a better quality than the other.

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The Roles of Metabolic Syndrome and Several Biomarkers in Incidence and Severity of Non-Alcoholic Fatty Liver Disease

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ABSTRACT

Backgrounds: The prevalence of NAFLD is increasing in the world. The mechanism of pathogenesis and severity has not been clearly understood. Metabolic syndrome and some biomarkers though to play a role in incidence and severity of NAFLD. **Objective:** To clarify the role of the metabolic syndrome and biomarkers: insulin resistance, adiponectin, plasma levels of TNF-α in the incidence and severity of NAFLD. We calculate diagnostic value of the metabolic syndrome with biomarkers and liver function test as non-invasive method of diagnosis severity of NAFLD. **Methods:** Conducted a case control study for risk factors of NAFLD and cross-sectional study for the diagnostic test severity of NAFLD. Cases were NAFLD patients and healthy subjects as controls. NAFLD diagnosis and severity classification based on liver biopsy with NAFLD activity score (NAS). Metabolic syndrome and insulin resistance evaluated based on IDF classification and HOMA IR index. Levels of insulin, adiponectine, and TNF-α were measured by ELISA. **Results:** Eighty cases and 75 healthy controls were included in the study. The independent risk factors for NAFLD significantly were hypo-adiponectinemia, metabolic syndrome, of insulin resistance and high plasma levels of TNF-α consecutively. Hypo-adiponectinemia was proven as independent risk factor and might be potential as diagnostic test severity of NAFLD while the metabolic syndrome, insulin resistance, high plasma levels TNF-α, were not proven as risk factors severity of NAFLD **Conclusions:** The presence of metabolic syndrome, insulin resistance, hypo-adiponectinemia high levels of plasma TNF-α was risk factors of NAFLD, while hypoadiponectinemia was proven to be risk factor and might be as diagnostic test severity of NAFLD.

Keywords: *metabolic syndrome, adiponectine, TNF- α, NAFLD*

Non-Alcoholic Fatty Liver Disease (NAFLD) was the spectrum of lesions in the liver that showed hepatic component of the metabolic syndrome (type 2 diabetes, insulin resistance, dyslipidemia, and hypertension). NAFLD is typically characterised by steatosis macrovesicular, from simple steatosis to the inflammation and persistant lesion will progress into fibrosis and cirrhosis.(1)(2)(3)

Several studies indicated that the prevalence rate of NAFLD and NASH have increased ranging from 17 to 33% for NAFLD and 5.7 to 17% for NASH.(4) Hasan Irsan (2002) reported that NAFLD was found in 30% of the population in Jakarta (5) In dr. Kariadi Hospital Semarang, the

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number of patients with fatty liver has increased by the year. The percentage of fatty liver was 4% in 2005, 4,5% in 2006, 5% in 2007, 6% in 2008 and 7% in 2009.(6) The prevalence is expected to increase in accordance with increasing population of obesity, metabolic syndrome, and development of diabetes.

NAFLD severity is defined as the most severe form of NAFLD with histopathology showing NASH characterized by fatty infiltration of lobular inflammation, hepatocyte ballooning with or without fibrosis (NAFLD activity score ≥ 5).(7) Currently NAFLD progression is also associated with various complications outside the liver. Cardiovascular complication is one of the many conditions associated with NAFLD.(8)

Pathogenesis of NAFLD have not yet clearly understood. The currently accepted explanation is the "Multiple hits hypothesis". Metabolic syndrome and several biomarkers such as: insulin resistance, adiponectin, plasma levels of TNF-α and polymorphisms of promoter gene of TNF-α may be involved.(9)(10)(11)(12)

Recent studies indicated that hypoadiponectinmia was responsible for the hepatic fat accumulation and insulin resistance. Complexity of NASH pathogenesis involves reciprocal role between adiponectin and proinflammatory cytokines produced by mononuclear cells of peripheral blood and infiltration of lymphocytes and macrophages, which are embedded in white fat tissue.(10)(13)

Insulin resistance is a major mechanism in the pathogenesis and progression of NAFLD. However, only a minority of patients with risk factors for NAFLD develop NASH, fibrosis and cirrhosis. It was still unclear why not all patients with insulin resistance develop NASH, allegedly specific changes in the liver responsible for the development of fatty accumulation and progression to inflammation. Studies in families suggest that genetic factors have a role in determining susceptibility to NASH.(14)(15)(16)

The role of TNF-α in NAFLD is crossroad of many pathogenic pathways. Specific differences between patients with hepatic steatosis and NASH is the serum level of TNF-α, which is usually higher in patients with NASH. However, this difference did not always reach statistical significance.(14)

The roles of metabolic syndrome and several biomarkers, such as insulin resistance, adiponectin, plasma levels of TNF-α in the pathogenesis of NASH require further investigation.

Aim of this study was to clarify the roles of metabolic syndrome, insulin resistance, adiponectin and plasma levels of TNF-α as risk factors for the presence and severity of NAFLD, and to analyze the diagnostic value of the metabolic syndrome with several biomarkers as non-invasive methods of diagnosis and early detection of NASH.

MATERIALS AND METHODS

Study designs were case-control to clarify risk factors of NAFLD and cross sectional for diagnostic test of NASH. Study was held in outpatient clinic of dr. Kariadi General Hospital Semarang on January 2009 – December 2011.

Inclusion criteria for case group includes: aged > 14 years old having metabolic syndrome components and proven to have NAFLD based on abdominal ultrasound. Subjects will be excluded if known to have hepatitis A, B, C virus infection (AST and ALT > 5 times higher than normal with positive IgM anti HAV or positive HBsAg or positive anti HCV); autoimmune hepatitis (positive ANA test); alcoholic hepatitis; history of alcohol consumption (> 30 gr/ day for male and > 20 gr/day fpr female); history of taking drugs causing fatty liver (glucocorticoid, estrogen, tamoxifen, amiodarone, metothrexate, valproate, diltiazem).

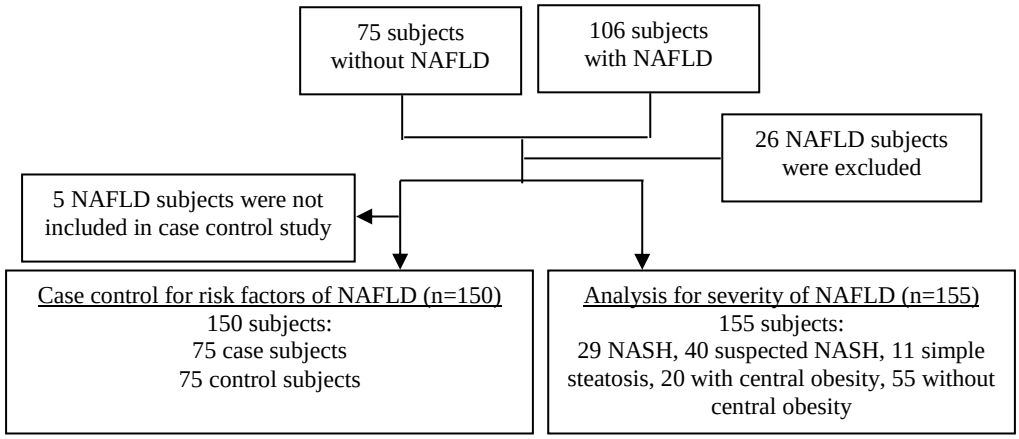
Inclusion criteria for control group includes no history, and clinical symptoms of liver disease (with normal AST and ALT, negative HbsAg, negative anti HCV) and no liver abnormality found on abdominal ultrasound. Subjects will excluded if known to have liver disease by anamnesis, physical examination and laboratory findings; hepatitis virus infection (A,B,C); autoimmune hepatitits, alcoholic hepatitis; history of alcohol consumption; and history of taking drugs causing fatty liver.

Histopathology of liver biopsy in classification according to NAS (NAFLD activity score) was performed in case group to assess the diagnosis and severity of NAFLD.(7) Diagnosis of metabolic syndrome was based on IDF classification,(17)(18) insulin resistance by index HOMA IR,(17)(18) measurement of the levels of insulin, adiponectin, and TNF-α using ELISA method.(19)(20)(21)

RESULTS

Clinical characteristics

During the study period 80 NAFLD cases and 75 controls were enrolled. To analyze risk factors for NAFLD, only 75 subjects in each group were involved in the study (figure 1). Analysis for severity of NAFLD involved all 155 subjects (figure 1).



Characteristic distribution of subjects in case and control groups were showed sex, age years and height did not differ significantly between both groups. (44,7 ±10,28 vs 44,3 ± 9,98 years old, 162,1±8,37 vs 161,1±6,31 Cm). Mean weight, body mass index and waist circumference of subjects in case group was found significantly higher than that of control group 77,0 (54,0 - 115,0) vs 56,0 (41,0 - 74,0) kg, 29,0 (22,0 - 47,4) vs 22,5 (17,3 - 27,5)kg/M2, 99,0 (77,0 - 134,0) vs 80,0 (64,0 - 98,0) cm (p< 0,001) consecutively.

Metabolic syndrome and several biomarkers of NAFLD

Distribution level of 5 components metabolic syndrome (waist circumference, blood pressure, fasting blood glucose , HDL cholesterol, fasting triglyserides) in case and control groups were significantly different (p<0,001). There was a significant relationship between each MS component and the frequency of NAFLD incidence (p <0.001). The majority of subjects in the case group (77.0%) had central obesity, 83.8% hypertension, 100% fasting blood glucose> 100 mg / dl, 78.6% low-level HDL cholesterol and 87.3% hypertriglyceridemia. Based on the number of MS components in the case group with MS, most (42.1%) had four, 44.4 % had three and 17.5% had 5 MS components. Presence of insulin resistance was greater significant in cases (86,2%) compared to the control (13,8%) group. (p<0,001) Median HOMA-IR index and insulin level in case group were higher than that of control group, 3,67 (0,40-29,00) vs 0,62 (0,02-23,58) , 12,40 (0,80-116,56) uIU/ml vs 2,74 (0,09-86,95) uIU/ml consecutively. Statistical test showed a significant correlation between insulin resistance status and presence of NAFLD (p <0,001).

Mean plasma levels of TNF-α in case group was significantly higher than in control group 7,98 (0,00 - 332,56) vs 2,24 (0,04 -77,65) pg/ml (p <0,001), while mean serum level of adiponectin in case group was significantly lower than that of control group, 2148,40 (493,80-9766,40) vs 8351,20 (1080,50 - 22364,00) (p <0,001). Cut off level for TNF-α and adiponectin as risk factors for incidence of NAFLD was > 3645 ng /cc, and < 4512 ng/cc respectively. There was significant correlation between serum levels of TNF-α and adiponectin with incidence of NAFLD (p <0,001).

Risk factors of incidence of NAFLD

Metabolic syndrome, insulin resistance, level of TNF-□ and adiponectin were analyzed for risk factors of incidence of NAFLD. With 95% confidence interval, all four variables were statistically significant as risk factors for the incidence of NAFLD. Highest to lowest odds ratio (OR) consecutively were level of adiponectin < 4511,94 ng/ml, OR 60,4 (21.4 - 170.4), presence of metabolic syndrome, OR 44,3 (15.5-126.8), presence of insulin resistance, OR 21,6 (9.1-51.6) and high level of TNF-□ >3,645 pg/ml,OR 10,8 (5.1-23.0).

Multivariate regression test for risk factors of NAFLD was performed as shown in table 1. Adiponectin < 4511,94 ng/ml and metabolic syndrome were two biggest risk factors for the incidence of NAFLD with adjusted OR 35,5 and 25 respectively

Table 1. Analysis of multivariate regression test for risk factor for NAFLD

Risk factors	*Crude*§ OR	*Adjusted* OR□ (95% confidence interval)	p*
Metabolic syndrome	44,3	25,0 (4,7 - 133,6)	< 0,001
Insulin resistance	21,6	12,4 (2,5 - 61,8)	0,002
TNF- α > 3,645	5,0	6,1 (1,3 - 19,9)	0,020
Adiponectin < 4511,94	60,4	35,5 (7,5 - 167,5)	<0,001

§ OR from bivariate test
□ OR from multivariate logistic regression test
* p value of multivariate logistic regression test

Severity of NAFLD

Severity of NAFLD was based on Histological Scoring System for Non Alcoholic Fatty Liver Disease Score and Fibrosis Staging (NASH activity score = NAS). Histopathology biopsy of the liver was performed in all subjects in case group (75 subject) and the results were NASH (38,7%), Possible NASH (50,7%) and Simple Steatosis (10,6 %). Simple steatosis and possible NASH in case group combined with the normal healthy control group were later categorized as non NASH.

Risk factors of severity of NAFLD

Bivariate analysis showed all clinical characteristics and biomarkers had significant correlation with severity of NAFLD (incidence of NASH) (table 2). Further multivariate regression test revealed that the most significant parameters correlated to the severity of NAFLD were adiponectin <2230,50 ng/ml, Exp(B)=11.5 (3.2-41), p=0.000, ALT >59,5 IU/L, Exp (B) = 8.6 (2.8-26), p =0.000 and waist circumference >95,5 cm, Exp(B) = 6.0 (1.8-20)p=0.003

Table 2. Correlation between clinical characteristic, biomarkers and severity of NAFLD

Clinical characteristic and biomarker	**Group**		**p***
	NASH n=29 n (%)	**Non NASH n=126 n (%)**	
Waist circumference (cm)			
- >94,5	23 (39,7%)	35 (60,3%)	
- ≤94,5	6 (6,2%)	91 (93,8%)	<0,001
Fasting blood glucose (mg/dL)			
- > 102,5	16 (28,1%)	41 (71,9%)	
- ≤102,5	13 (13,3%)	85 (86,7%)	<0,020
Triglycerides (mg/dL)			
- >142,5	23 (32,4%)	48 (67,6%)	
- ≤142,5	6 (7,1%)	78 (92,9%)	<0,001
HDL (mg/dL)			
- <43,5	23 (30,3%)	53 (69,7%)	
- ≥43,5	6 (7,6%)	73 (92,4%)	<0,001
ALT (IU/L)			
- >59,5	20 (54,1%)	17 (45,9%)	
- ≤59,5	9 (7,6%)	109 (92,4%)	<0,001
AST (IU/L)			
- >27,5	24 (37,5%)	40 (62,5%)	
- ≤27,5	5 (5,5%)	86 (94,5%)	<0,001
Homa IR			
- >2,21	23 (34,8%)	43 (65,2%)	
- ≤2,21	6 (6,7%)	83 (93,3%)	<0,001
TNF-α (ng/mL)			
- >9,41	18 (39,1%)	28 (60,9%)	
- ≤9,41	11 (10,1%)	98 (89,9%)	<0,001
Adiponectin (ng/mL)			
- <2230,50	25 (44,6%)	31 (55,4%)	
- ≥2230,50	4 (4,0%)	95 (96,0%)	<0,001

* x^2 test

Risk factor of severity of NAFLD was analyzed by comparing several risk factors: metabolic syndrome, insulin resistance, level of TNF-α and adiponectin. All parameters were statistically significant as risk factors of severity of NAFLD with prevalence ratio as listed in table 3.

Table 3. Analysis for risk factor of severity of NAFLD

Risk factors	Group		Prevalence ratio (95% confidence interval)	p
	NASH n=29 n (%)	Non NASH n=126 n (%)		
Metabolic syndrome (MS)				
- MS (+)	23 (35,9%)	41 (64,1%)	5,4 (2,3 - 12,6)	< 0,001*
- MS (-)	6 (6,6%)	85 (93,4%)		
Insulin resistance (IR)				
- IR (+)	23 (34,3%)	44 (65,7%	5,0 (2,2 - 11,7)	< 0,001*
- IR (-)	6 (6,8%)	82 (93,2%)		
TNF- α (pg/mL)				
- > 9,41	18 (39,1%)	28 (60,9%)	3,9 (2,0 - 7,5)	< 0,001*
- ≤ 9,41	11 (10,1%)	98 (89,9%)		
Adiponectin (ng/mL)				
- < 2230,50	25 (44,6%)	31 (55,4%)	11,0 (4,0 - 30,1)	< 0,001*
- ≥ 2230,50	4 (4,0%)	95 (96,0%)		

* x^2 test

Multivariate regression test for risk factor of severity of NAFLD is shown that Matabolic syndrome Adj OR (95% CI) 3.3 (0.9-11.5), p = 0.060, HomaIR >2.21 AdjOR 1.4 (0.4-4.8), p = 0.600, TNF-TNF- α > 9,41, AdjOR 1.6 (0.5-4.5) , p=0.400, and Adiponectinin < 2230,50 ng/ml, AdjOD 9,6 (2,6 - 34,4),p=0.001. Based on the test result, adiponectin < 2230,5 ng/ml was the most significant risk factor of severity of NAFLD. Results of analysed among clinical characteristic and biomarkers for diagnostic test severity of NAFLD (presence of NASH) proved that adiponectin < 2230 ng/ml has Sensitivity 86 %, Specificity 75%, Positive predictive value 45%, Negative predictive value 96 %, and Accuracy 77%.

DISCUSSION

This study proved the presence of metabolic syndrome, insulin resistance, low adiponectin levels, high plasma levels of TNF-α as independent risk factors for the incidence of NAFLD. Various studies have shown that obesity, type 2 diabetes, dyslipidemia, hypertension and insulin resistance (metabolic syndrome) were associated with NAFLD.[2,3,23] Multiple hit hypothesis that explained the pathogenesis of simple fatty liver to the occurrence of progression to inflammation (NASH, fibrosis and cirrhosis) involves thorough metabolic syndrome components. The first hit is the presence of fatty liver as results from imbalance formation and breakdown of triglycerides.(22)(23)(24)(25)(26) Mitochondrial dysfunction is likely; disorder of beta-oxidase in mitochondria is the main cause of fatty liver. Insulin resistance is a key abnormality underlying the metabolic syndrome, and progression to NASH. Insulin resistance will enable secretion of several adipocytokines (TNF-α, IL-6), if excessive will be dangerous for the liver tissue, changes the speed and transports triglyceride synthesis in hepatocytes and finally

increases lipolysis in adipose tissue that causes exposure to the liver tissue with more free fatty acids.(15)(22)(27)

Other factors such as inflammatory and oxidative stress will aggravate the progression of fatty liver to NASH, fibrosis, and necrosis. Metabolic syndrome is closely related to inflammation and oxidative stress conditions, individuals with metabolic syndrome, there were an increase in lipid peroxidation, IL-6, some adipocytokine eg TNF- α, reactive oxygen species (free radicals) decrease of adiponectin, and will activate stelate cells and liver fibrogenesis.(2)(3)(27)

Multivariate analysis of risk factors for the severity variables of NAFLD showed that only adiponectin which was proven to be an independent risk factor of severity of NAFLD. In accordance with these results, Hui et al (2004) also reported that adiponectin levels of NASH patients were significantly lower compared to patients with simple fatty liver,(28) although there were differences in the criteria used NAFLD degrees where the Hui, et al using Brunt criteria and this study used NAFLD activity score (NAS). Another study by Lemoine et al (2009) also reported a significant difference in adiponectin levels between NASH group with simple fatty liver.(29)

Adiponectin concentrations decreased in patients with obesity, insulin resistance, and type 2 DM and NAFLD, correlated negatively with the degree of fatty liver(30)(31) Hyperinsulinemic condition will lower adiponectin receptor expression and biological activity. Adiponectin has anti-lipogenic effects that would protect non-adipose tissues such as liver and muscle. Adiponectin was hepatoprotective by increasing the sensitivity of liver cells to insulin, which leads to increased adenosine monophosphat protein kinase (AMPK) activity of fatty acid oxidation and the inhibition of the inflammatory process and lowering sterol regulatory element,(32)(33) hepatic cell proliferation and increased apoptosis thus lowering severity NAFLD and antagonists against TNF-α .(34)(35)(36) Decrease in adiponectin causes an increase of inflammatory response characterized with the increased of TNF-α level and fibrosis of the liver tissue.(34)(35)(36)(37)(10)

Adipose tissue is a major presence of macrophage accumulation as the main source of local expression of TNF-α.(37) TNF- α is one of adipocytokine known to have antagonistic effects with adiponectin and contribute to insulin resistance, and recent studies have also alleged role in the metabolic syndrome and the progression / severity of NAFLD.(10)(38) TNF- α in animal model proved to play an important role in obesity and insulin resistance, there was also a link between levels of TNF- α and mitochondrial dysfunction that underlying liver abnormalities in NAFLD and NASH.(14)(39)

This study suggest that adiponectin may potential it is a non-invasive diagnostic parameter for determining the existence of NASH. Although liver biopsy is the gold standard for diagnosis NASH, not easily be done in clinical practice for a variety of reasons accompanying. Expected future examination of adiponectin may be one option for diagnosis NASH.

