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Prolonged Kidney Ischemia-Reperfusion Injury Associates with Inflammation, Vascular Remodelling, and Myofibroblast Formation

Nur Arfian*, Hilma Kholida Ats-tsani, Pratiwi Indah Sayekti, Dwina Agrila Lakabela, Amelia, Toni Febriyanto, Hana Rutyana Putri Antonio, Dian Prasetyo Wibisono, Dwi Cahyani Ratna Sari
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ABSTRACT

Prolonged kidney ischemia-reperfusion injury (IRI) is the important risk factor for leading to chronic kidney disease (CKD). Persistent hypoxia and inflammation are considered as the main pathogenesis of chronic injury, followed by myofibroblast expansion and fibrosis process. Tubular injury, cell proliferation, and vasoconstriction, as acute compensatory responses, are restored in chronic phase. The aim of the study was to investigate the relation between inflammation, vascular remodeling, and myofibroblast formation as response to ischemia injury after prolonged kidney ischemia-reperfusion (I/R). Fifteen male Swiss mice aged 3-4 months were used as kidney I/R injury model after bilateral pedicle renal clamping. Rats were divided into 3 groups with five rats in each group i.e. control group (sham operation/SO), acute I/R model (IR1), and chronic I/R model (IR12). PAS staining was used for scoring tubular injury. Fibrosis was assessed using sirius red and α-SMA immunostaining for myofibroblast expansion. PCNA and CD68 immunostaining were used for identifying cell proliferation and macrophage infiltration. RT-PCR was conducted for assessing MCP-1, HIF-1α, and ppET-1 expression, which were quantified using ImageJ software. Data were analyzed using one way ANOVA and Kruskal-Wallis test with significance level of $p<0.05$. Significantly increase of tubular injury score ($p<0.001$) and PCNA positive cell ($p<0.001$) in IR1 group compared to SO were observed, otherwise HIF-1α of IR12 enhanced ($p<0.05$). Macrophage cell count ($p<0.01$) and MCP-1 expression ($p<0.05$), were significantly increase in IR1 and IR12 injury, compared to SO. Wall thickness of arteries was significantly increase ($p<0.05$) as well as decrease of vascular lumen area ($p<0.05$), followed by enhancement of ppET-1 expression ($p<0.01$) in IR1 group and restored significantly ($p<0.05$) in IR12 group. Fibrosis fraction-area and myofibroblast expansion were significantly increase gradually from IR1 to IR12 injury ($p<0.01$). In conclusion, prolonged kidney I/R injury induces the sustainability of hypoxia and inflammatory response, which promotes myofibroblast formation, and decrease the response of vascular remodelling.

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ABSTRAK

Perpanjangan cedera iskemik-reperfusi ginjal (*kidney ischemia-reperfusion injury*/IRI) merupakan fakor risiko penting terjadinya penyakit ginjal kronis (*chronic kidney disease*/CKD). Inflamasi dan hipoksia berkepanjangan diduga merupakan pathogenesis utama cedera kronik, diikuti ekspansi miofibroblas dan kejadian fibrosis. Cedera tubulus, proliferasi sel dan vasokontriksi sebagai respon balik akut terjadi pada fase kronik. Tujuan penelitian ini adalah mengkaji hubungan antara inflamasi, remodelling vaskular dan pembentukan miofibroblas sebagai respon cedera iskemik setelah perpanjangan iskemik/reperfusi (I/R) ginjal. Lima belas mencit Swiss jantan berumus 3-4 bulan digunakan sebagai model cedera setelah dilakukan penjepitan *bilateral pedicle renal.* Tikus dibagi menjadi tiga kelompok dengan 5 ekor setiap kelompok yaitu kelompok *sham operation* (SO), kelompok model IR akut (IR1) dan kelompok model IR kronis (IR12). Pengecatan PAS digunakan untuk menilai cedera tubulus. Terjadinya fibrosis diukur menggunakan pengecatan imunologi merah sirius dan α-SMA untuk ekspansi miofibroblas. Pengecatan imunologi PCNA dan CD68 digunakan untuk mengidentifikasi proliferasi sel dan infiltrasi makrofag. RT-PCR dilakukan untuk mengkaji ekspresi MCP-1, HIF-1α dan ppET-1 yang diukur dengan program ImageJ. Data dianalisis menggunakan ANAVA satu jalan dan uji Kruskal-Wallis dengan tingkat signifikansi 0,05. Kenaikan secara nyata terjadi pada skor cedera tubulus ($p<0,05$) dan sel positif PCNA (0,05) pada kelompok IR1 dibandingkan SO, selain itu terjadi kenaikan HIF-1α pada kelompok IR12. Jumlah makrofag ($p<0,01$) dan ekspresi MCP-1 ($p<0,05$) meningkat secara nyata pada kelompok IR1 dan IR12 dibandingkan kontrol. Ketebalan dinding arteri meningkat (p0,05) diikuti penurunan area lumen vascular ($p<0,05$) dan kenaikan ekspresi ppET-1 ($p<0,01$) pada kelompok IR1 dan pulih secara nyata ($p<0,05$) pada kelompok IR12. Fraksi daerah fibrosis dan ekspansi miofibroblas meningkat nyata secara bertahap dari IR1 ke IR12 ($p<0,01$). Dapat disimpulkan, perpajangan cedera I/R ginjal menginduksi hipoksia dan respon inflamasi berkelanjutan yang menyebabkan pembentukan miofibroblas dan penurunan respon pemodelan kembali vaskular.

Keywords: ischemia-reperfusion injury - kidney – inflammation - vascular remodelling - myofibroblast.

INTRODUCTION

Kidney ischemia-reperfusion injury (IRI) is sudden temporary impairment of blood flow to the kidney, which is characterized by blood supply restriction to kidney and followed by restoration of blood flow and re-oxygenation (perfusion).[1] Kidney IRI is a major cause of acute kidney injury (AKI) and 70% of AKI progress to chronic kidney disease (CKD).[2] CKD is the chronic consequence of ischemia injury and thought to be related to glomerulo-interstitial fibrosis and persistent kidney dysfunction.[3]

The pathophysiology of kidney IRI is complicated. There are 3 stages of tissue response to ischemia injury, that are initiation, extension, and maintenance.[4] In early period or initiation phase, microvascular damage causes hypoxia in corticomedullary junction which is characterized by obstruction, inflammation, and coagulopathy. Then persistent hypoxia and inflammatory responses stimulate extension phase in 24 hours after initiation phase.[4] Loss of tubular brush border, exfoliation, and tubular obstruction are found in this period.[4] On day-3, maintenance phase is began. In this phase, there are repair process, migration, apoptosis, and proliferation to restore and maintain cellular and tubular integrity.[4]

Inflammation has the important role in early stage. Chemokines are major mediators of the inflammation that regulate pro-inflammatory cytokine, adhesion molecule expression, leukocyte activation and infiltration to the tissue.[1] Inflammatory mediators, reactive oxygen species (ROS), intracellular adhesion molecule (ICAM-1), and P-selectin can promote leukocytes and neutrophil infiltration into post-ischemic tissue.[1] The infiltration of leukocyte, including macrophages, may play an important role in development of kidney injury which is facilitated by chemotactic factors and/or adhesion molecules.[5] Monocyte chemoattractant protein-1 (MCP-1) is a potent chemokine that stimulates migration of monocyte into the intimate layer of arterial walls and organs.[5]

Enhancement of leucocyte-endothelial interaction can cause endothelial injury, then leads to decreasing blood flow to the tissue which aggravate ischemia.[1,6] Hypoxia tissue stimulates hypoxia