CONCLUSION

This study showed that the risk factors proven to influence the incidence of NAFLD were metabolic syndrome, insulin resistance, plasma adiponectin levels <4511 ng / ml, plasma of TNF-α levels > 3,645 pg / ml consecutively.

Risk factors proven to affect the severity of NAFLD / NASH events in patients with simple fatty liver was plasma adiponectin levels <2330,5 ng / ml, whereas metabolic syndrome, insulin resistance, plasma levels of TNF- α > 9,41 pg / ml were not proven statistically. Plasma adiponectin levels <2230,5 ng / ml have potential can be used as a non-invasive diagnostic tests of NASH.

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Skipping and Running Improve Short-Term Memory in Young Adults

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ABSTRACT

Background: Aerobic exercise has been reported to improve memory function. Skipping and running are known as sports that easy to do and like by young adults. However, their effect to improve short-term memory in young adults has not been studied yet. **Objective:** to know the effect of skipping and running to improve short-term memory in young adults. **Method:** This study used quasi experimental research design with pre- and post-test unequivalent group. The subjects were medical students of Diponegoro University (n = 80), aged 18-22 years old, who were selected by purposive sampling and divided into 4 groups: skipping 3 times a week for 8 weeks (n=20), group without exercise as a skipping control (n=20), running with music for 30 minutes (n=20), and running only as its control. Short-term memory in pre- and post-test were measured using Scenery Picture Memory Test and data were analyzed using paired t-test, unpaired t-test, Wilcoxon, and Mann-Whitney. **Results:** There was a significant difference in short-term memory ($p<0.05$) after skipping or running either with music or not ($p=0.000$). Short-term memory in skipping and running with music groups were significantly increase ($p<0.05$) compared with its each control, 3.6±2.63 vs 0.95±3.12, and 5.0±2.66 vs 3.05±1.76, respectively **Conclusion:** Short-term memory can be improved by regular skipping exercise and running particulalrly with music in young adults.

Keywords: *aerobic exercise, short term memory, skipping, running*

Memory is the storage of acquired knowledge for later recall. It is one of the fundamental cognitive functions for adapt to human environment.[1] It can be classified into two types: short-term and long-term memory. Short-term memory has limited capacity, but has been proved to present one's intelligence.[2] Other study also proven that short-term memory affects long-term academic achievement.[3] Young adults, for example, college students are known to require good memory function. Short-term memory is required for learning as the beginning of long-term memory formation.[1,4,5]

Previous studies reveal that short-term memory functions can be improved through aerobic exercise.[6,7,8,9] However, about 81% of adolescents aged 11-17 years and 23% of young adults aged 18 years and over in the world are still classified to have lack physical activities.[10] This sedentary life lifestyle may interfere the process of learning in student. Many factors affect the process of memory formation, such as age, genetics, nutrition, hormones, gender, disease, psychology, drugs, and stimulation.[11,12,13–18] One of stimulation that can affect the process of memory formation is aerobic exercise.

Aerobic exercise increases working memory in healthy adults, by increasing vascularization to the brain so that will cause the viability of nerve cells in the brain.[19,20,21] Previous study proves that there is a neurotropic stimulation such as BDNF serum that can increase the volume of hippocampus, the part of the brain that plays a role in memory formation. The other brain structures like gray matter, white matter, and brain glia also show changes.[22]

Skipping and running are included in aerobic sports activities that has been known by all ages group and are favored because of the easy and simple method. Skipping exercises is quite easy, can be done anywhere and anytime. This sport does not depend on location, time or weather, so it

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can be one of the alternative sports in the young age group to improve the quality of lifestyle. [23,24] Running is proven to improve the cardiovascular system function and cognitive function.[25,26,27] Music, especially motivational music with a tempo >120 bpm, has ergogenic properties that is proved to relieve fatigue, improve sports performance, and affect brain activity.[28,29] In this study, we analyzed the effects of aerobic exercise in the form of skipping, running, and running with music as a factor affecting short-term memory as an alternative to aerobic exercise for young adults.

METHODS

This research used quasi experimental method with pre- and post-test unequivalent group design. Subjects were divided into 4 groups; skipping 3 times a week for 8 weeks (n=20), with group without exercise as control (n=20), running with music for 30 minutes (n=20), and running without music as control. This study was done in March-May 2017 among medical students of Diponegoro University, who are selected by purposive sampling with following inclusion criterias: aged 18-22 years, no sedative drugs and such, Body Mass Index 18.50-24.99 kg/m^2, and approving to be the subject of this research. Subjects for skipping and no exercise (control) groups should commit to not doing other sports other than skipping during the period of this research, and able to perform the exercise, i.e. skipping 3 times a week for 8 weeks. Otherwise, the subjects for running with and without music should already used to take regular exercise at least twice a week, and have a normal score on Depression Anxiety Stress Scale 42 (DASS-42), which is 0-9 for depression, 0 - 7 for anxiety, and 0 - 14 for stress scale. The exclusion criteria were subjects with a history of psychiatric disorders, head trauma, systemic infections and diseases, epilepsy, injury or limb disability, and consuming electrolyte and caffeine drinks within 2 hours before a short-term memory test.

Skipping

Skipping has been done indoors with subjects wearing uniform shirts, shoes, and skipping ropes. Warming up and cooling down steps were done for 5 minutes before and after skipping. There were 4 sets of skipping exercises on 1st - 4th week, while on 5-8th week were done as many as 5 sets. 1 set of exercises consisting of 2 cycles. Each cycle was done 30 seconds of skipping and 30 seconds of break. Each odd and even set have different period of rest, i.e. rest for 1 minute on odd set and 5 minutes on even set.

Running

Running has been done outdoors for 30 minutes at Diponegoro University Stadium with moderate intensity or conversational pace of speed, i.e. running by a normal conversation with a little effort comfortably. Running group was divided into 2 groups; running with music and running without music. The music in this research was motivational music type that were selected by subjects (Self-selected music) with Brunel Music Rating Inventory-2 (BMRI-2). BMRI-2 was used to select the motivational quality of the music, with score range was on 36-42 (highly motivated musics). Playlist of 15 selected musics were made on the phone or iPod and played during running for 30 minutes using headsets or headphones with 50% volume.

Scenery Picture Memory Test (SPMT)

Short-term memory was measured using SPMT. It has some advantages, i.e. time effectiveness and ease of understanding from respondents, therefor did not cause floor and ceiling effect. Before the measurement, a forward digit span test is done to outwit the subjects.[30]

Subjects were asked to memorize the picture in 1 minute and mention 23 objects contained on the picture. After 1 minute, a forward digit span test was done to outwit the subject by mention sequence of 1-7 digits, then the subjects were asked to recall those number. Then, subjects were asked to mention objects previously memorized on the SPMT test. Short-term memory on running groups were done before and after exercise, while skipping and its control groups were examined before and after 8 weeks.

Data Analysis

Saphiro-Wilk test was used to analyze the distribution of short-term memory score before and after treatment in all groups. Then, to analyze short-term memory difference between before and after skipping, running, and running with music, Wilcoxon test was used because the post-test data of short-term memory showed non-normal distribution. Meanwhile, unpaired t-test was used to analyze the difference of short-term memory in no exercise group. We analyzed the difference of short-term memory increase between control and treatment groups using Mann-Whitney test because post-test data showed non-normal distribution. The data showed significantly difference if the value of $p<0.05$.

Table 1. Characteristic of subject

Characteristic	Skipping and No Exercise Group		Running and Running with Music Group	
	n(%)	Mean±SD; Median (Min-Max)	n(%)	Mean±SD; Median (Min-Max)
Gender:				
- Male	20(50)		20(50)	
- Female	20(50)		20(50)	
- Total	40(10)		40(10)	
Group				
- Treatment	20(50)		20(50)	
- Control	20(50)		20(50)	
	40(10)		40(10)	
Age		20.53±0.72; 20.50 (19-22)		20.65±0.949; 21 (18-22)
Height		161.88±8.95;160.00 (140-180)		162.335±7.32; 162 (152-182)
Weight		54.65±7.44; 54.00 (42-68)		57.14±8.04; 55.00 (42-78)
Body Mass Index		20.72± 1.59; 21.05 (18.59-22.99)		21.65±1.89; 22.05 (18.6-24.7)
History of psychiatric disorder				
· Yes	0(0)		0(0)	
· No	40(10)		40(10)	
Brain Abnormalities				
· Yes	0(0)		0(0)	
· No	40(10)		40(10)	

Note.SD = Standard deviation; Min = Minimum; Maks = Maximum; n= Total subjects

RESULTS

Characteristic of subjects can be seen in table 1.

Skipping vs without exercise

Short-term memory was measured using SPMT scores in eight-weeks aerobic skipping study. The average of group that performs routine skipping at the initial test is 18.00 ± 3.08 and after routine skipping is 21.60 ± 1.67, with significantly difference (p = 0,000). The control is without exercise group, had an initial SPMT score of 17.75 ± 2.81 and increased after 8 weeks became 18.70 ± 2.68. This group showed a non-significant increase (p = 0,189, see figure 1). The delta of pre-test and post-test between treatment and control group showed a significant difference (p = 0.008, see figure 2).

Running vs running with music

Short-term memory was also done through aerobic treatment which was running with music for 30 minutes and runing for 30 minutes as control. The treatment group had an initial value of 16.45 ± 2.188 and became 21.45 ± 1.932 after running with music for 30 minutes. The Wilcoxon test showed significant difference of before and after exercise score (p = 0,000). The group that runs for 30 minutes also showed an average difference in the initial score of 16.75 ± 2.77 to 19.80 ± 2.387. This difference is significant based on the Wilcoxon test (p = 0.000, see figure 1). Delta between the treatment and control group was tested with Mann-Whitney and showed a significant difference (p = 0.015, see figure 2).

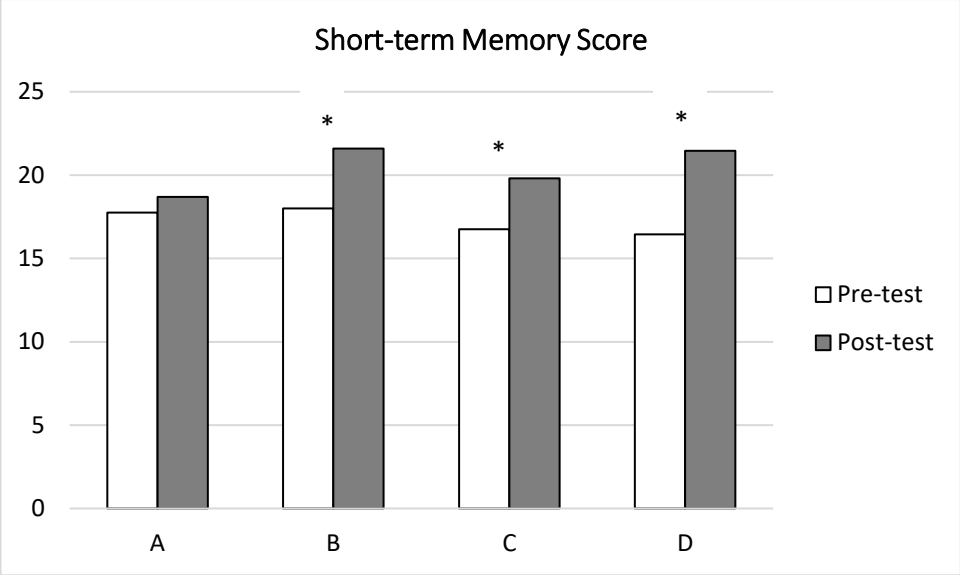

Figure 1. The difference of short-term memory score measured by SPMT between groups; A. No exercise, B. Skipping, C. Running, D. Running with music. *p<0,05

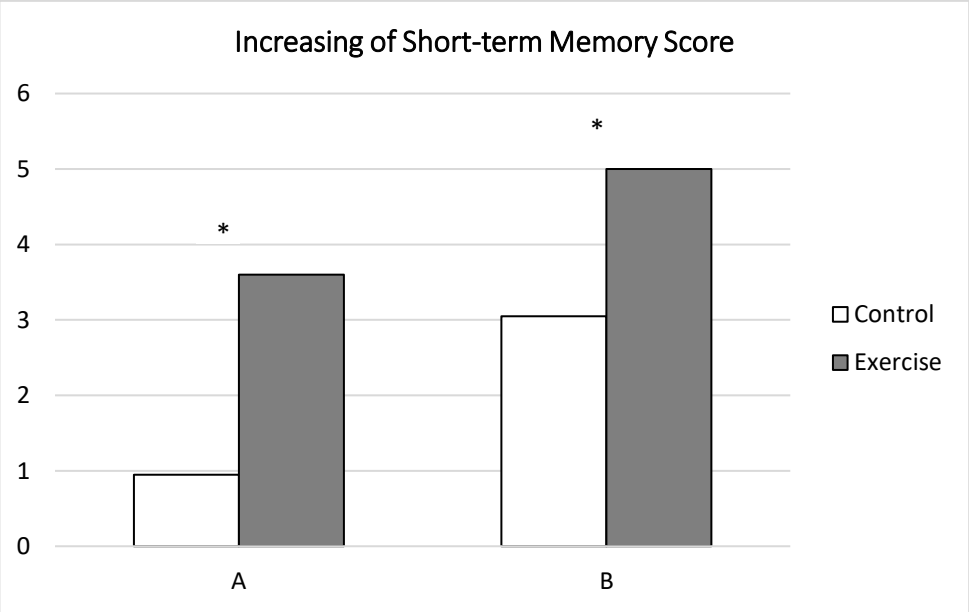

Figure 2. The increasing of short term memory measured by SPMT; A. No exercise (control) and Skipping (exercise), B. Running (control) and Running with music (exercise). *p<0,05

DISCUSSIONS

Results showed significant increase in short-term memory on skipping, running, and running with music group after tested using Wilcoxon and unpaired t-test. This result was in accordance with the hypothesis, which is there is an increase of short-term memory score measured with SPMT before and after treatment. Mann-Whitney and test was done to compare both groups, which showed that skipping group has significantly higher results compared to control group, and running with music group has significantly higher results compared to running as control group.

In this study, both skipping 3 times a week for 8 weeks and 30 minutes of running, as aerobic exercise, significantly improved short-term memory. This was in accordance with previous studies which stated that aerobic exercise, both routine and acute, can improve cognitive function. This happened because aerobic exercise increases cardiovascular function, thus increasing the peripheral and cerebral blood flow. Increased blood flow in hippocampus and anterior cortex will improve neural connections between these areas. As the blood flow increased, oxygen will optimally distributed to neurons and resulting in increasing the viability of neurons.[7,31,32] Aerobic activity of skipping and running also affects the hippocampus as a brain structure that plays a role in memory and learning. Aerobic activity has been shown to increase serum BDNF, neurotropin, which helps improve cognitive function including memory.[8]

This study also showed that 30 minutes running with music significantly improved short-term memory of young adults, and was significantly higher compared to control group. This is in accordance with some previous researches that discusses the influence of music during physical exercise. Motivational music used in this study, which has more than 120 beat per minute, can increase ergogenic effects during physical exercise through increased hormone levels, including cortisol and norepinephrine.[33] Increased cortisol in the blood leading to the catabolism in the muscle, adipose, and connective tissue, so that energy will be produced by the metabolism. Increased norepinephrine levels will stimulate the cardiovascular system to increase heart rate and both peripheral and brain blood flow.[34,35]

The limitation of this study is monitoring other daily life factors that may affect short-term memory, for example, learning activities, playing games, long term nutritional factor, hormonal factor, and history of cardiovascular disease.

CONCLUSION AND SUGGESTION

In conclusion, short-term memory can be improved by regular skipping exercise and running particularly with music in young adults.

This study determines that there is an effect of regular skipping and running in short-term memory on young adults, therefore these exercise can be recommended to young adult group in improving short-term memory performance. Further studies regarding the investigation of the detail mechanisms how skipping and running effect in short-term memory improving are required.

CONFLICT OF INTEREST

Author declare no conflict of interest.

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The Effectiveness of Phaleria Macrocarpa and Chemotherapy in Increasing Caspase 3 and Apoptotic Index in Epidermoid Carcinoma

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ABSTRACT

Chemotherapy management of epidermoid carcinoma of the skin is relatively expensive and has toxic effects on vital organs. *Phaleria macrocarpa* is a medicinal plant that is often used as an anti-cancer. This study aims to prove the effectiveness of the *Phaleria marcocarpa* extract*, paclitaxel-cisplatin* chemotherapy and the combination of both as a *neoadjuvant* to increase caspase-3 expression and apoptotic index in epidermoid carcinoma of the skin in Swiss male mice. This study used male mice of the Swiss strain, after induction of epidermoid carcinoma cells, 4 group randomization, K (control), P1 (Phaleria macrocarpa 0.0715 mg/ day), P2 (paclitaxel 175 mg/m^2 and cisplatin 50 mg/m^2), P3 (the combination of Phaleria macrocarpa and chemotherapy). The caspase-3 expression was examined with immunohistochemical stain and the apoptotic index examination was done by staining the Tunel. The research shows that there is an increase in caspase-3 expression; the order of the increase (from the most significant to the less significant) is P3, P2, and P1 with significant result ($p<0.05$) compraed to the control group. There is a significant correlation between caspase-3 expression and apoptotic index with a very strong positive correlation. It can be concluded that the *Phaleria marcocarpa* extract*, paclitaxel-cisplatin* chemotherapy and the combination of both increased the caspase-3 expression and the apoptotic index of epidermoid carcinoma cell; there was no significant differences between P1 and P2 but there was a synergy effect from the administration of P1 and P2 combination, and there was significant correlation between the caspase-3 expression and apoptotic index with a very strong positive correlation.

Keywords: *Phaleria marcocarpa,* caspase 3, apoptotic index, epidermoid carcinoma of the skin.

Epidermoid carcinoma (squamous cell carcinoma) is a malignant proliferation of epidermal keratinocytes. It is one of the most common skin cancers occur after basalioma. The incidence is estimated at 25% of all skin malignancies. Epidermoid carcinoma is more common in white skin than in color and occurs more in males than in females, particularly people over the age of 40. Incidence of epidermoid carcinoma is elevated with increasing age.(1)(2) The incidence of metastasis as a whole is 2% - 3%. Predisposing factors of epidermoid carcinoma include ultraviolet radiation, arsenic, hydrocarbon, temperature, chronic radiation, scarring, virus.(2)(3)

According to the National Cancer Institute study, the local recurrence rate after primary therapy from epidermoid carcinoma reaches 3% - 23% depending on the location of the anatomy. Approximately 58% of local recurrences manifested in 1 year, 83% in 3 years, and 95% in 5 years .(4) According to the American Cancer Society, the rate of recurrence of epidermoid carcinoma is still high at 2% and 8.9% after extensive excision with excision limit at 2cm from tumor edge, post radiotherapy 7% - 50% and 20% after curettage and electrodesection.(5)

Modality of epidermoid carcinoma therapy is surgery with wide excision and if KGB (+) is found, KGB dissection is performed. Primary radiation is indicated in an inoperable case. Radiation adjuvant is performed on the condition: the boundary incision is not free from tumor, the incision limit is located near the tumor, there is a contamination of the surgical field by tumor cells, lymph nodes contain more than one metastases, and the diameter of the

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KGB > 3cm, and there is a high-grade malignancy.(2)(3)

Primary chemotherapy is performed in cases with indication of distant metastases, inoperable or patients who fail to be treated surgically and radiotherapy. Currently there is a tendency to use neoadjuvant chemotherapy before surgery or radiotherapy to reduce tumor volume and optimize healing. The chemotherapy commonly used is cisplatin, 5-fluorouracil, bleomicin, doxorubicin and paclitaxel. The rate for partial response is 40%-50% and for the complete response is 28%-31% depending on the given regimen.(2)(3)

The management of epidermoid carcinoma of the skin is relatively expensive and there are often toxic effects that can impair the function of some human vital organs.(6) Currently, many alternative therapies are developed in the form of immunotherapy, namely by modulating the immune system against tumors that are expected to kill cancer cells spread systemically after the local definitive treatment.(7) A number of substances which are tested for cancer therapy influence the expression factor and or activity that regulates apoptosis. In the process of cell apoptosis, caspase 3 is the main implementer that can be activated through extrinsic and intrinsic signal pathways.(8)(9)(10)

Phaleria macrocarpa (*mahkota dewa*) is an Indonesian medicinal plants that have been widely used as an anti-cancer drug plants which have been sold on the open market at 5 gram dose per day. *Phaleria macrocarpa* contains *alkaloids, flavonoids, polyphenols, resins, tannins* and others substances that are efficacious as antihistamines, antioxidants, and medicine for rheumatism, diabetes, high blood pressure, and cancer (cytotoxic). Empirical evidence of its usefulness has been found in many societies, but the scientific proof is still very limited and further studies are required.(11)(12)

The purpose of this study is to prove the effectiveness of the *phaleria marcocarpa* extract, *paclitaxel-cisplatin* chemotherapy and the combination of both as a *neoadjuvant* for caspase-3 expression and apoptotic index of epidermoid carcinoma in Swiss mice.

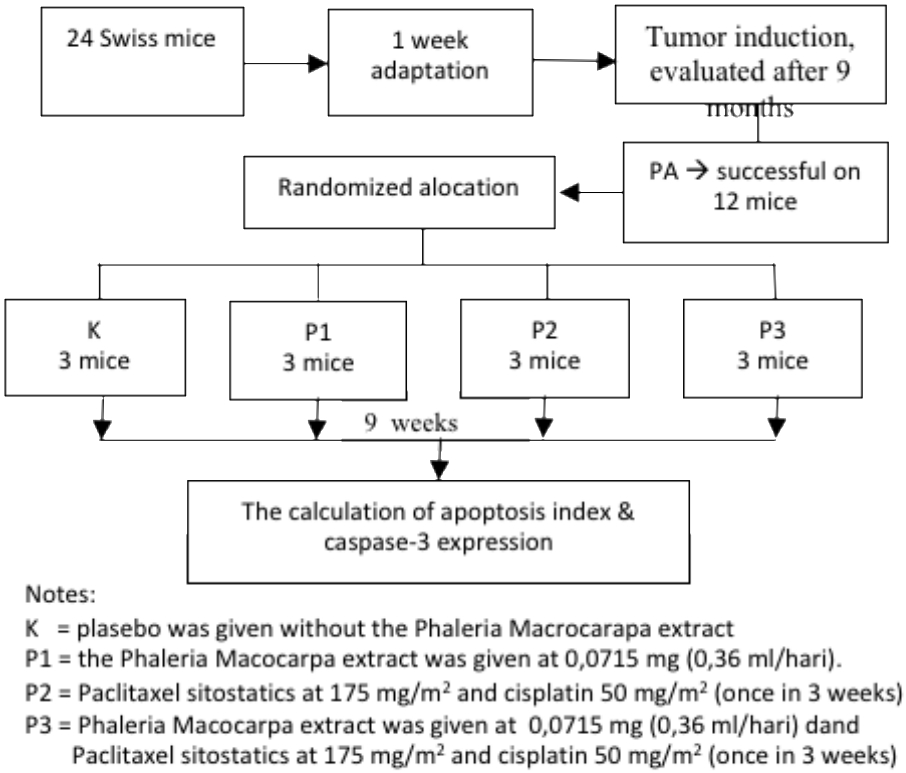
MATERIALS AND METHODS

Twenty four male Swiss mice aged 3 months and weighed 20-30 grams were individually stacked and fed with standard feed for one week with *ad libitum* (at the mice's pleasures) system. Epidermoid carcinoma was induced in the mice's skin by clean shaving the hair in interscapular area for 1.5 x 1.5 cm. The carcinogen used was 9. 12-dimethyl-1.2-benzanthracene (DMBA) 100 nmol (0.025 mg), dissolved in 0.1 ml of acetone reagent per mouse. DMBA was applied 2 times per week for 2 weeks, then continued with topical 12-o-tetradecanoylphorbol-13-acetate (TPA) application in the interscapular region at a dose of 1.7 nmol (0.001 mg) in 0.1 ml aceton per mice tail 2x per week for 22 weeks.

The mice which successfully inoculated and performed PA biopsy with epidermoid carcinoma results, was divided into 4 groups, then they were given treatment for 3 weeks. After the treatment was completed, the mice in anaesthesia with ether and then the mice were exterminated by means of cervical dislocation and the tumor tissues were taken and processed into histologic preparations. Furthermore, Tunel was stained to examine the index of apoptosis and immunohistochemical staining to observe the caspase-3

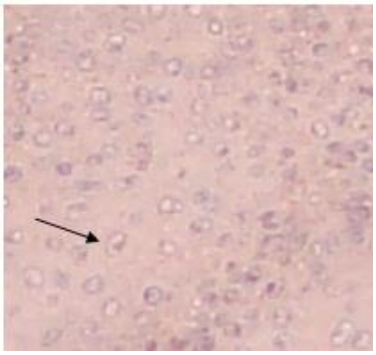
RESULTS

Figure 1. Consolidated research report

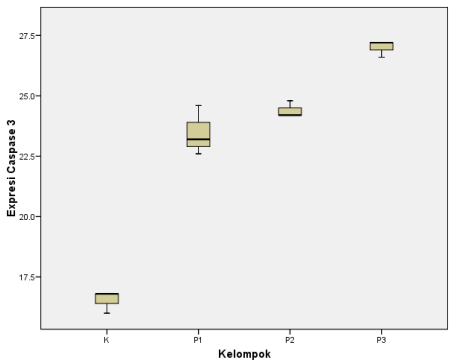


After the treatment, the tumor tissue was taken, and histological examination with immunohistochemical staining with *Caspase 3 (CPP32) Ab-4 rabbit polyclonal antibody RB-1197-PO, NeoMarkers* was conducted to calculate the index of caspase 3 and Tunel staining was performed to calculate apoptotic index (13,14). With immunohistochemical staining on caspase 3, cellular features are called positive if there is a dominant brown apoptotic cell in the cytoplasm and some in the cell nucleus, as well as compared with positive control.(13) Cell provides an overview of apoptosis microscopically on Tunel staining in the form of the apoptotic cells will turn into green fluorescent if observed using a fluorescence microscope with magnification of 400x.(14) Histological examination results are as follows

Figure 2. Histological features of caspase expression 3 (arrow)



Grafic 1. *Box plot* of caspase-3 expression



The normality test using *the Shapiro-Wilk* test shows abnormal data. Furthermore, the *Kruskal Wallis* test shows p value <0.05 or significant, then to observe the difference between groups, *Mann Whitney* test was performed

Table 1. *Mann Whitney* caspase 3 expression

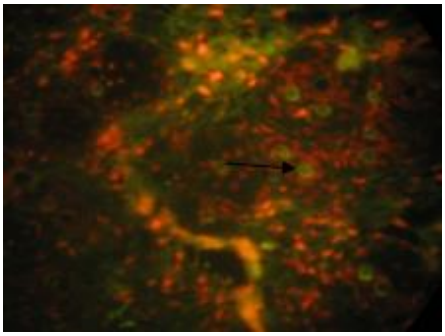
Group	P1	P2	P3
K	0.046*	0.043*	0.043*
P1	–	0.268	0.046*
P2		–	0.043*

note:

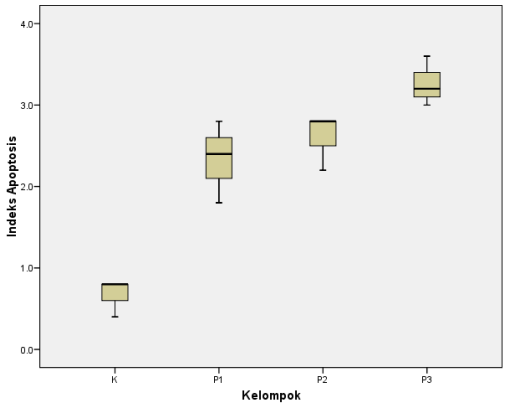
* Significant p<0.05

From table *Mann Whitney* test, it was found that a significant difference (p <0.05) exists between the control group (K) and treatment 1 (P1), treatment 2 (P2) and treatment 3 (P3), between the treatment group 1 (P1) and treatment 3 (P3), between treatment 2 (P2) with treatment 3 (P3)

Figure 3. Histologic picture of apoptosis (arrow)



Grafic 2. *Box plot* of apoptotic index



From the data normality test of apoptotic index with *the Shapiro-Wilk* test, it was found that the obtained data are not normal. Then, *Kruskal Wallis* test shows p value = 0.022 (p <0.05) or significant to determine the difference between groups, *Mann Whitney* test was conducted.

Table 2. *Mann Whitney* Test of Apoptotic Index

Group	P1	P2	P3
K	0.046*	0.043*	0.046*
P1	–	0.487	0.049*
P2		–	0.046*

Note:

* Significant p<0,05

The Mann Whitney test shows a significant difference (p <0.05) exist between the control group (K) and treatment 1 (P1), treatment 2 (P2) and treatment 3 (P3), between the treatment group 1(P1) and treatment 3 (P3), between treatment 2 (P2) and treatment 3 P3).

Meanwhile, regarding with the correlation between the caspase-3 expression and the apoptotic index, the data normality test with *Shapiro Wilk* test shows that caspase 3 expression has p value = 0.025 (p <0.05), which means that the data distribution is not normal, so that for the next test, non-parametric *spearman* correlation test was used

Table 3. *Spearman* correlation of caspase 3 expression and the apoptotic index

Variable	r	P
Expresi Caspase 3 Indeks Apoptosis	0.961	< 0.001*

note :
* Significant p<0,05

The *Spearman* correlation test table shows p value = <0.001 (p <0.05) and the value of r = 0.961, so it can be concluded there is a significant correlation between caspase 3 expression and the apoptotic index with a very strong positive correlation.

DISCUSSION

Phaleria macrocarpa is one of Indonesia's traditional medicinal plants that have anti-cancer effects. However, there is relatively few scientific reference in terms of pharmacology and phytochemical regarding this plant. M*acrocarpa Phaleria* was used as a medicinal plant for anti-cancer and anti-microbial. The empirical evidence of its usefulness has been found in many societies, but the scientific proof is still very limited, so further evidences are needed.(11)(12) As an attempt to prove the effects of *Phaleria macrocarpa* as anti-cancer drugs particularly on the epidermoid carcinoma, this study was conducted. This study aimed to prove the effect of *Phaleria macrocarpa* with indicator caspase 3 expression and apoptotic index of epidermoid carcinoma cells in Swiss mice.

The result shows that there was an increase in caspase 3 expression in all treatment groups which were given *Phaleria macrocarpa* extract*, Paclitaxel-cisplatin* chemotherapy, and the combination of both. The order of increased caspase-3 expression from the most significant increase to the least significant increase is the group which was given a combination of *Phaleria macrocarpa* extract and chemotherapy *paclitaxel-cisplatin* (group P3), followed by the group which was given the chemotherapy *paclitaxel-cisplatin* (group P2) and the group which was given the extract of *Phaleria macrocarpa* (P1). The significant result *(p <0.05)* occurred in all treatment groups compared to the control group (K).

The significant results in the treatment group which were given *Phaleria macrocarpa* extract are closely related to *polyphenols* contained in medicinal plants; *polyphenols* are reported to have the effect of inducing apoptosis through TNF-α (extrinsic pathway*/ extrinsic pathway)* .(10)(15) *Pholyphenol, which constitute as* active substances, contained in *Phaleria macrocarpa* such as *gallic acid* and *flavonoids* play a role in inducing apoptosis; A study using esophageal cancer cells (TE-1) shows that the *gallic acid (GA: 3,4,5-trihydroxybenzoic acid)* would increase the protein pro apoptosis Bax and will decrease Bcl-2 anti-apoptotic protein.(16) Meanwhile, *flavonoids* work by inhibiting the activity of DNA topoisomerase I/II, modulation of signaling pathways, decreasing gene expression of Bcl-2 and Bcl-XL (anti-apoptotic), increasing gene expression of Bax and Bak (pro-apoptotic) as well as the activation of endonucleuses.(17) Topoisomerase, an enzyme that serves to cut the tight winding of DNA is as a result of the opening of *double strand* DNA by enzyme helicase, a U-turn and then connect it again. The enzyme works on the extension of DNA replication. If there is inhibition of topoisomerase activity, there will be stabilization of the cut topoisomerase-DNA complex, resulting in a permanent damage of *double-stranded* DNA which then activates p53.(18)(19) Activation of p53 in response to DNA damage would stop the cycle of mitosis in the G1 phase, so that the cell cannot enter S phase when DNA damage is not repaired, and if the DNA damage cannot be repaired, the p53 will activate protein pro-apoptotic *Bcl-2 Family* as Bid, Bax and Tub which will subsequently lead to caspase-3 inactivation which eventually leads to cell apoptosis.(20)(21)(22)

Bax, Bak, Bcl-2 and Bcl-xl is a Bcl-2 *family* proteins. Bax and Tub are proapoptotic proteins whereas Bcl-2 and Bcl-xl are antiapoptotic proteins. Bcl2 attaches to the outer membrane of the mitochondria thus blocking the release of cytochrome c whereas Bcl-xl binds to Apaf-1. Cytochrome c and Apaf-1 are required in the process of apoptosis through an intrinsic pathway by activating caspase-9. The function of survival is offset by the cell death function mediated by Bax and Bak. Bax may bind to the outer membrane of the mitochondria thus inducing cytochrome c out of mitochondria while Bak may bind with Bcl-xl to release Apaf-1. If the expression of Bax or Bak is raised and Bcl-2 or Bcl-xl is lowered, cell regulation towards the cell death by caspese 9 activation will lead to caspase-3 inactivation leading to cell apoptosis.(15)(16)(21)(22)(23)

Phaleria macrocarpa extract, *Paclitaxel-cisplatin* chemotherapy, and the combination of both is also shown to enhance the apoptotic index when compared with controls. The increase in apoptotic index in the order from large to small were seen in the group which were given a combination of *Phaleria macrocarpa* extracts and *Paclitaxel-cisplatin* chemotherapy (group P3), given *Paclitaxel-cisplatin* chemotherapy (group P2) and the group given the extract *Phaleria macrocarpa* (P1). The significant result *(p <0.05)* occurred in all treatment groups compared to the control group.

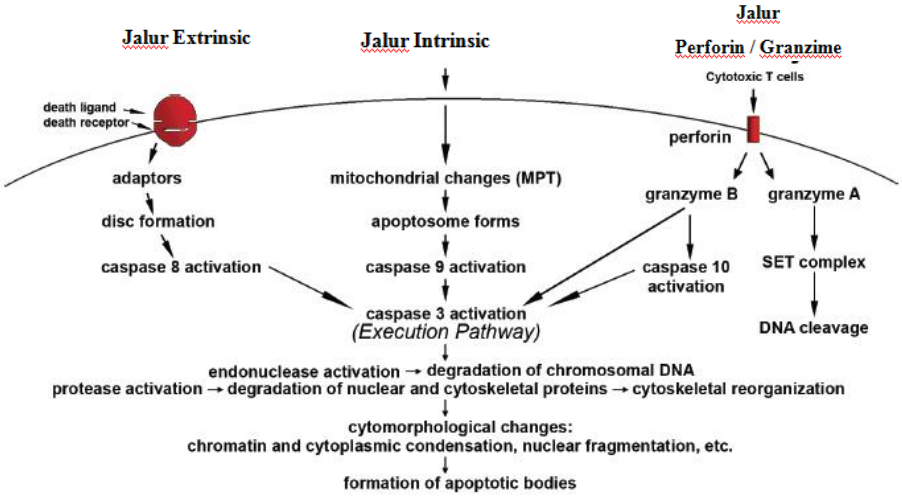
The increase in apoptotic index in the treatment group, apart from the *gallic acid* and *flavonoids* to increase the caspase-3 expression as described above, *gallic acid* and *flavonoids* can also stimulate the production of interferon-γ (IFN-γ) in a population of immunosit, which is very important in promoting the activation of CTL and NK cells in the immune system of immune cells against cancer cells. When CTL and NK cell is active and a lot more going on the *killing* of the tumor cells that cause a lot of apoptosis of tumor cells.(10)(22) Besides the effector function of NK cells to kill cancer cells by secreting *perforin* and *Granzyme* which then induce apoptosis of target cells; releasing IFN-γ which enhances macrophage phagocytosis work; bonding on *the target-cell death receptor,* such as FAS (CD95) or FAS ligand (FasL) in opsonized cancer cells that causes cancer cell to be programmed as apoptosis; As well as breaking/ hydrolyze substrate specific proteins including caspase causing target cells to undergo apoptosis.(10)(15)(16)

Paclitaxel, in addition to inhibit cell proliferation, may also increase apoptosis by reducing expression of Bcl-2 and Bcl-xl, and raise proapoptosis Bax protein.(24)(25) Meanwhile, in addition to inhibit the cell cycle, *cisplatin* also increase apoptosis by inducing P53, P53 will induce proapoptosis Bax protein that will ultimately lead to cell apoptosis. Therefore, a synergistic effect between the extract *Phaleria macrocarpa* and *Paclitaxel-cisplatin* chemotherapy is obtained.(16)(19)(26)(27)

The result of correlation test which was performed to analyze the correlation between the caspas-3 expression and the apoptotic index using *Spearman's* test shows a significant correlation with p = <0.001 (p <0.05) and the value of r = 0.961, so it can be concluded that there is a significant correlation between caspase-3 expression and apoptotic index with a very strong positive correlation. This is in accordance with the three mechanisms of apoptosis:

1. Extrinsic pathway where there is a *death receptor* activation (DR) on the surface of the cell membrane by ligands which then activates caspase 8 and eventually cause the activation of caspase 3.
2. intrinsic pathway where there is *cellular stress* (oxidative stress, radiation, cytotoxic drugs) which will cause the mitochondria to synthesize *cytocrom c* binding to Apaf-1 and pro-caspase 9 forms *apoptosome* which will cause the activation of caspase 9 and in the end causing caspase 3 to be activated. Intrinsic pathway can also be activated by caspase 8*)* through protein breakdown Bid.
3. *Granzime B pathway*s that are sensitive to the target cell .(15)(21)(28)

Figure 4. Apoptosis path (26)



CONCLUSION

Based on this study, it can be concluded that *Phaleria marcocarpa* extract, chemotherapy *paclitaxel* *cisplatin* and the combination of both increased caspase-3 expression and the apoptotic index of epidermoid carcinoma cell; it was not found significant differences between P1 and P2; a synergy effect was obtained from the administration of the combination of P1 and P2; there is a significant correlation between caspase-3 expression and apoptotic index with a very strong positive correlation.

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Effects of Neo Automatic Code on the Accuracy of Chest Compression Depths in Cardiac Arrest Patients

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ABSTRACT

Objective: to analyze the effects of manual chest compression using neo automatic code on the accuracy of compression depths. Methods: This study used a quantitative, post test quasi-experimental design with a control group. The samples were 74 cardiac arrest patients in two hospitals in Surakarta and Klaten, who were selected using purposive sampling technique. The data were analyzed by the Mann-Whitney test. Results: the mean of accuracy of compression depth in the control group was 68.10 ± 17.60%, and in the treatment group was 83 ± 6.04%. The result of statistical analysis showed that there were differences in the accuracy of compression depth in the intervention and control group with p-value of 0.000. Conclusion: there were effects of manual chest compression using neo automatic code on the accuracy of compression depths. Neo automatic code could improve the accuracy of chest compression depths.

Keywords: *cardiac arrest, chest compression, compression depth accuracy, neo automatic*

The burden of cardiac arrest in health sectors is substantial with an estimated 424,000 and 275,000 cardiac arrests occuring each year in the USA and in Europe respectively. Moreover, a number of patients who survived cardiac arrest in North America and the UK in 2016 were less than 10% [(1)]. However, the incident of cardiac arrest in Indonesia has not been reported though the prevalence of cardiac diseases and cerebro vascular accidents in 2013 were reported high of 3,180,408 and 2,137,941 respectively. These high numbers would potentially result in high incidents of cardiac arrest [(2)]. Therefore, timely and high quality cardiopulmonary resuscitations (CPRs) are needed to increase cardiac arrest survivors.

Following the highlights of the 2015 American Heart Association (AHA) guidelines update for CPR, it recommends rescuers to compress the chest of adult cardiac arrest victims at 100 to 120 compression rates per minute with the depth of 5 to 6 cm, to allow full chest wall recoil, and to minimize interruptions of chest compressions [(3)]. Professional rescuers, however, often do not deliver high quality CPRs regarding compression rates and depths [(4–8)]. Avoiding excessive compression rates may lead to more compressions of sufficient depths [(6)]. This problem can be solved using metronome [(9)].

The metronome is a tool to guide the CPR providers in keeping an accurate chest compression rates [(9).] A higher rates of recommended chest compression is needed for metronome-guided CPRs to obtain high quality chest compressions [(10)]. The new generation of metronome is The Neo Automatic Code.

Neo Automatic Code is an android based application tool to control CPR chest compressions at the rates of 100 rates per minute. It has two guiding modes of CPR which are standard CPR and compression-only CPR. Moreover, it allows the rescuers to give rescue breaths and to check pulses without interrupting compressions. Neo automatic code enables the rescuers to control their breathings as they do not need to count the compressions, and to prevent them from being exhausted. Therefore, recommended chest compression depths may higly be obtained [(11)]. A preliminary study of Neo Automatic Code-guiding CPRs in a mannequin showed that Neo Automatic Code produced 2253 (86.70%) recommended chest compressions out of 2598 compressions [(12)]. However, there is still no evidence regarding the effect of Neo Automatic Code on the cardiac arrest patients. This study, thereby, aimed to analyze the effect of manual chest compression using Neo Automatic Code on the accuracy of compression depths. Our hypothesis was that there were effects of manual chest compression using Neo Automatic Code on the accuracy of compression depths.

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METHODS

This study used a quantitative, post test quasi-experimental design with a control group. The sample size was counted according to the previous studies was idris et al [5] resulting in 74 cardiac arrest patients. The respondents were divided into treatment and control groups who were selected using purposive sampling techniques in two hospitals in Surakarta and Klaten from May to June 2017. Inclusion criteria for the samples were that patients were resuscitated on a solid surface, patients were attached with bed site monitors, chest compressions was done by certified emergency nurses or certified emergency/anaesthesiology physicians with more than 20 of body mass index (BMI). Exclusion criteria for analysis were do-not-resuscitate cardiac arrest patients. The treatment group was given Neo-Automatic-Code guiding chest compressions while the control group was given standard chest compressions. The accuracy of chest compression depths was measured from the number of R waves with the height more than 10mV.

Ethics

This study obtained an ethical clearance from The Health Research Ethics Committee of Faculty of Medicine, University of Diponegoro with ethical clearance number of 176 / EC / FK-RSDK / IV / 2017. This study also granted permissions to undertake research from two hospitals in Surakarta and Klaten. Research ethic priciples were maintained during the study by giving informed consents to the patient's families, delivering standard CPRs as AHA guideline to the treatment and control groups, using initials for respondents, keeping respondent's personal details confidential, no coercion to participate in the study, and using certified emergency practitioners as the CPR providers to minimize the harm.

Statistics

For every compression, the bed site monitors automatically showed R waves. Researchers recorded the number of R waves and the number of chest compressions performed using hand counters. The accuracy of compression depths was measured by the percentage of the number of R waves with the height more than 10 mV on the bed side monitor with the total chest compression performed. Data on age, sex of the patient, presenting heart rhythm and the main illnes were extracted from medical records. The data were analyzed by the Mann-Whitney test.

RESULTS

by group as can be seen from table 1. The result showed that the majority of respondents in intervention and control group were 41-60 years and female. The most common rhythm in intervention and control group shortly before cardiac arrest was unshockable rhythm which were 37 respondents (100%) and 32 respondents (86%) respectively. The leading cause of cardiac arrest patients in intervention group was non cardiac (n=32, 86%) followed by cardiac (n=5, 14%) and in control group was non cardiac (n=29, 79%) followed by cardiac (n=8, 21%).

The result showed (table 2) that intervention group had on median of accuracy of compression depth was 85% $\pm$ IQR 77.5 and control group was 72% $\pm$ IQR 53.5. Figure 1 showed means of accuracy of compression depth in intervention group was 83% ± 6.04 and means of accuracy of compression depth in intervention group was 68% ± 17.60%. The range of chest compression depth accuracy in the treatment group were 70-92% while the accuracy in the control group ranged 29-96%. This meant that the treatment group had all fraction of accuracy above 60% compared to the control group which half of them were below 60%.

The total 74 cases met the inclusion criteria, and complete data. Demographic characteristics for the study population were stratified

The result of statistical analysis showed p value of 0.000 meaning that there were differences in the accuracy of compression depths in the intervention and control group (p-value<0.05). There was a significant effect of manual chest compression using neo automatic code on the accuracy of compression depth.

DISCUSSION

The study showed that, during hospital resuscitation by professional rescuers, the accuracy of compression depth was 29-96%. The means and standard deviation of accuracy of compression depth in intervention group 83% ± 6.04 greater than control group 68% ± 17.60%. The accuracy of compression depths in the intervention group which used Neo Automatic Code was far higher than the control group. Neo Automatic Code allows the rescuers to deliver compression rates constantly at 100x/min, so that appropriate compression depths could be achieved. Rescuers only follow codes for compressions, so that did not need count and makes a sound. Recuers can breathe freely, so

that did not easily tired and make optimal compression depths [11].

On the other hand control the group did not use neo automatic code. An absence speed regulator controlling compression rates yields chest compression faster than using neo automatic code. An increase of chest compression rates causes fatigue among the rescuers, which consequently decrease the accuracy of compression depth [4–6,13,14].

Field et al [14] show that faster compressions lead to reduced compression depths. Monsieurs et al [6] state that high compression rates are common and can be caused by stress or by the inability of rescuers to assess and control the compression rate. Very low compression rates were uncommon and may be associated with specific activities potentially interrupting chest compressions such as aspiration, intubation and defibrillation [6].

Other than compression rates, accuracy of chest compression depth related to the physical fitness of the rescuer. The weight of the rescuer is an important factor in the compression depth (4). Perkins et al [15] find that bed height affects maximal compression forces, and makes effects on accuracy of compression depth. Jantti et al [16] showed the surface under the patient may affect the cardiopulmonary resuscitation (CPR) quality. The result of statistical analysis showed that there were differences in the accuracy of compression depth in the intervention and control group (p-value<0.05). This is due an absence of speed regulator to control compression rates resulting in differences in the accuracy of compression depth.

Limitations of this study was that core diseases in this study were heterogen.

Table 1. Demographicss and study characteristics stratified by group

Characteristics	Intervention group	Control group
Age		
18-40 years	5 (14 %)	6 (16 %)
41-60 years	19 (51%)	17 (46 %)
>60 years	13 (35 %)	14 (38 %)
Gender		
Male	11 (30 %)	17 (46 %)
Female	26 (70 %)	20 (54 %)
Hearth rhythm		
Shockable	0 (0%)	5 (14 %)
Unshockable	37 (100%)	32 (86%)
Main illness		
Cardiact	5 (14 %)	8 (21 %)
Non Cardiact	32 (86 %)	29 (79 %)

Table 2. A*ccuracy of compression depth* stratified by group

Group	Median (Q2)	Q1	Q3
Intervention	85,00%	77,50%	87,50%
Control	72,00%	53,50%	81,00%

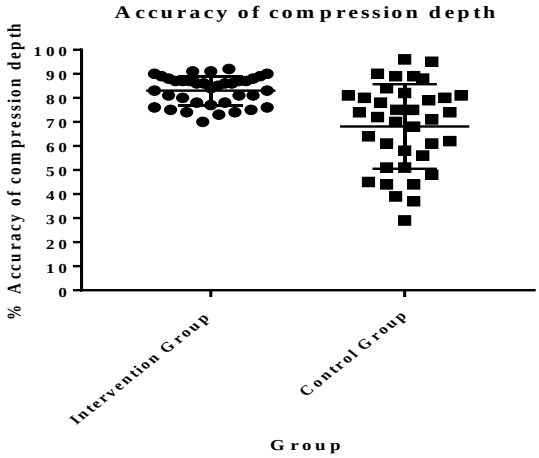

Figure 1 Accuracy of compression depth

CONCLUSIONS

There were effects of manual chest compression using neo automatic code on the accuracy of compression depth. Neo automatic code could improve the accuracy of chest compression depth. Rescuer of cardiac arrest patient must have knowledge about factors that can be improve chest compressions depth such as weight of rescuer, hard board, and chest compressions rate.

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Autism phenotype in fragile X premutation males is not associated with *FMR1* expression: a preliminary evaluation

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ABSTRACT

To explore the association between autism phenotype and *FMR1* protein (FMRP), *FMR1* mRNA and CGG repeat length in 31 male *FMR1* premutation carriers aged 3.0 to 27.9 years old (mean 13.0 ± SD 6.5) using the ADOS communication, social interactive and total scores. FMRP levels were determined using the sandwich Enzyme-linked Immunosorbent Assay (ELISA) method, *FMR1* mRNA expression levels were measured by qRT-PCR, and CGG repeat size was determined using Southern blot and PCR analyses. There was no significant difference in FMRP, CGG repeat length, and *FMR1* mRNA between fifteen subjects without (ASD / PDDNOS / autism and sixteen subjects with ASD / PDDNOS / autism. ADOS scores were not significantly associated with either FMRP or *FMR1* mRNA, This preliminary evaluation found that autism phenotype is not associated with the level of expression of either *FMR1* mRNA or FMRP. However, CGG was significantly negative associated with both ADOS communication score (p= 0.0173) and ADOS total score (p= 0.0358).

Key-words: Autism, CGG, *FMR1* mRNA, FMRP, Fragile-X Premutation

The expansion of the CGG repeat in the premutation range (55-200 CGG repeats) of the fragile X mental retardation 1 gene (*FMR1*) can lead to a range of clinical involvement, including psychological problems [1,2]; fragile X-associated primary ovarian insufficiency (FXPOI) [3,4]; immune-mediated disorders [5,6]; hypertension [7]; fragile X-associated tremor/ataxia syndrome (FXTAS) [8–10] and neurodevelopmental disorders, such as autism spectrum disorders (ASD) and attention deficit hyperactivity disorder (ADHD) [11,12]. Some of behaviours associated with autism such as avoidance of eye gaze, hand flapping, repetitive behaviours, and speech perseverations have been reported in more than 60% of all individuals with fragile X syndrome (FXS) [13–15].

A lack or deficiency of the *FMR1* protein (FMRP) in individuals with the full mutation (>200 CGG repeats) leads to the clinical features of FXS[16]. However, FMRP may be also mildly deficient in some individuals with the premutation, particularly those with CGG repeats in the upper premutation range as well as the premutation CGG Knock-In (CGG KI) mouse model [17–20]. In addition, elevated level of *FMR1* mRNA, which rises with increased CGG-repeat number, is the most consistent molecular abnormality observed in both human and mouse premutations [20–23]. Elevated mRNA also leads to central nervous system (CNS) toxicity and neurological disease, such as FXTAS and psychopathology in older carriers [1,2,24].

Although most individuals with the premutation are unaffected by intellectual disability, a subgroup of children experience ASD, ADHD, anxiety, seizures, and learning difficulties or intellectual disability [12,13,25–29]. The prevalence of ASD in boys with the premutation whose parents sought medical attention for their sons' behaviour problems in the clinic (probands) is

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high (73%); however, if the premutation is identified through cascade testing, brothers with the premutation (non-probands) are then found to have a lower prevalence of ASD (14%)[11]. A questionnaire study of more than a thousand families demonstrated a prevalence of autism or ASD of 13% in boys with the premutation and 1% in girls with the premutation [28].

The cause of the developmental problems in some premutation children is likely related to mild deficits of FMRP [12,17] in addition to the toxic effect of elevated mRNA [30–32]. A recent report by Chonchaiya et al. found that seizures were common in male probands with the premutation and were significantly associated with ASD and intellectual disability in these boys [29]. A study demonstrated that 11.3% had seizures of 57 premutation boys from a family survey [28]. In the CGG KI mice, the CGG premutation animal model, neurons appear to die more easily in culture than in control neurons [33], and these neurons have an enhanced frequency of electrophysiological spikes [34]; thus the premutation neurons appear to be more vulnerable to seizures or other environmental toxicity. There are case reports of carriers who have been exposed to environmental toxins, including chemotherapy [35] or industrial contaminants [36], and have suffered more severe neurological problems than typically seen in carriers.

The prevalence of the fragile X premutation in the general population is estimated at one in 130 to 259 females and one in 250 to 813 males [37,38]. Because the premutation is relatively common, some of the problems in premutation carriers may be exacerbated by other factors, in addition to low FMRP or to the RNA toxicity of elevated mRNA. The association between autism and *FMR1* expression has been studied in the full mutation range such an association has not been reported in those with premutation. In this study, we utilized enzyme-linked immunosorbent assay (ELISA) to measure FMRP levels in blood [39] and assessed the association between FMRP, *FMR1* mRNA, and CGG repeats and three ASD scores: communication score, social interactive score, and total score.

SUBJECTS AND METHODS

Subjects

Subjects participated in studies at the MIND Institute at the University of California, Davis, between 2006 and 2011. The subjects included 31 male premutation carriers with mean age 13.0, SD 6.5 (3.0 to 27.9 years). Parents and participants older than 8 years signed consent approved by our institutional review board. Premutation status was confirmed in all participants, as described below. Subjects underwent a full medical evaluation, which involved a medical history and a physical / neurological examination by one of the authors (RJH), an expert in neurodevelopmental disorders. The medical history involves a detailed review of systems and questions regarding medical conditions, such as history of development, and medical problems, including seizures. We classified the subjects into probands and non-probands. Probands were individuals who presented to the clinic because of their medical or developmental problems, while individuals who were identified by cascade testing named as non-probands.

During the clinical evaluation, assessment of specific measures for ASD were completed by licensed psychologists. The diagnostic algorithm of the Autism Diagnostic Observation Schedule (ADOS) module 1,2,3 and 4 were used to examine the profile of autism. [40] From the ADOS examination, the subjects were diagnosed with ASD, PDDNOS or autism. The participants were then divided into two groups, group A with no ASD/ PDDNOS/autism or were in typical range and group B with ASD/PDDNOS/autism

Molecular Measures

FMRP was determined using the sandwich ELISA method published previously [39]. *FMR1* mRNA expression levels, measured by qRT-PCR were as described [41]. CGG repeat size was determined using Southern blot and PCR analyses, as previously described [37].

Statistical Analysis

Descriptive statistical analysis was based on Fisher exact test for categorical variable and student's t test for continuous variables. To test association between ASD scores and molecular biomarkers (FMRP, *FMR1* mRNA, CGG repeats), the analysis of covariance (ANCOVA) was used to with covariates group effect and FMRP or *FMR1* mRNA or CGG repeat. Differential association between ADOS variables depending on whether premutation carriers in group A or B were assessed with inclusion of interaction term with group; however, the all interaction terms were not statistically significant. All statistical analysis was conducted in SAS version 9.2.

RESULTS

Characteristics of study subjects

The initial cohort included 31 males who carried an *FMR1* allele with CGG repeats number from 55 and 186 (Mean 101, SD 40). Relative FMRP levels were measured from blood lymphocytes with levels ranging from 0.65 to 2.27 (Mean 1.57, SD 0.5), where a value of 1.0 is the mean FMRP value derived from controls with normal alleles. The analysis cohort included 31 subjects based on ADOS overall diagnosis: 8 (25.81%) subjects had PDDNOS; 7 (22.58%) subjects had autism; 1 (3.23%) subject had ASD, and 15 (48.39%) subjects either did not have ASD/PDDNOS/autism. Among participants, three individuals (9.67%) experienced seizures and all of them had been diagnosed with ASD.

Although FMRP levels were negatively correlated with CGG repeat length (p= 0.0465). FMRP levels in group A (N 15, Mean 1.53, SD 0.52) were not significantly different (p= 0.7782) from values for the group B (N 16, Mean 1.59, SD 0.59).

Group A consisted of 9 (60%) subjects who were identified as probands and 6 (40%) as non-probands. Group B consisted of 11 (68.75%) subjects who were identified as probands and 5 (31.25%) as non-probands. Among the probands in group A, 7 (64%) had ADHD, 3 (27%) individuals had learning difficulties, and 1 (9%) individual had intellectual disability. The prevalence of probands was not significantly different between the two groups (p=0.716). As expected, ADOS total, communication and social interactive scores differed between the two groups.

Table 1 summarizes the study cohorts, including molecular variables, and ADOS outcome score

		A= No ASD/PDDNOS/autism			**B=ASD/PDDNOS/autism**			
Variable		**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**P-value**
Age		15	13.50	7.70	16	12.56	5.70	0.7014
CGG		15	92 (56-144)*	29	16	109 (55-186)*	48	0.233
FMR1 mRNA		15	3.23	1.29	16	3.21	1.84	0.9613
FMRP		15	1.53	0.52	16	1.59	0.59	0.7782
Seizures								
Proband		**N**	**%**		**N**	**%**		
	Yes	9	60.00		11	68.75		0.716
	No	6	40.00		5	31.25		

Association between ADOS communication, social interactive and total score with, FMR1 expression and CGG expansion

Table 2 summarizes the primary analyses to assess the association between ADOS scores with FMRP, *FMR1* mRNA, and CGG among premutation groups (A= No ASD/PDDNOS/autism, B = ASD/PDDNOS/autism).

There was no significant association between the three ADOS scores (communication, social interactive, total score) and either FMRP or *FMR1* mRNA expression. CGG repeat length was significantly negatively associated with both ADOS communication score (p=0.0173) and ADOS total score (p=0.0358); see Table 2. For example, the total ADOS score declined by a modest 0.0394 unit for one CGG repeat increase (Table 2); thus, the model average ADOS total scores for an individual with 60, 90 or 130 CGG repeats are 5.1, 3.9, and 2.3, respectively (for premutation carriers without ASD).

Table 2 ADOS outcome score

Variable	Parameter	Estimate	Standard Error	P-value
Model #1: ADOS score= Group* + FMRP				
ADOS Communication Score	Intercept	0.1465	0.9106	0.8736
	Group=B	2.5085	0.5920	0.0003
	FMRP	0.5119	0.5358	0.3498
ADOS Social Interactive Score	Intercept	0.8415	1.3301	0.5335
	Group=B	5.1733	0.8647	<.0001
	FMRP	0.8998	0.7827	0.2627
ADOS Total Score	Intercept	1.0685	2.2496	0.6393
	Group=B	6.8448	1.4227	<.0001
	FMRP	1.8700	1.3108	0.1671
Model #2: ADOS score= Group + *FMR1* mRNA				
ADOS Communication Score	Intercept	1.8301	0.8217	0.0365
	Group=B	2.4577	0.5843	0.0004
	FMR1 mRNA	-0.2778	0.2111	0.2017
ADOS Social Interactive Score	Intercept	3.4861	1.2147	0.0089
	Group=B	5.1296	0.8637	<.0001
	FMR1 mRNA	-0.3934	0.3120	0.2206
ADOS Total Score	Intercept	6.7056	1.9556	0.0023
	Group=B	6.7464	1.4064	<.0001
	FMR1 mRNA	-0.8645	0.5170	0.108
Model #3: ADOS score= Group + CGG				
ADOS Communication Score	Intercept	2.5550	0.7503	0.0025
	Group=B	2.7371	0.5271	<.0001
	CGG	-0.0177	0.0069	**0.0173**
ADOS Social Interactive Score	Intercept	3.4725	1.2191	0.0093
	Group=B	5.4328	0.8564	<.0001
	CGG	-0.0139	0.0112	0.2274
ADOS Total Score	Intercept	7.4645	1.8688	0.0006
	Group=B	7.5273	1.3513	<.0001
	CGG	-0.0394	0.0177	**0.0358**

*Group: A= No ASD, as reference; B = ASD/PDDNOS/Autism

DISCUSSION

ASD is common in the general population and occurs in as many as one in 88 individual.[42] The etiologies of autism are numerous, including a number of known genetic disorders, though FXS is the most common single-gene disorder associated with autism.[43] FMRP regulates the translation of many other proteins important for synaptic plasticity and whose genes are also associated with autism when mutated; when FMRP is absent, many of these gene products are dysregulated. [44] Fragile-X carriers especially boys who have CGG repeat number in premutation range can present with ASD although their etiology may be heterogeneous similar to idiopathic autism. [45] In this study, we investigated the role played by *FMR1* molecular measures in leading to the ASD phenotype among premutation boys and found no correlation between autism phenotypes and both the level of FMRP and *FMR1* mRNA. However, we observed that CGG repeat number was significantly negatively associated with both ADOS communication score and ADOS total score; although the effect size was clinically modest. A variety of mRNA transcripts arise from

a combination of alternative splicing, alternative transcriptional start sites selection and differential usage of polyadenylation sites. In fact, these events generate the vast majority of diversity of gene expression and have been described for over 180 000 mouse transcripts. Besides alternative splicing, which can produce extraordinary protein diversity, regulation at the level of the 5′- and 3′-UTRs modulates mRNA processing, nuclear export, stability, subcellular localization and translational efficiency. Such processes are crucial for differential expression of a gene during development, tissue differentiation and under certain pathological conditions. Since our analysis cohort only had a small sample size of 31 subjects in total, the results need to be interpreted with caution. Larger studies are needed to provide more accurate estimate and to confirm the observed association reported here. Another important limitation of this preliminary evaluation is this association may be confounded by other factors that we may not have in the study, potentially genetic and environmental factors. Those with low CGG-repeat numbers are the most common group of premutation males and perhaps most likely to have autism for additional causes, confirming that the etiology of their autism may be multifactorial or similar to idiopathic autism. A publication demonstrates that approximately 20% of premutation carriers with ASD, ID or neurological problems can have a second genetic hit that may be additive to the premutation specifically a copy number variants (CNV) that may also be associated with autism or ID [46].

The study population reported here included boys who were diagnosed with autism or ASD either before or after premutation status was established. Some presented as the proband of their family, and others were identified by cascade testing once a proband was diagnosed with FXS or premutation involvement. Because of the inclusion of probands with the premutation, our study is biased towards clinical involvement, particularly ASD in this group of patients. However, this is an optimal population of carriers to see if the levels of FMRP or mRNA are related to this type of clinical involvement. We observed a significant negative correlation between FMRP and CGG repeats. However, FMRP was not significantly associated with ADOS measurements (communication score, social interactive score, and total score). The large range of FMRP values in these premutation boys is similar to what has been reported in individuals with normal CGG repeats [39]. Lowered FMRP levels have been seen in the blood and brain tissue of some premutation patients with developmental problems [12,17], and in both blood and brain of premutation mice[18]. Brain levels of FMRP may be significantly lower than blood levels in premutation carriers, particularly in areas of the brain important for social deficits such as the amygdala and insula. Therefore, low levels of FMRP may be present in the brain even when FMRP is not low in blood [47]. Fatemi et al have demonstrated low levels of FMRP in the brain in individuals with idiopathic autism without an *FMR1* mutation, and in those with neuropsychiatric disorders such as schizophrenia[48]. Both age of onset and IQ correlates with level of FMRP in the blood of those with schizophrenia who do not have an *FMR1* mutation [48]. Therefore, FMRP may be important for the clinical phenotype in a number of neuropsychiatric disorders.

Premutation neurons lose viability more rapidly than control neurons in cell culture [33], and display an increase in spike-wave discharges [34] compared to controls. Therefore, environmental factors, such as infections or toxins, associated with autism would be more likely to cause deleterious effects in premutation neurons. There is also evidence of mitochondrial dysfunction in premutation carriers [49], which has been seen in fibroblasts and brain tissues from premutation carriers with and without FXTAS [34]. Mitochondrial problems are also commonly seen in idiopathic autism [50]. Mitochondrial abnormalities could possibly be related to the autism or ASD phenotype linked to RNA toxicity, even without lowered FMRP levels. A variety of environmental factors may elevate FMRP. Jeon et al. have reported that HeLa cells exposed to cell-death inducer etoposide up-regulated FMRP levels.[34] This effect was synchronized with phosphorylation of Akt, a known cell-survival-related signaling molecule. Induction of FMRP apparently plays a protective role against the stressed status of cells and, with reduced FMRP, cell survival is compromised. The premutation is associated with higher rates of seizures in both the current premutation group and I n reports in the literature[28]. Chonchaiya et al. have shown that the presence of seizures is closely associated with the diagnosis of autism or ASD in premutation boys [29]. On a cellular level, premutation neurons demonstrate enhanced spikes in culture, so it appears that RNA toxicity can predispose to seizures in premutation carriers [34]. Seizures in turn can lead to neurochemical changes in the CNS that can further exacerbate developmental problems [51] and seizures can pull FMRP away from the dendritic spines and into the cell body thereby depleting the dendrites of the regulatory effects of FMRP [52].

We do not yet know why some individuals with the premutation have developmental problems or ASD and others do not. The reasons are not simple, and they not seem to be related to FMRP levels or *FMR1* mRNA levels in blood. There are likely additive and multifactorial effects that involve both background genetic and environmental effects. Further studies of neurotoxicants are warranted, and more detailed genomic studies are needed to better understand the neurodevelopmental effects of the premutation.

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The Evaluation of PMP22 and Protein 0, Examinations for Early Disability Detection in Leprosy Patients

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Introduction: Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* that has a predilection for peripheral nerves, especially Schwann cells. Leprosy medications may only eradicate the bacteria without preventing or recovering peripheral nerve damage. Previous studies proved that Krox-20 could be a useful diagnostic tool for early peripheral nerve damage detection in leprosy.n**Objective:** To analyse and to determine PMP22, and P0 cut-off points as diagnostic tools of early disability in leprosy. **Methods:** We examined ambulatory patients at Kediri Leprosy Hospital, Indonesia. We employed WHO's criteria to assess the degree of disability and measured the study variables using ELISA. We then determine the cut-off value using Receiver Operating Characteristic curve. **Results:** From overall patients (n=79), 36 patients had 0-degree of disability, and 43 patients had 1-degree of disability. The ROC curve analysis revealed cut-off values for PMP22 and P0 at 4,42 pg/mL and 11,39 pg/mL, respectively. The mean value for all variables in patients with 0-degree of disability were higher than that in patients with 1-degree of disability at 12,56 pg/mL vs 4,24 pg/mL ($p<0,05$) and at 9,85 pg/mL vs 2,86 pg/mL, respectively ($p<0,05$). **Conclusion:** Leprosy is a chronic infectious disease that brings forth many degrees of disability secondary to peripheral nerve invasion, particularly Schwann cells. Hence, early detection of peripheral nerve damage becomes crucial. The evaluation of PMP22 and P0 examinations is useful to identify early peripheral nerve damage in leprosy.

Keywords: *leprosy, degree of disability, PMP22, P0*

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* and has a predilection for the skin and peripheral nerves, especially in Schwann cells [1-4]. Furthermore, leprosy is also cited as the most frequent aetiology of peripheral neuropathy.[4] Approximately 2-3 million people globally have suffered from defects secondary to the disease. A large number of global leprosy incidents in the world is caused, amongst many, by inadequate access to health services and the challenges faced by health workers to reach the patients in isolated areas. Owing to late diagnosis and management common in the countryside, many have become disabled, both due to the past and the newly acquired infections. Additionally, the vaccine for leprosy remains unavailable until now [1].

Even now, leprosy treatment is fundamentally aimed only at eradication of bacteria, without being able to prevent or to cure the damage to the peripheral nerve and its components. Early detection is hence crucial to determine the presence of acid-fast bacteria in Schwann cells [5, 6]. *Mycobacterium leprae* causes peripheral nerve damage resulting in disability and deformity. Anti-leprosy medications can indeed eradicate the bacteria albeit unable to restore the defects and the deformities that have occurred, such as recovering the function of a nerve. By understanding the mechanism of nerve damage caused by *Mycobacterium leprae*, we can better prevent nerve damage [7].

The WHO divides the levels of disability in leprosy patients into 3 degrees, ranging from the absence of symptoms to visible damage or apparent disability (8). However, peripheral nervous disorders may already occur without specific leprosy symptoms.

Schwann cells reside within the peripheral nerves. The expression of specific transcription factors governs their differentiation. Immature Schwann cells will increase the expression of

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multiple transcription factors such as NFκB, Oct-6, and Brn2 after receiving signals from axons, including NRG1.

Mycobacterium leprae enter Schwann cells, and as self-defense, the cells produce and excrete cytokines such as IFN-γ, TNF-α, Erk 1/2 signalling, Ras, Raf and other inflammatory cytokines. Erk 1/2 activate c-Jun, which inhibits myelination through Krox-20 down-regulation. Krox-20 is a gene crucial in myelin sheath synthesis by activating neuregulin (NRG) and Neuron Growth Factor (NGF) [9]. The initial signs of myelin sheath formation in peripheral nerve cells are the generation of Myelin Protein Zero (MPZ) or protein 0 (P0), and Peripheral Myelin Protein 22 (PMP22).

Previous research indicated that Krox-20 was valuable as an early diagnostic tool for peripheral nerve defect,[10] and thus, we assumed that PMP22, P0, NGF, and NRG1 might also show positive correlations as early indicators of disability in leprosy patient.

PMP22 is a tetraspan protein required for maintaining myelination stability. Amongst many strategies to determine the presence of early peripheral nerve damage following Mycobacterium leprae infection is to assess Schwann cell behaviour by measuring the changes in the myelin sheath synthesis markers produced by Schwann cells. These include P0, and PMP22. However, up to date, there is still no published research that discusses the early detection of disability in leprosy patients.

Early identification remains an impediment in establishing the diagnosis of disability as leprosy sequelae secondary to peripheral nerve cell demyelination. It is impossible to restore the condition once demyelination begins; therefore precautionary endeavours are necessary. An understated approach is an early detection of possible nerve damage that may occur so that the patient can receive immediate preventive managements.

With this research, the authors sought to determine the values of PMP22, and P0 as early diagnostic tools for disability in leprosy patients.

AIMS

The study aimed to investigate the validity as well as to determine the cut-off points of PMP22 and P0 as early diagnostic tools of disability in leprosy patients.

METHODS

We took the samples from ambulatory patients visiting the outpatient polyclinic of Kediri Leprosy Hospital, Indonesia, during the period of August-December 2014. The inclusion criteria comprised of everyone who showed 0- to 1-degrees of disability, aged between 14 to 50 years old, and were willing to participate in the research.

We establish leprosy based on cardinal signs, i.e., anaesthetic skin disorder, peripheral nerve enlargement with autonomic, sensory and motor function abnormalities, and the detection of acid-fast bacilli on skin scrapings or ear lobes. We based the determination of the degrees of disability on the WHO criteria published in 1970. ELISA examination provided by the Biochemistry-Biomolecular Laboratory, Faculty of Medicine, Brawijaya University, Malang, Indonesia measured the PMP22, P0, NGF, and NRG1 levels.

We analysed the data obtained using SPSS 17 compatible with Windows® operation system.

RESULTS

We conducted the study on Multibacillary leprosy patients (n=79) with 0- and 1- degrees of disability (n=36 and n=43, respectively). The time span required for the subject collection at Kediri Leprosy Hospital, Indonesia, was five months. We briefed each multibacillary leprosy patient who met the inclusion criteria about the research. After obtaining a signed informed consent, we took a biopsy of the skin lesion. We sampled peripheral blood for PMP22, and P0 level measurements. Two-tailed T-test results showed F = 58.869 with p = 0.000 (p <0.05). The differences between plasma PMP22 examination results in patients with 0 versus 1-degrees of disability were significant (Figures 1 and 2). The result of 2-tailed T-test in P0 level showed F value = 9.909 with p=0,000 (p<0,05). This showed a significant difference of P0 plasma level in MB leprosy patients grade 0 and grade 1. The cut off value for P0 is 11,40 pg/mL.

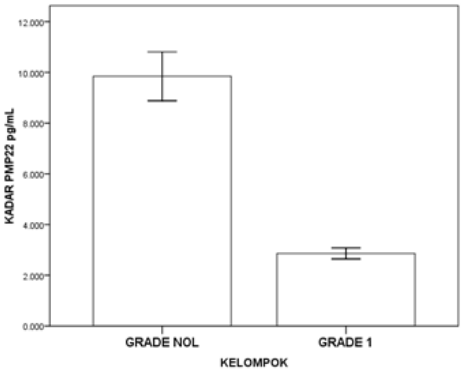

Figure 1. Plasma PMP22 levels in leprosy patients with 0- and 1-degrees of disability

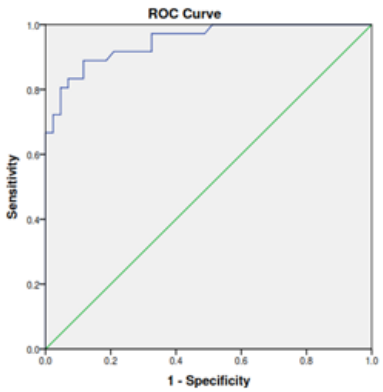

Figure 2. ROC curve of plasma PMP22 levels

Examination of P0 levels in blood revealed significant differences in plasma P0 levels between multibacillary leprosy patients with 0 versus 1-degrees of disability. The two-tailed T-test results further showed F = 9.909; p = 0,000 (p <0.05) (Figures 3 and 4).

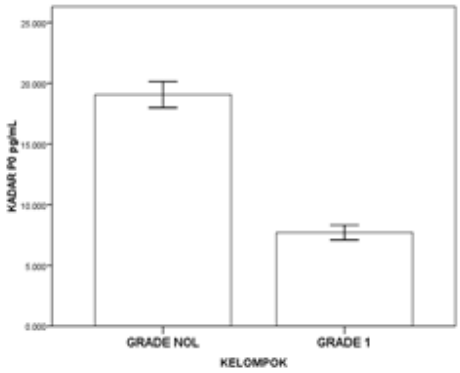

Figure 3. Plasma P0 levels in leprosy patients with 0- and 1-degrees of disability

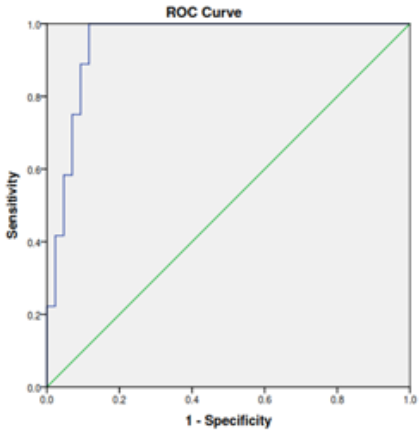

Figure 4. ROC curve of plasma P0 levels

DISCUSSIONS

This research involved samples taken from 36 leprosy patients with 0-degree of disability and 43 leprosy patients with 1-degree of disability. We settled on these group based upon the assumption that peripheral nerve disorder, which was a myelinated disorder caused by *Mycobacterium leprae* infection, would have begun to develop in leprosy patients with 1-degrees of disability.

The sample collection required a period of between October - December 2014 in the Leprosy Department outpatient clinic at Kediri Leprosy Hospital, Indonesia. The age distribution of leprosy group with the highest degree of disability ranged from 26 to 35 years old (n=13; 36%), whilst 1-degree of disability was predominant between 36 to 45 years (n=17; 40%). A Brazilian study by Nardi et. al. revealed that of 232 patients who had completed their leprosy treatment, 32% suffer from 1- and 2-degrees of disability as per the WHO criteria [11].

Women comprised the highest gender distribution (56%) amongst the leprosy patients studied with 1-degree of disability. This was in accordance with an earlier study by Van Brakel, et. al. conducted in Indonesia, which revealed that of the overall 1358 patients enrolled, 31.8% are leprosy patients with 1-degree of disability [12].

We employed the WHO criteria (see Table 2) to determine the degrees of disability, i.e. 0-, 1-, and 2-degrees of disability. The study examined patients with merely 0- and 1-degrees of disability due to its diagnostic nature. We identified 43.54% people who suffered from 1-degree of disability out of the overall study population (n=79) in the Leprosy Department outpatient clinic at Kediri Leprosy Hospital, Indonesia. The rest were those with 0-degree of disability. The definition of 1-degree of disability involved anaesthesia of the hands and feet in the absence of deformity. Visual acuity decrease ought to be no worse than 6/60, or a pass in 60-metre finger count test.8 A study by Ramos et. al. in 210 leprosy patients on therapy at Ethiopia Leprosy Centre revealed that during 1999-2009, 128 patients (61.5%) had suffered disability. Within the group, 26% experienced 1-degree of disability, whereas 35.6% developed 2-degree of disability. Moreover, 13.5% acquired eye defects, 44.5% were physically handicapped, and 44.7% procured defects of the lower limbs [13].

PMP22

Myelin protein expression regulation is an intricate process, reflecting the essential tasks of the myelin sheath in the nervous system. There is a halt in nerve cell proliferation during the development and subsequently following an insult to the peripheral nervous system; the myelin formation ensues thereafter. Myelin protein synthesis correlates tightly with that of myelin formation during the developmental periods in both central- and peripheral nervous system.

Following a peripheral nervous system lesion, myelin protein expression lagged, probably due to transcriptional regulation secondary to axonal contact loss. Hence, myelin protein synthesis progressing on trauma-damaged nerves at a given time is tantamount with the remyelination of a regenerated axon. Thus, the expression of the myelin protein exhibits the same regulatory exemplars both at the developmental stages and during nerve regeneration [14].

PMP22 is a 22-kDa myelin protein expressed by Schwann cells exclusively in the peripheral nervous system and acts as an indispensable element of all myelin sheaths therein. Furthermore, the PMP22 expression corresponds closely with myelin formation during the development of the peripheral nervous system and thus is closely linked to myelin degradation and remyelination processes during the peripheral nerve cell regeneration. The central nervous system does possess PMP22 albeit in significantly minor amounts compared to that within the peripheries [14].

Within our study, two-tailed T-test revealed $F = 58.869$; $p = 0.000$ ($p < 0.05$), signifying significant differences in plasma PMP22 levels between MB leprosy patients with 0- and 1-degrees of disability. It confirms that emerging nerve deterioration in patients with 1-degree of disability resulted in minute amounts of NGF, in contrast with those of 0-degree of disability. Snipes et. al. confirmed that PMP22 resided exclusively within the peripheral nerve myelin sheaths and was a product of Schwann cell activities.16 Consequently, PMP22 is requisite in the myelin synthesis in the peripheral nervous system.

P0 Levels

Protein P0 is the principal structural protein of myelin in the peripheral nervous system. It belongs to Type-I glycoprotein with a single immunoglobulin-like domain and has a molecular weight of 30 kDa. The precise mechanism by which the mesaxon membrane transforms into myelin remains obscure, yet it is clear that the membrane insertion with P0 and subsequent homophilic bonds in the cis- and trans-orientation excludes Myelin Associated Glycoprotein (MAG) and brings forth the formation of intact myelin. A single myelinating Schwan cell is capable of generating several square millimetres of surface membrane. The process requires the transcription of the myelin protein gene and an exact measure of correctly translated protein. The duplication of myelin protein genes may occur either naturally or inducibly and subsequently triggers demyelination that often leads to heavier phenotypes compared to null mutations in the same genes. It reflects the importance of having the appropriate dose of myelin protein gene throughout the myelination process [15].

High levels of P0 are characteristic in the differentiation of myelinating Schwann cells. The expression of P0 mRNA peaks during the period of active peripheral nerve myelination i.e. during the first three weeks postpartum in rodents, and is maintained at lower sedentary levels unto adulthood. P0 gene expression exists during Schwann cell development along with the expressions of other genes that code for specific myelin-forming proteins, such as MBP, PMP-22, and MAG. It is worthy to clarify a common misconception that oligodendrocytes produce P0 in the central nervous system; instead, Schwann cells in the peripheral nervous system execute the task exclusively. Increased P0 biosynthesis manifests early in the myelination process secondary to signals associated with the axonal surface. cAMP potentially enhances these signals for intracellular transduction.

In this study, we found that two-tailed T-test results showed $F = 9.909$; $p = 0.000$ ($p < 0.05$), implying a significant difference of plasma P0 levels between MB leprosy patients with 0- and 1-degrees of disability. Figure 3 displays a significant decrease ($p < 0.05$) of P0 level amongst the 1-degree of disability group compared with the 0-degree of disability group. As per aforementioned, P0 is a transmembrane glycoprotein expressed specifically by Schwann cells that occupy the whole myelin. Besides, P0 is an adhesion molecule that belongs to the IgG gene superfamily and is responsible for maintaining the integrity of the myelin membrane through interactions between the intracellular and extracellular domains. Proteins P0 and PMP22 collectively form a complex on the myelin membrane periphery and in the eukaryotic system in vitro. One may observe mutations of the P0 and PMP22 genes in hereditary peripheral neuropathy diseases e.g. CMT1A or CMT1B [16].

These diseases emerge from heterozygous genetic mutations and express only low levels of sound protein. They exhibit properties in which initially, there is normal myelin formation. However, subsequent loss of self-integrity that leads to eventual demyelination soon follows. It suggests that the aforementioned small number of functional proteins are incapable of keeping the myelin sheath intact. Previous studies support this assumption by revealing that heterozygous transgenic mice with only one single copy of the P0 or PMP22 genes exhibit relatively slow onsets of myelin deficiency.11 Thus, P0 is a myelin-forming protein produced solely by Schwann cells in the

peripheral nervous system and plays a vital role in the myelination process.

CONCLUDING REMARKS

Examination of PMP22, P0 levels is invaluable in recognising the risk of early disability in leprosy patients ahead of clinical symptom substantiations, and hence one may employ a variety of preventive endeavours for irreversible disabilities that morbidly influences numerous aspects of patient life. The former appears to display higher degrees of sensitivity and specificity compared to the latter examination. The exploration of other roles of PMP22, P0, NGF, and NRG1 in leprosy patients relevant to peripheral nerve damage in subclinical and paucibacillary leprosy, amongst many, by examining PGL-1 demands further studies.

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Homocystein Levels and Lipid Profile on Non-DM and DM Individuals with and without Cardiovascular Complications

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ABSTRACT

Background: Homocysteine is suspected to increase the risk of diabetes mellitus (DM) complications and is associated with CV disease. While dyslipidemia and DM are risk factors for death due to CV disease, studies on relationship between homocysteine and glycemic control were inconsistent. **Objective**: To analyze the difference of Hcy level and lipid profile on non-DM (Group I), DM with CV (II) and without CV (III). **Methods:** This crosssectional study was conducted in Dr. Kariadi Hospital Semarang from April to October 2016. Samples were recruited consecutively, of which group I consists of 26 persons, group II (30) and III (30). All of the samples aged 30-75 years old, with long DM duration of more than 5 years. Fasting and 2 hours PP blood glucose, lipid profile was analyzed with *auto analyzer*, while Hcy was analyzed with ELISA. Data were analyzed using independent t test. Significance is expressed at $p < 0.05$. **Results:** A significant difference on Hcy level was found between group I and III (p=0.000), but not between group I and II or II and III. No significant difference was observed on total cholesterol (TC) and LDLC in all groups. Significant differences were found on HDLC level between group I and II (p=0.009); II and III (p=0.000); I and III (p=0.033). Triglyceride level on group I was significantly different compared to group II and III (p=0.030 and 0.013 respectively), but was not significantly different compared to group II and III. **Conclusion:** The highest Hcy level, the lowest HDL-C and the highest triglyceride level were found in DM patients with cardiovascular complication.

Keywords: DM, Homocysteine, Lipid Profile, CHD

WHO estimated that the number of DM patients in worldwide scale will increase to as much as 194 million people in the year 2003, and about two-thirds of these people lived in developing countries.(1)(2) [14,16] DM will have an impact on the quality of human resources and a substantial increase in health costs.(3)

DM individuals are two to four times more likely to develop vascular disease than non-DM. Homocysteine (Hcy) is an interesting topic on arteriosclerosis and cardiovascular disease in recent years.(4) Homocysteine is an amino acid (AA) with AA derivatives of essential sulfhydryl group of methionine and source of animal protein.(5) Hyperhomocysteinemia/HHcy is predicted to increase the risk of DM complications and is often associated with retinopathy, nephropathy and cardiovascular disease (CV).(6)

Hypotheses on rising levels of Hcy is due to the acceleration of glucose as a trigger of oxidative stress on endothelial cells have been proven in animal study, it is evidenced that Hcy is a more real trigger of endothelial dysfunction in diabetes than in non-DM.(5) The mechanism on how Hcy can increase the risk of vascular disease is not clear yet. In one of the studies, it is mentioned that HHcy stimulates the growth of vascular smooth muscle cells, causing intimal thickening of the artery walls, inhibiting oxygenation of the vascular wall and increasing oxygen-free radicals with tissue damage. Reduction of 2 O_2 molecules on Hcy oxidation will produce free radicals and hydrogen peroxide and cause endothelial cell damage. Hyperhomocysteinemia may increase oxidative stress and, alongside with other mechanisms, induce vascular endothelial dysfunction and vascular smooth muscle cell proliferation, atherogenesis then occurs and increases the risk of vascular thromboembolic disease. (4)

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Dyslipidemia is a risk factor of death due to CV disease, this risk will increase in patients with type 2 DM (DMT2). Hyperglycemia, diabetic dyslipidemia, insulin resistance and hypertension will produce atherogenic environment in circulation. However, atherosclerosis also occur in patients without CV dyslipidemia risk factors, smoking, diabetes or hypertension, and this led us to think towards the other risk factors such as Hcy. Low HDL-Cholesterol (HDL-C) levels in DMT2 indicate an inverse relationship between HDL-C and the incidence of cardiac abnormalities. Increased level of LDL-C has been known as an independent risk factor. The increase in LDL-C spur increased oxidized LDL which plays a role in heart vascular. Atherogenic. Hyperglycemia in DMT2 increases the risk of coronary arterial disease (CAD) incident. Increased levels of triglycerides will cause an increase in VLDL-C and LDL-C independently, and causes atherogenity LDL-C through triglycerides oxidized lipoproteins. The ratio of total cholesterol/HDL-C was higher in DMT2 and is stronger predictor of CAD than total cholesterol or HDL-C in single.(7) Recent studies have examined Hcy as a cardiovascular risk factor in DM patients, but the results of research on the association of Hcy levels with glycemic control are still inconsistent and require further investigation.(8)

The purpose of this study is to analyze differences in homocysteine levels and lipid profiles in non-DM individuals, as well as DM patients, with and without cardiovascular complications

MATERIALS AND METHODS

The research was conducted after obtaining research permission from Health Research Ethics Commission Faculty of Medicine UNDIP/ Dr Kariadi Hospital Semarang. Before the study was conducted, all subjects were requested written informed consent.

This is a descriptive analytic study with cross sectional approach. It was conducted in Dr. Kariadi Hospital Semarang, from April to October 2016. The subjects for DM patients with and without CV complication were taken in DM and Cardiology clinic (30 patients each) and for non-DM subject were students of Faculty of Medicine (26 respondents). The inclusion criteria were age 30-75 years old, not DM (for the non-DM group), suffering DM > 5 years (for DM group without CV complication), or already suffering from DM over 5 years and had complications CHD based on minimal electrocardiography (ECG) examination (for DM group with cardiovascular complications), not taking any medications for lowering lipids, and willing to take part in this study. Patients with acute myocardial infarction were rejected to participate in this study. Blood were taken from cubital vein (5 ml) and analyzed for fasting and 2 hours PP glucose levels using auto analyzer methods. Serum Hcy levels were analyzed using ELISA were analyzed using SPSS version 21.0. Each numerical variable was tested for normality, on which abnormal data were transformed to search for a normal distribution, and parametric test was done by using independent t test. Significance is expressed at $p<0.05$.

RESULTS

Description of result

The subjects of this study consisted of non-DM individual groups: 26 persons, consisting of 12 men (46.2%) and 14 women (53.8%). DM subjects without CV complications were 30 persons, consisting of 10 men (33.3%) and 20 women (66.7%) and DM subjects with CV were 30 persons, consisting of 23 men (76,7%) and women 7 people (23.3%) as can be seen in table 1.

The mean age of non-DM group is 32.7 ± 6.3 years, DM without CV complication 56.2 ± 9.7 years and DM group with CV is 59.3 ± 7.8 years. Subject smokers were more common in the DM group with complications of CV counted 19 people (63.3%). Mean body mass index DM group with CV was higher than the other group that is 25.6 ± 2.2 µml/dl. The mean duration of DM was longer in the DM group with CV complications (9.0 ± 3.5 years) than in the DM without CV (6.9 ± 3.2 years). Mean fasting blood glucose level of non-DM group was 84.1 ± 9.1 mg/dl, DM group without CV was 167.7 ± 52.4 mg/dl and DM with CV complication was 186.9 ± 90.0 mg/dl. However, the mean of 2 hours PP glucose levels of both DM groups with and without CV complications were similar. (See table 1).

The mean Hcy levels were the lowest in control group (7.7 ± 1.7 mg / dl). Groups of DM with and without CV complications were similar, 13.1 ± 3.7 vs. 13.1 ± 15.7 mg / dl, respectively.

The mean of total cholesterol levels of the non-DM and DM groups with no similar CV complications were (183.2 ± 32.2 mg / dl and 183.2 ± 34.9 mg / dl). DM group with lower CV complications (165.0 ± 50.5 mg / dl). The highest mean triglyceride rate was found in the DM group with CV complications, followed by DM without the complications of CV and the lowest in the non-DM group (202.5 ± 178.9 vs 183.2 ± 34.9 vs. 104.6 ± 78.2 mg/dl).

The mean of LDL cholesterol level was highest in the DM group without complications of

CV, followed by the non-DM group and the DM group with CV complications (115.2 ± 34.9 vs 109.7 ± 30.2 and 105.7 ± 47.4 mg/dl). The highest HDL cholesterol average level was found in the non-DM group, followed by DM without CV complications and DM group with CV complications (42.4 ± 6.3 vs 37.6 ± 7.0 vs. 34.3 ± 4.1mg / dl).

Table 1. Description of the subject

Parameter	Mean±SD Min-maks	Control (n=26)	DM without CV (n=30)	DM with CV (n=30)
Age(years)	Mean±SD	32.7 ± 6,3	56.2 ± 9.7	59.3 ± 7.8
	Min-maks	22-51	36-75	40-73
Sexuality (total/in %)	Man	12 (46.2)	10 (33.3)	23 (76.7)
	Woman	14 (53.8)	20 (66.7)	7 (23.3)
Smoking (total/in %)	Yes	7 (26.9)	6 (20.0)	19 (63.3)
	No	19 (73.1)	24 (80.1)	11 (36.7)
BMI	Mean±SD	23.3 ± 4.5	22.8 ± 2.5	25.6 ± 2.2
	Min-maks	17.6-39.8	18.4-27.6	22.4-29.8
Long suffering from	Mean±SD		6.9 ± 3.2	9.0 ± 3.5
DM(years)	Min-maks		3-15	5-20
Fasting blood glucose(mg/dl)	Mean ±SD	84.1 ± 9.1	167.7 ± 52.4	186.9 ± 90.0
	Min-maks	69-110	100-285	65-451
2 hours Post Prandial glucose	Mean±SD	89.4 ± 19.1	259.8 ± 69.3	258.2 ± 117.0
(mg/dl)	Min-maks	57-152	132-434	118-605
Total Cholesterol(mg/dl)	Mean±SD	183.2 ± 32.2	183.2 ± 34.9	165.0 ± 50.5
	Min-mak	145-298	110-267	110-267
Triglyseride (mg/dl)	Mean±SD	104.6 ± 78.2	151.2 ± 78.4	202.5 ± 178.9
	Min-maks	47-451	62-419	62-419
LDL (mg/dl)	Mean±SD	109.7 ± 30.2	115.2 ± 34.9	105.7 ± 47.4
	Min-maks	77-223	49-207	39-205
HDL (mg/dl)	Mean±SD	42.4 ± 6.3	37.6 ± 7.0	34.3 ± 4.1
	Min-maks	35-63	27-66	24-45
Homocysteine(µml/dl)	Mean±SD	7.7 ± 1.7	13.1 ± 15.7	13.1 ± 3.7
	Min-maks	5.0-13.0	4.0-93.9	6.4-26

Table 2 Differences in levels of homocysteine and lipid profiles between groups

Group	Hcy	Cholesterol	LDL-C	HDL-C	Triglyseride
Non-DM and DM without CV complication	0.086	0.999	0.531	0.009*	0.030*
Non-DM and DM wth CV complication	0.000*	0.111	0.710	0.000*	0.013*
DM without and with CV complication	0.993	0.111	0.383	0.033*	0.156

Differences in levels of Homocysteine and Lipid Profiles between Groups

Difference test for each variable between groups were done by using independent t test for Hcy level and lipid profile, and can be seen in Table 2.

Homocysteine

The results showed that there were significant difference on Hcy levels between non-DM group and DM group with CV complications of (p=0.000). However, there was no significant difference between non-DM group and DM group without CV complication (p=0.086), as well as between DM group without and with CV complication (p=0.993).

Lipid profile

The results of data analysis in this study showed that there is no significant difference between total cholesterol and LDL cholesterol in the non-DM group and DM without CV

complications (p=0.999 and p=0.531); non-DM and DM with CV complication (p=0.111 and p=0.710); DM without and with complications (p=0.111 and p=0.383)

We found there were significant differences in HDL cholesterol levels between the non-DM group and the DM group without CV complication (p=0.009), between the non-DM group and the DM group with CV complications (p=0.000), as well asq between the DM group without and with CV complications (p=0.033). In this study there was a significant difference in triglyceride levels between the non-DM group and the DM group without CV complications P=0.030) and with CV complication (p = 0.013). There was no significant difference of triglyceride level between the DM group without and with CV complication (p=0.156)

DISCUSSION

Obesity, DM, and duration of DM were the risk factors for cardiovascular disease, the mean duration of DM in this study was longer in the DM group with CV complications. The mean fasting blood glucose level was higher in the group with CV complications (186.9 ± 90.0 mg/Dl) compared to group without CV complications (167.7 ± 52.4 mg/dl), but the mean 2 hours PP blood glucose levels of both two groups were similar.

High triglyceride levels and low HDL levels are risk factors for cardiovascular disease. High Hcy levels are also risk factor for cardiovascular disease as found in the DM group with complications of CV.

Homocysteine

The majority of diabetes mellitus subjects with cardiovascular complication were: males (76.7%), the oldest age among the three groups, the highest fasting glucose level compared to the other two groups, smoking (63.3%), the highest BMI and longer suffering from DM (table 1). All of these factors are risk factors for cardiovascular disease. The risk factors of hyperhomocysteinemia encountered in this group are male, higher BMI, smoking and DM. As we know increasing levels of Hcy also a risk factor for cardiovascular disease. There was significant difference in Hcy levels between the non DM group and the DM with cardiovascular complication, eventhough still in the normal range.

Homocysteine is an amino acid which is a metabolic intermediate in the metabolism of essential amino acids methionine.[6] Homocysteine is found in daily diets, and high levels are common in atherosclerotic DM and CHD. Thus, Hcy is strong independent predictors of CHD occurrence. Association between Hcy and atherosclerosis remains unclear, suspected to be associated with free radicals, smooth muscle cell stimulation and changes in platelets and hemostasis. Hyperhomocysteinemia is a risk factor for endothelial dysfunction. Homocysteine levels are higher in patients DM and is a risk of CHD.(9)

The results of various studies on the effect of glycemic control on plasma Hcy were inconsistent. Previous studies suggested that there was no correlation between Hcy and glycemic control. Hoogoven et al reported that there is no association between Hcy and HbA1c. Another study conducted by Aghamohammadi et al, examined the association between Hcy levels and glycemic control in 70 men with type 2 DM and concluded that there was no correlation between Hcy on glycemic control.(10) Diabetes has a CVD risk of 2 to 4 times compared to non-diabetes and atherosclerotic also develops despite mild glucose tolerance disorder.

Homocysteine is an interesting topic in vascular disease in recent years, elevated Hcy levels can increase CVD and mortality in the population. Increased Hcy levels are also present in DM patients but their association with CVD remains unclear.

Studies in humans showed that Hcy spurred atherosclerosis, it is characterized by platelet accumulation and thrombus formation in the endothelial area of the lesion. Hcy stimulates endothelial injury in sub-endothelial matrices that will stimulate platelet activation.(11) Other researchers pointed out that Hcy is a risk factor for type 2 DM not only for coronary events but also deaths from cardiovascular, retinopathy and microalbuminuria.(12)

Several cross-sectional studies and case control studies showed that moderate HHcy increased risk factors for atherosclerosis and cardiovascular disease (CVD). Some researchers found significant Hcy effect on CVD. Recent studies mention that HHcy contribute 10% to the risk of Coronary artery diseases (CAD). Although possible mechanism explaining the relationship of plasma homocysteine level and CVD is inconclusive. The most possible hypothesis that elevated homocysteine levels is endothelium dysfunction due to enhanced oxidative stress and reduced the production and bioavailability of nitric oxide (a strong relaxing factor) of the endothelium .(13)

Lipid Profile

Abnormal lipids are often primarily found in DM type 2. The prevalence of dyslipidemia in DM

is 95%. Dyslipidemia is a major risk factor for CHD which is a cause of morbidity and mortality in DM patients due to elevated serum triglycerides (69%), cholesterol (56%), LDL-C (77%) and HDL-C decrease (71%). Hyperlipidemia is the most common complication of DM and predisposes to atherosclerosis and macrovascular complications. The most common abnormal lipids in DM are elevated triglycerides, LDL-C and decreased HDL-C. Good glycemic control will prevent the development and progression of abnormal lipids in DM patients.(14)

Several factors can affect lipid levels in patients with DM, such as interrelation between carbohydrates (CH) and lipid metabolism. Therefore, any interference on CH metabolism will cause disruption of lipid metabolism and vice versa. Insulin resistance is a primary defect in majority of DM type 2 and in non-DM insulin resistance and hyperinsulinism is a predictor factor of progression to DM type 2. Some studies indicate that insulin affects apolipoprotein production by the liver, regulates activity of enzyme lipoprotein lipase and cholesterol ester transfer protein (CETP) causing dyslipidemia in DM. In addition, insulin deficiency decreases hepatic lipase activity and some of the production stages of biologically active lipase protein.(15)

This study obtained no significant difference on Total Cholesterol and LDL-C between each group, this is probably due in DM patients with CV complications has been aware to keep his diet and is probably already taking oral diabetic medications and anti-lipidemia, despite trying dug this questioners. It is evidenced by mean cholesterol and LDL-C levels were higher in the DM group without CV complications compared with DM group with CV complication.

Pakard et al reported a decrease in HDL-C is a strong predictor of CHD, while Goldberg reported hyperglycemia progressive increase transfer of cholesterol esters from HDL-C to particles VLDL-C, then the LDL-C particle solids will acquire the majority of HDL ester and will reduce levels of HDL-C. Improved glycemic control will increase HDL-C, poor glycemic control will lower lipoprotein levels. Poor insulinization will increase lipolysis in adipose tissue which will increase fatty acid (FA) transport to the liver and increase VLDL-C. Insulin directly degrades Apo B (the main protein VLDL-C) and increases Apo B secretion.(15)

The highest level of HDL-C was found in Non DM group, followed by DM group without CV complication and the lowest in DM group with CV complication.

Decreased HDL-C levels are associated with an increased risk of CHD. HDL-C is cardio-protective. Decreased HDL-C is often accompanied by increased levels of trigliceride.(16) HDL-C decreases in DM due to decreased lipoprotein activity because the rate of HDL2 formation depends on the flux rate of the surface component of triglyceride rich lipoprotein mediated in part by lipoprotein lipase. When VLDL-C mediated by LPL catabolism is efficient, the availability of surface components for HDL-C transfer is increased, when undisturbed VLDL-C lipolysis will result in a decrease in HDL-C. Increased HDL-C catabolism due to hypertriglyceridemia, triglyceride transfer rate to HDL2 high and will result in triglyceride-rich HDL2 susceptible to catabolism by hepatic triglyceride lipase. Increased activity of cholesterol - ester transfer proteins (CETP), which modify pathological lipid composition of the sub-population of apoprotein B containing lipoproteins to form atherogenic b-like VLDL particles, so the increased activity of CETP are atherogenik.(3) Descriptive data in this study showed that the lowest average of fasting glucose level in the non-DM group and the highest in the DM group with CV complications, the same case was found in the triglyceride level, the highest level was found in the DM group with CV complications (Table 1).

Dyslipidemia increased in patients with elevated blood glucose levels. Hypertriglyceridemia is the most common lipid abnormality found in type 2 diabetes mellitus (73.3%)(15)(12)Other researchers attributed high triglyceride levels to poor glycemic control in patients with diabetes mellitus and obesity, thought to be associated with decreased lipoprotein lipase activity in muscle and adipose tissue.(15)

DMT2 is associated with elevated triglyceride and VLDL-C levels due to elevated glucose and FFA, VLDL-C and triglyceride clearance disorders resulting from decreased lipase activity of lipase proteins especially in moderate-severe hypertriglyceridemia indicating both deficiency and insulin resistance. In diabetes due to hypertriglyceridemia, large particles of triglyceride rich VLDL are secreted. Changes in VLDL composition have implications for increasing the propensity for atherosclerosis.(17)

Evidence of the relationship between plasma triglyceride levels and the risk of coronary arterial disease (CAD) is broadly based on epidemiological studies. Meta-analysis of 7 populations based on prospective studies found that each 1 mmol/ L increase in triglyceride levels increased the risk of 32% of coronary disease in men and 76% in women after corrected with HDL-C effects to 14% in men and 37% in women. The

direct atherogenic effects of triglyceride rich particle especially IDL-C and the remnant lipoprotein may independently contribute to plasma triglyceride levels as cardiovascular risk factors. It is said that dyslipidemia is associated with insulin resistance in DMT2 and is strongly associated with increased cardiovascular risk.(13)

CONCLUSION

The highest Hcy level, the lowest HDLC and the highest Triglyceride level found on DM with CV complication.

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Sojagol from Mung Beans as A Potential Antagonist of Mineralocorticoid Receptor

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ABSTRACT

This bio-computational study aimed to explore phytochemicals derived from Indonesian plants which inhibited the mineralocorticoid receptor (MR) for cardiovascular diseases treatment. A total of 516 phytochemicals was used in this study which was derived from the HerbalDB database and screened with Lipinski Rule of Five. Three dimensional structure of MR was obtained from a protein data bank (access code 3VHU) and the structure of aldosterone antagonists (spironolactone and eplerenone) as standard ligand was obtained from the ZINC database (ZINC03977913 and ZINC72187491) respectively. MR-standar ligand binding complexes were validated using AutoDock Vina 1.1.2 software three times. Interaction between MR and phytochemicals was molecularly dock with the same software and visualized using Chimera 1.9 software. Spironolactone had -9.5 kcal/mol docking score and MR binding site at Gln^{776}, Arg^{817}, and Cys^{942}. Whereas -9.7 kcal/mol docking score was observed in eplerenone and it had binding site at Arg^{817} and Thr^{945}. There were six phytochemicals with lower binding score againts MR than the standards but only Sojagol interacted with MR at Gln^{776} and Arg^{817} residues. More over, Sojagol had a lower molecular size (336.338 Da) compared with the standards and was commonly found in mung beans. In conclusion, Sojagol might become in silico antagonist of aldosterone.

Key words *: Mineralocorticoid receptor, Aldosterone antagonist, Spironolactone, Phytochemical*

In recent years, cardiovascular and circulatory diseases have become the world health burden and the leading causes of death worldwide. In 2013, there were up to 54 million deaths globally and cardiovascular diseases were attributable to 32% of these deaths (17 million).(1) Moreover, 80% of cardiovascular diseases mortality occurs in low-income and middle-income countries. It is estimated that cardiovascular diseases will give rise to 23.6 millions of death in 2030.(2)

Aldosterone has been known for many years as an endogenous hormone that plays an important role in the pathogenesis of heart disease.(3)(4) Growing evidence had indicated that aldosterone was involved in the progression of end-organ damage since it induced vascular smooth muscle hypertrophy, vascular matrix impairment (remodelling) and endothelial dysfunction.(5)(6) Inhibition of aldosterone and other mineralocorticoid steroids to the MR has been demonstrated to have beneficial therapy in various cardiovascular disease.(7) Hence, aldosterone antagonist has become a new therapeutic agent, based on its sodium retention properties in the management of cardiovascular diseases.(8)(9)

Spironolactone is firstly generated as MR antagonist. It is able to inhibit not only the MR but also other families of steroid receptor. Therefore, it is not surprised if long term administration of spironolactone has unwanted effects like progestational and antiandrogenic activities.(3)

Virtual screening which uses molecular docking methods is one of the most effective ways to explore new agents or phytochemicals as a novel candidate of aldosterone antagonist.(10) Molecular docking is a computer program that can predict the complex structure of two molecules in silico efficiently.(11) So, this study aimed to explore phytochemicals of Indonesian herbal plants which were able to interact with

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mineralocorticoid receptor (MR) and potentially developed as a new aldosterone antagonist.

MATERIAL AND METHODS

Protein preparation

Preparation of MR protein was performed using AutoDockVina program 1.1.2 version.(12) Three dimensional structure of MR was downloaded from Protein Data Bank (http://www.rcsb.org/pdb/) with access code 3VHU. This MR protein was crystallized with spironolacton and had 2.11 Å diffraction pattern. In addition, spironolacton was interacted with MR at Gln^{776}, Arg^{817}, and Cys^{942} residues.(13) Before running the molecular docking, spironolactone and water were removed from the MR structure. Hydrogen was then added to MR in order to increase polarity in the binding pocket. After that, grid box was made by selection of some amino acid residues that surrounded the binding pocket. Hence, three binding site of spironolactone to the MR had to be located in the center of grid box.

Standard ligand preparation

Aldosterone was used as the standard ligand agonist of MR while spironolactone and eplerenone became the aldosterone standard antagonists. Their three dimensional structures were obtained from ZINC database (zinc.docking.org) with access code ZINC03830183, ZINC03977913 and ZINC72187491 respectively. They were saved in *mol2 and *sdf format. Validation of MR-standard ligand interaction was molecularly docked with AutoDock Vina 1.1.2 software three times to obtaine the binding energy (Table 1). Visualisation of MR-standard ligand interaction was done using Chimera software (version 1.9, The Resource for Biocomputing, Visualization, and Informatics, University of California) (Fig.1). Their binding sites were compared with the binding sites of Hasui's study.(13) The validation results were then used as the reference to explore candidates of aldosterone antagonist.

Phytochemical preparation

Phytochemicals of Indonesian herbal plants were obtained from HerbalDB data base which was Indonesian herbal data base and developed by Department of Pharmacy, Universitas Indonesia http://herbaldb.farmasi.ui.ac.id/. The phytochemical structures were downloaded from a public data base at https://pubchem.ncbi.nlm.nih.gov/. All phytochemicals of Indonesian herbal plants were then screened using Lipinski's rule of five criteria(14) and 517 phytochemicals were used as the research samples. All selected phytochemicals were modified and prepared using PyRx program. They finally saved in *.pdbqt format file.

Table 1. Validation results of MR interacted with aldosterone, spironolactone or eplerenone

	Validation 1	Validation 2	Validation 3	Average of binding energy (kcal/mol)	Binding site
Aldosterone (ZINC03830183)	-9.3	-9.3	-9.3	-9.3	Asn^{770}, Gln^{776}, Arg^{817}
Spironolactone (ZINC03977913)	-9.5	-9.6	-9.5	-9.5	Gln^{776}, Arg^{817}, Cys^{942*}
Eplerenone (ZINC72187491)	-9.7	-9.7	-9.7	-9.7	Arg^{817} and Thr^{945}

*The first two binding sites were the same as binding sites of Hasui's work

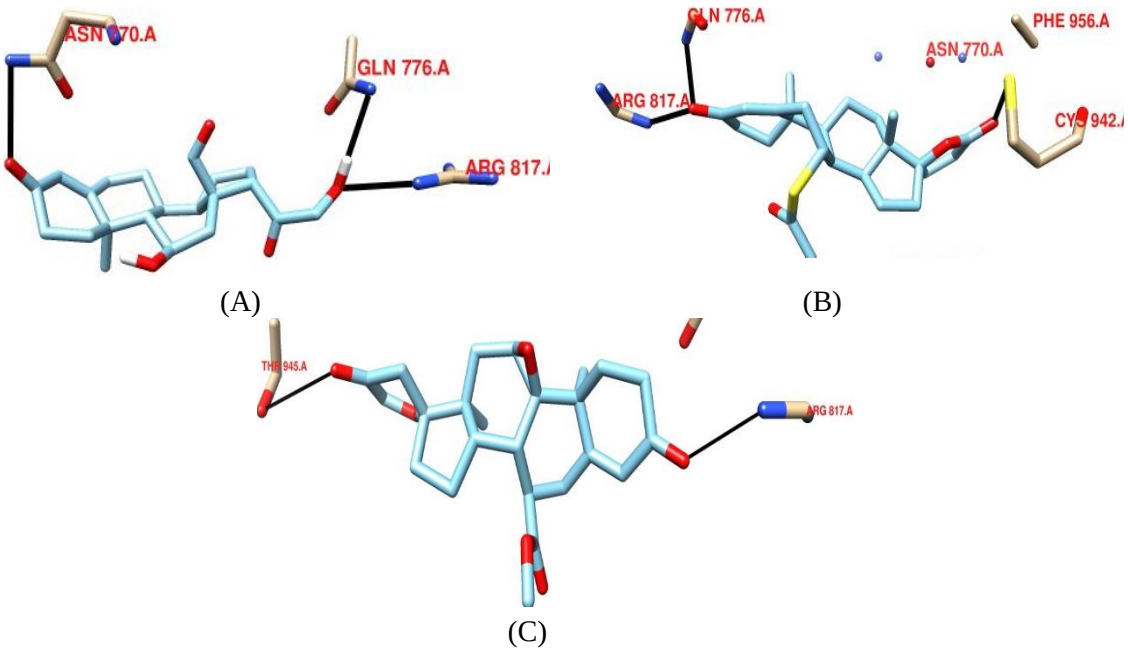


Fig 1. MR-standard ligand binding complexes were visualized using Chimera 1.9 software.

A). Aldosterone interacted with MR at Asn^{770} , Gln^{776} and Arg ^{817} residues. B). Spironolactone interacted with MR at at Gln^{776}, Arg^{817} and Cys^{942} residues while (C) eplerenone interacted with MR at Arg^{817} and Thr^{945}.

Docking and visualisation of herbal phytochemical

A total of 517 selected phytochemicals was molecularly docked with MR using AutoDockVina 1.1.2 program to analyze their binding energy. Molecular interaction of MR and phytochemicals was then visualized using Chimera 1.9 program. MR-phytochemical binding complexes which had lower binding energy than standard ligand antagonist and had similar binding sites were considered as a new candidate of MR antagonist.

RESULTS

Validation of standard ligands

Figure 1 showed that binding sites of MR agonist and antagonist. Aldosterone interacted with the MR at Asn770, Gln776 and Arg817 residues. Whist, MR antagonist (spironolactone) bound to MR at Gln776, Arg817 and Cys942 residues. These binding sites were different from the previous study which Asn770 was substituted with Cys942 residue. Arg817 and Thr945 residues were found in Eplerenone-MR interaction.

Molecular docking between MR and phytochemicals of Indonesian herbal plants

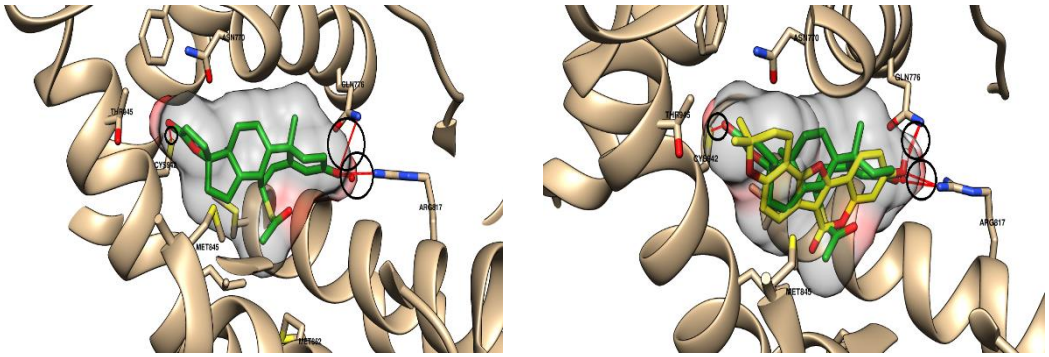
From 517 phytochemicals which full filled Lipinski's criteria, only 6 phytochemicals had lower binding energy than MR standard agonist and antagonists (Table 2). Progesterone had the lowest binding energy (-11.5 kcal/mol) whilst strychnine had the highest binding energy (-9.8 kcal/mol). Eurycomalactone and sojagol shared similar binding energy. In terms of binding site, there was only sojagol that had similar binding sites (Gln776 and Arg817) to aldosterone and spironolactone (Figure 1 and Table 1). Other phytochemicals interacted with MR only at one amino acid residue (Asn770, Leu810, Arg817, or Cys942). Additionally, molecular weight of all phytochemicals was lower than molecular weight of MR agonist and antagonists. The lowest molecular weight was observed in gentisin (258.226 Da) while the highest molecular weight was eurycomalactone (348.390 Da). Strychnine had molecular weight as similar as sojagol (approximately 335 Da).

Docking visualisation between MR and Sojagol

Figure 2 showed that sojagol occupied the MR binding pocket as similar as spironolactone except Cys^{942}. Sojagol bound to the MR at Gln^{776} and Arg^{817} residues and had similar conformation to spironolactone.

Table 2. Result of docking between phytochemical of Indonesian herbal plant with MR

Phytochemical name	Average of binding energy (kcal/mol)	Binding site	Molecular weight (Da)
Aldosterone	-9.3	Asn^{770}, Gln^{776}, Arg^{817}	360.45
Spironolactone	-9.5	Gln^{776}, Arg^{817}, Cys^{942}	416.583
Eplerenone	-9.7	Arg^{817}, Thr^{945}	414.498
Progesterone	-11.5	Arg^{817}	314.461
Strychnine	-9.8	Asn^{770}	334.412
Eurycomalactone	-10.2	Cys^{942}	348.390
Strigol	-10.6	Cys^{942}	346.374
Gentisin	-9.9	Leu^{810}	258.226
Sojagol	-10.1	Gln^{776}, Arg^{817}	336.338



A B

Fig 2. A. MR-Spironolactone binding complexes were visualized using Chimera 1.9. B. Overlay of MR-spironolacton/Sojagol binding complexes. Green colour was spironolactone and Sojagol was yellow. Black circles were designated binding sites.

DISCUSSION

In this study, we found some differences of binding sites between aldosterone /spironolactone and MR using different docking methods. Aldosterone is the endogenous steroid hormone that occupies the ligand binding domain (LBD) of MR at Asn^{770}, Gln^{776} and Arg^{817} residues. Our results differ from other studies that reported aldosterone interacted with MR at Asn^{770}, Ser^{767}, Cys^{942}, Thr^{945} and Glu^{955} residues.(15) Asn^{770} residue is importantly required for stable binding to activate MR by which recruits some coactivators.(8) The two remaining residues (Gln^{776} and Arg^{817}) were reported to play an important role in stabilization of hydrogen bound with the MR.(9) Whereas, Ser^{767}, Cys^{942}, Thr^{945} and Glu^{955} residues are also needed to make hydrogen bond with activation function-2 of MR in helix 3 and 10.(15)

Spironolactone is passive MR antagonist because this compound has labile interaction with the LBD of MR and prevent recruitment of coactivators.(16) Spironolactone occupies Gln^{776} and Arg^{817} residues of MR binding pocket to block aldosterone-MR interaction. In our study, spironolactone also binds to the MR at Cys^{942} residue to make a lipophilic bond and to restrict the volume of binding space.(8) In contrast to our results, Hasui and co-workers reported that spironolactone has Asn^{770} instead of Cys^{942} residue to interact with MR. Asn^{770} is useful for binding stability and partial MR agonist or antagonist.(15) Further investigation is required to solve these different results.

From this bio-computaional study, we have demonstrated that sojagol was a new candidate of *in silico* MR antagonist regarding to binding energy, binding sites and molecular weight. A lower binding energy observed in sojagol will has a higher affinity to interact with the MR

compared with the standard MR antagonists. In addition to binding affinity, the lower energy also stabilizes sojagol-MR binding complexes. In general, binding score in molecular docking programs is calculated by summing up all molecular interactions like hydrophobic, hydrogen, van de Walls, electrostatic and solvation effect.(17)(18) Therefore, stronger molecular interaction will has lower binding energy and more stable ligand binding complex.

In our study, sojagol has two residues (Gln^{776} and Arg^{817}) which are very important for interaction with the LBD of MR. Although sojagol does not have Asn^{770} binding site, it will give a beneficial effect which lead to inactivation of MR.(8) This natural compound is different from some synthetic compounds that created by Hasui's lab center. The synthetic compounds bind to the MR at Asn^{770}, Gln^{776} and Arg^{817} residues that they probably have side effects as same as spironolactone for long term use although their selectivity is higher than spironolactone.

The next advantage of sojagol properties is its molecular weight. Sojagol has lower molecular weight compared with the existing MR antagonists. Lower molecular size of drug molecules will increase their absorption and bioavaibility in blood circulation.(19)

Sojagol is a secondary metabolite which is found in *Phaseolus radiates* plant (mung bean). One study has reported that administration of mung bean extracts lowers blood pressure in mice and exerts anti-inflammatory and antioxidant effects.(20) Another study indicated that mung bean extracts suppressed inflammation-induced by lipopolysaccharide in macrophage cell line.(21) Moreover, cardioprotective effect appears in rat model with cardiac damage which was given mung bean extracts. In the end of treatment. It significantly improved the integrity of heart tissues.(22) Overall, sojagol may potentially to be developed as a new MR antagonist. In conclusion, six phytochemicals have lower binding energy and molecular size than spironolactone and eplerenone but only sojagol has similar binding sites to the MR antagonist. Sojagol might become in silico MR antagonist. Further investigation should be performed to investigate whether or not sojagol has MR antagonist activity.

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Diagnostic Value of Fractional Anisotropy in Detecting Hippocampal Sclerosis: A Study on Intractable Mesial Temporal Lobe Epilepsy with Normal MRI

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ABSTRACT

Purpose: This study aimed to investigate whether Fractional Anisotropy (FA) is reliable in detecting hippocampal sclerosis on intractable Mesial Temporal Lobe Epilepsy (MTLE) with normal MRI as measured from the degree of of neuronal loss, gliosis and axonal sprouting. **Method:** Twenty-three MTLE patients underwent surgery and 10 healthy volunteers were involved in this study. The MTLE diagnosis was based on semiology and ictal EEG, while hippocampal sclerosis was diagnosed using standardized MRI, followed by DTI FA. Histopathological analysis of hippocampus was performed with NeuN, GFAP, and NPY staining to detect the neuronal loss, gliosis, and axonal sprouting. Correlation and Diagnostic test was done to asses of diagnostic value FA. **Result:** Ten MTLE patients showed normal MR, 4 with hippocampal sclerosis and 6 were with FCD. The value of FA was significantly lower compared with healthy subject. The cut-off point of FA in detecting hippocampal sclerosis was 0.17 (AUC=0.89). The sensitiviy, specificity, positive predictive value, and negative predictive value of FA were 81.8%, 72.3%, 64.3%, and 89.56% respectively. There was significantly correlation between FA with the degrees of neuronal loss and gliosis. The concurrence between FA with EEG 7 out of 10 patients. **Conclusion:** Fractional Anisotropy has a good diagnostic value in detecting hippocampal sclerosis on normal MRI patients. In addition, this technique also shows a moderate association with degrees of neuronal loss and gliosis.

Keywords: *intractable MTLE; normal MRI; DTI; fractional anisotropy; hippocampal sclerosis.*Mesial

Temporal Lobe Epilepsy (MTLE) is one type of epilepsy appeared in adults that most often turns into intractable epilepsy. Hippocampal sclerosis is the most common cause of intractable MTLE. In this case, surgery will give a more satisfying result [1-3]. Surgery therapy needs diagnosis precision in determining the location as well as lateralization of epileptogenic zone in order to achieve a better surgery result. High Tesla MRI is a noninvasive examination has an excellence anatomic resolution and sensitive in detecting microstructural lesions, has sensitivity about 85-100% [4]. However, in about 20-30% intractable focal epilepsy patients undetected using standardized MRI [5].

Advanced Diffusion Tensor Imaging (DTI) -MRI technique can visualize a complex brain's tissue structure [6]. MD and ADC of DTI correspond to diffusivity whereas FA corresponds to anisotropy. Previous studies indicates a significant increase of mean diffusivity and a decrease of FA on ipsilateral hippocampal formation compared with the contralateral side, and also when compared with healthy subjects [7]. DTI role in visualizing a larger cerebral network, including extra temporal involvement in epilepsy with

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hippocampal sclerosis, which marked by the increase of diffusivity on epileptic hippocampal and ipsilateral temporal structure followed by the decrease of anisotropy along the temporal lobe. A decrease of diffusivity on hippocampal sclerosis, amigdala and contralateral temporal pole as well as an anisotropy decrease on ipsilateral posterior extra temporal region [8]. An MTLE study with hippocampal sclerosis shows that there is a correlation between histopathology and in vivo DTI i.e. an increase of extra axonal fraction and a cumulative decrease on both axon membrane circumference and myelin area at fornix fimbria [9]. There is an increase of FA along with the increase density of axonal mossy fiber sprouting on histopathology analysis using Timm staining [10]. There are no available data yet on the correlation between FA and axonal mossy fiber sprouting histopathology on humans.

There are only a few researches that study the use of DTI on epilepsy patients with normal MR and the results were inconsistent. Previous study finds diffusion abnormality on only 27% patients (8 out of 30 patients) with partial seizure cryptogenic [10]. In this research 6 out of 8 patients with diffusion abnormality and increase in diffusion correlate with epileptiform abnormality. Another study finds 64.3% (9 out of 14 patients) with diffusion change that is consistent with its intracranial EEG [8]. There is a significant increase in MD on 13 out of 15 subjects, an increase on FA (2 out of 15), and a decrease on FA (5 out of 15). In this study, the diffusion change is compared with healthy subject controls [8]. The changes of DTI (MD and FA) parameter can show a change in and around hippocampal and also on normal MRI epilepsy [12]. There has been no research concerning DTI diagnostic test on MRI-normal MTLE patients with histopathological analysis as a gold standard. Therefore, this study aimed to investigate whether Fractional Anisotropy (FA) is reliable in detecting hippocampal sclerosis on intractable Mesial Temporal Lobe Epilepsy (MTLE) with normal MR as measured from the degree of neuronal loss, gliosis and axonal sprouting.

MATERIAL AND METHODS

Patients

Twenty-three MTLE patients and ten healthy subjects were involved in this study. The criteria for healthy subject were who do not have any history of neurological and psychiatric disorder and have normal conventional MRI. The mesial temporal lobe epilepsy diagnosed according on ILAE criteria: 1) semiologically consistent with MTLE, included: epigastric, autonomic, or psychic auras followed by behavioral arrest, progressive clouding of consciousness, oroalimentary and manual automatisms, and autonomic phenomena; 2) unilateral or bilateral anterior, and temporal mesial of interictal spikes; 3) electroencephalogram video monitoring with seizure onset especially from temporal lobe; 4) an intractable TLE defined as response failure of 2 antiepileptic drug (AED). All MTLE subjects meet the following inclusion criteria: age ≥ 7 years; able to fulfill MRI examination requirements; and able to provide hippocampal surgery specimen for histopathological analysis. Drop out criteria when the surgery's specimen cannot be analyzed histopathologically.

Magnetic Resonance Imaging

All subjects were scanned on 1,5T MR scanner (Signa HDxt 16 Ch, GE Milwaukee USA) at Department of Radiology dr. Kariadi General Hospital and St. Elisabeth Hospital, Semarang Indonesia.
MRI epilepsy standard conducted on MTLE were T1, T2, FLAIR (Fluid Attenuation Inversion Recovery) sequence which perpendicular with hippocampal axis.

DTI

DTI were acquired using a single-shot echoplanar diffusion weighted (SE-EPI) applied simultaneously along 6 directions (b= 1000 s/mm2) as well as an acquisition without diffusion weighted (b=0 s/mm2). Moreover 30 contiguous axial slices were acquired with a 3 mm slice thickness with no gap. The acquisition parameters were: repetition time (TR):6100ms, echo time (TE):106 ms, number of excitation (NEX)= 2, flip angle 900, matrix 128x128, FOV (field of view) 230x230 mm. The total acquisition time was 4,5 min. All scans were reviewed by an experienced radiologist.

Image analysis

Data were transferred to independent workstation to measure FA and MD. FA and MD were calculated with available software application provided by manufacturer. Region of interest (ROI= 20-30 mm2) were selected on the axial section from a small oval region drawn in 4 locations (2 at head, 1 at body and at tail of hippocampus) then calculating the average. Adjacent cerebrospinal fluid-containing pixels were avoided in ROIs to reduce the partial volume effects. (Figure 1)

Histopathological staining

Haematoxyllin-Eosin (HE), NeuN, GFAP and Neuropeptide Y (Biocare) staining are to analyze the neuronal loss (NeuN), gliosis (GFAP) and axonal/Mossy Fiber sprouting (Neuropeptide Y). The score of lesion was categorized as mild grade when < 70% neuronal loss and there is no intensely stained for gliosis or axonal sprouting. Lesion score criteria categorized as severe grade when > 70% neuronal loss and intensely stained for gliosis or axonal sprouting.

Ethic
This study was approved by the local ethics committee. Informed written consent to participate in the study was also obtained from the patients and healthy volunteers.

Statistical analysis
Accuracy FA, in detecting hippocampal sclerosis, included sensitivity, specificity, positive predictive value, negative predictive value, and the association between FA with neuronal loss, gliosis and axonal sprouting.

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RESULTS

Clinical characteristics
The mean of MTLE group age was 24 years, the youngest was 14 years and the oldest was 41 years. Age mean in the healthy volunteer group was 31 years (ranging from 26 to 36 years). The age of healthy volunteer group showed no significant difference (p=0.19). In MTLE group 12 subjects were females (52.17%) and 11 were males (47.83%). As in healthy volunteer group most subjects were females (70%). The average duration of illness was 12.9 years ranging from 2 to 32 years. The average age of onset was 11.1, with onset range from 1 year to 22 years. Table 1, 2 and 3, showed data for patient group and the healthy control subject, along with the relevant medical history and DTI measurement

EEG and standardized MRI T2
The details of EEG lateralization were as follows: right 8, left 12, normal 2, and undetermined 1. Next, EEG video monitoring and intracranial were carried out towards 2 normal and 1 undetermined of EEG. From here it was found that there were 10 right and 13 left EEG lateralization. (Table 1)Based on T2/FLAIR parameter on 23 MTLE subjects, it was found that 11 hippocampal sclerosis (43.48%), 1

dysplasia, and 1 heterotopy. MRI T2/FLAIR right lateralization was 4 subjects, 5 with left lateralization, 10 subjects were normal, 1 subject bilateral, and 2 subjects' lateralization to the right (dysplasia and heterotophy). On 10 TLE cases with normal MRI, lateralization of 9 patients was determined by EEG monitoring and 1 case intracranial EEG. Furthermore, the location of lesion was identified by PET examination.

Table 1. Clinical characteristics of subjects and histopathological findings

Patient/ Sex/ Age (Y)	Age at onset (Y)	Duration of illness (Y)	Scalp EEG	Video EEG	Surgery	Histopathological Finding
1/ F/ 25	8	17	Lt		Lt SAH	Hippocampal sclerosis
2/M/30	18	12	Rt		Rt ATL	Hippocampal sclerosis
3/ F/19	9	10	N	Lt	Lt SAH	Hippocampal sclerosis
4/ M/26	5	21	Lt		Lt SAH	Hippocampal sclerosis
5/ F/28	22	6	Lt		Lt SAH	Hippocampal sclerosis
6/ F/17	1	16	Lt		Lt ATL	Hippocampal sclerosis
7/ M/17	12	5	N	Rt	Rt ATL	Hippocampal sclerosis
8/F/24	22	2	Lt		Lt SAH	Hippocampal sclerosis
9/ F/20	6	14	Lt		Lt ATL	Hippocampal sclerosis
10/ M/24	12	12	Rt	Lt	Lt ATL	Hippocampal sclerosis
11/ F/40	20	20	Lt		Lt SAH	Hippocampal sclerosis
12/ M/14	9	5	Rt		RT ATL	Focal Cortical Dysplasia
13/ M/41	15	26	Rt		Rt ATL	Focal Cortical Dysplasia
14/ F/34	9	25	Rt		Rt ATL	Focal Cortical Dysplasia
15/ M/18	16	2	Rt		Rt ATL	Focal Cortical Dysplasia
16/ M/16	5	11	Lt		Lt SAH	Focal Cortical Dysplasia
17/M/24	4	20	N	Rt	Rt ALT	Focal Cortical Dysplasia
18/ M/17	14	3	Rt		Rt ATL	Focal Cortical Dysplasia
19/F/20	7	13	Rt		Rt ATL	Focal Cortical Dysplasia
20/F/22	12	10	Lt		Lt SAH	Focal Cortical Dysplasia
21/ M/37	5	32	Lt		Lt SAH	Focal Cortical Dysplasia
22/F/20	15	5	Rt		Rt ATL	Focal Cortical Dysplasia
23/F/19	10	9	Lt		Lt SAH	Focal Cortical Dysplasia

MRI DTI FA analysis

FA on FCD and hippocampal sclerosis group were significantly lower compared to the normal subject group. There were a significant difference between FA values in hippocampal sclerosis group and FCD compare control group. (Table 4) However, there was no difference found between hippocampal sclerosis group and FCD. On MTLE with normal MRI, the FA showed a compatible lateralization compared to EEG (7/10).

Histopathological analysis

A complete structure of hippocampal was not found in all samples, however an enthorinal cortex structure, subiculum and CA1 were found. Neuronal loss and gliosis were prominent on CA1 of all subjects (probably hippocampal sclerosis). Based on the histopathological analysis of the surgery result, there were 11 subjects with hippocampal sclerosis and 12 with Focal Cortical Dysplasia (FCD).

MTLE with normal MRI on 10 subjects, 4 (40%) was considered as hippocampal sclerosis, with the following level of immunohistochemistry examination: 1 subject with mild gliosis, 50% neuronal loss, severe axonal sprouting, 1 subject with mild gliosis, 70% neuronal loss, mild axonal sprouting, 2 subjects with severe gliosis, 80% neuronal loss, severe axonal sprouting. Meanwhile, 6 subjects (60%) were histopathologically showed FCD. In 6 FCD, 4 subjects showed hyperintensity of hippocampal.

The diagnostic value of FA

A diagnostic test was conducted based on the result of COP FA. AUC of ≥ 0.7 was considered suitable for an analysis. In this study the size of AUC FA was 0.89, with cutoff point (COP) = 0.17. The sensitivity, specificity, positive predictive value, and negative predictive value of FA were 81.8%, 72.3%, 64.3%, and 89.56% respectively (Table 5). There were significantly negative correlation between FA with degree of neuronal loss ($r = -0.557$; $P = 0.006$) and gliosis ($r = -0.438$; $P = 0.037$ Spearman test).

Table 2. Summary of subject population evaluated with DTI

Patient/ Sex/ Age (Y)	MR T2	FA		MD (x10-3)	
		L	R	L	R
1/ F/ 25	Lt	0.19	0.18	1.29	1.19
2/M/30	N	0.17	0.16	1.31	1.29
3/ F/19	N	0.13	0.16	1.44	1.25
4/ M/26	Lt	0.13	0.15	1.47	1.24
5/ F/28	N	0.17	0.19	1.09	1.2
6/ F/17	N	0.22	0.15	1.15	1.21
7/ M/17	Rt	0.17	0.14	1.09	1.29
8/F/24	Lt	0.17	0.15	1.36	1.33
9/ F/20	Lt	0.12	0.13	2.13	1.38
10/ M/24	Lt	0.12	0.26	1.44	1.27
11/ F/40	N	0.18	0.19	1.3	1.23
12/ M/14	Rt Displasia	0.20	0.23	1.22	1.31
13/ M/41	Rt Heterotopia	0.19	0.25	1.21	1.20
14/ F/34	Rt	0.20	0.17	1.39	1.23
15/ M/18	N	0.19	0.16	1.31	1.31
16/ M/16	Lt	0.20	0.19	1.22	1.26
17/M/24	Rt	0.15	0.16	1.22	1.30
18/ M/17	N	0.16	0.15	1.21	1.24
19/F/20	Rt	0.20	0.17	1.23	1.25
20/F/22	N	0.19	0.18	1.27	1.29
21/ M/37	N	0.16	0.16	1.23	1.22
22/F/20	N	0.21	0.19	1.26	1.38
23/F/19	N	0.18	0.17	1.31	1.37

DISCUSSION

In this study FA has a good diagnostic value in detecting hippocampal sclerosis. Besides, FA value in MTLE case with MRI negative is lower than the one in control group. It also shows 70% lateralization capability in accordance with EEG. It is different from the increase in MD which was caused by myelin damage, FA decrease was caused by axonal degeneration axonal degeneration, the decrease of FA correlates significantly with the perimeter of total axon in an area [9]. Axonal sprouting and synaps reorganization with neuronal loss is the characteristic of hippocampal sclerosis on dentate gyrus neuron. The decrease of CA 3 hippocampal pyramidal cell is related to the enlargement of axonal sprouting that can decrease the inhibition function of dentate gyrus. In a bigger scale, spreading also happens in white matter. The result of correlation test in this study also finds a moderate correlation among the degrees of neuronal loss, gliosis and axonal sprouting. A study using DTI on Wistar rat also indicates the increase of FA along with the increase of axonal mossy fiber sprouting density on histopathological analysis with Timm staining [10]. Arfanakis et al study shows a significant increase in radial diffusion and decrease of FA on internal capsule, as well as anterior and posterior corpus callosum. In his research, Rugg Gunn indicates that the decrease of FA and the increase of MD have lateralization capability on MTLE. A change of white matter happens in both MTLE patients with hippocampal sclerosis and those without hippocampal sclerosis.

Table 3. Summary of the healthy control evaluated with DTI

Control/ Sex/ Age (Y)	FA		MD (10-3)	
	L	R	L	R
1/ M/ 36	0.20	0.20	1,17	1,17
2/M/35	0.21	0.25	1,07	1,18
3/ F/26	0.22	0.23	1,07	1,20
4/ F/34	0.23	0.29	1,08	1,04
5/ F/26	0.22	0.24	1,16	1,18
6/ F/35	0.26	0.23	1,15	1,15
7/ F/29	0.22	0.26	1,21	1,18
8/M/33	0.25	0.24	1,11	1,13
9/ F/29	0.20	0.25	1,13	1,11
10/ F/30	0.23	0.23	1,20	1,20

The diffusion process of free water molecule in brain MRI is likely to measure the microstructure (restricted diffusion due to cross fiber, the large number of cells, demyelinization, or gliosis). Some factors also affect diffusion, such as fibre diameter and density, membrane permeability, and myelinization that can influence the direction and the size of the shift of water. FA is a water diffusion deviation index of spherical random movement. Meanwhile, MD is the scalar marker of diffusion size in each voxel. FA has a large spreading pattern, whereas MD anomaly has quite bigger restricted distribution [9, 15].

Table 4. FA MRI DTI of hippocampal sclerosis, FCD (histopathology) and healthy volunteers

		Mean (SD)	
Parameter FA	Hippocampal Sclerosis	FCDHealthy volunteers	P
Right	0.17 (0.03)	0.18 (0.03)0,24 (0.02).	0.001[a]
Left	0.16 (0.03)	0.18 (0.02)0.22 (0.02)	0.000[a]

Table 5. Diagnostic value of FA and MD to detect hippocampal sclerosis

MRI Parameter	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuration (%)
FA	81.8	72.3	64.3	89.5	78.8
MD	81.8	68.2	56.2	88.2	72.7

Normal MRI can be caused by unclear bilateral and unilateral atrophy as well as symmetrical hippocampal. The etiology of epilepsy is a complex process of interaction which involved genetic and environment factor which affects treatment plan and prognosis. In intractable epilepsy, surgery is still the main option. The process of intractable epilepsy surgery relied on the information of the location of the focal epileptogenic zone. Thus, MRI examination is a reliable method as an early screening to diagnose any structural defects regarding epileptogenic zone. A clearly measurable lesion is mesial temporal sclerosis (hippocampal sclerosis) which is the main cause of most MTLE cases.

Besides hippocampal sclerosis, intractable epilepsy can be caused by extratemporal epilepsy, which covers almost 30% of cases. MRI examination cannot identify this lesion (Non Lesional Neocortical Epilepsi/NLNE). NLNE caused by FCD is often undetected by regular MRI examination. Currently, pathology specimens are still unable to give a good surgery outcome compare to those who present a clear epilepsy lesion. Epilepsy cases with normal MRI examination needs multimodality treatment in order to determine the epileptogenic zone.

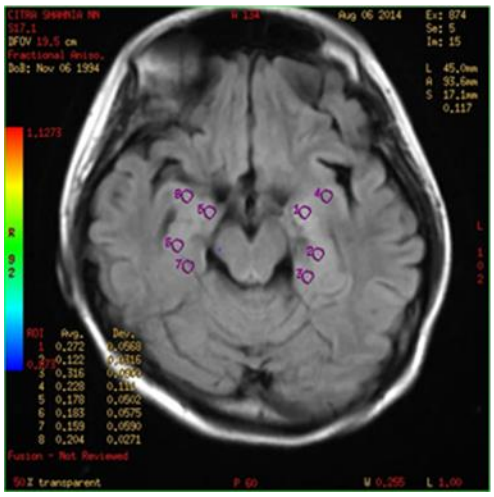

Fig.1 ROI's placement in hippocampal location

Qualitative MRI T2 examination which measured qualitatively is showed by hyperintense or non-hyperintense. Qualitative assessment is very subjective. Therefore, the ability to diagnose is not good enough, especially to T2 which able to be covered with LCS, especially on T2 that can be interfered with Liquor Cerebro-Spinal (LCS) hyperintense. In this case T2 is confirmed by FLAIR examination, which will show LCS as a hypodense so FLAIR can confirm in this examination, which will show LCS as a hypointense structure thus will look contrast if there is a hyperintense lesion in hippocampal.

The previous study shows a pathological finding on ELTI with normal MRI, i.e. 50% gliosis, microdisgenesis and cortical dysplasia are found [15]. The occurrence of FCD is due to the cortical malformation development, which is considered the most often cause of intractable MTLE in children and the second and third place in adults. The cause of intractable MTLE in adults is hippocampal sclerosis. A new classification shows a modification of Palmini classification [16]. FCD type 1 shows mild symptoms and slower onset usually occur in adult age and result in changes in temporal lobe. The second clinical symptom which is more severe is found in children. An extensive change is seen outside the temporal lobe with predilection of location in the frontal lobe. The third type of the new classification is the combination of either type 1 or 2 with other pathology, for example hippocampal sclerosis, tumor, vascular malformation or other pathology found during the early life phase [17]. MRI also able to show a clear pathology especially in FCD type 2. However, in type 1 pathology is often unclearly seen, and thus having a normal MRI examination.

CONCLUSIONS

The diagnostic value of FA MRI DTI in detecting of hippocampal sclerosis are 81.8%, sensitivity, 72.3% specificity, 64.3% positive predictive value and 89.5% negative predictive value respectively. Lateralization concordance between FA and EEG is 7 out of 10 patients with normal MRI. Therefore, FA shows a moderate significant association among neuronal loss, and gliosis.

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CONTENTS

Hiroshima J. Med. Sci.
May 2018 volume 67 Special Issue

CONTENTS

Hiroshima J. Med. Sci.
May 2018 volume 67 Special Issue

CONTENTS

Hiroshima J. Med. Sci.
May 2018 volume 67 Special Issue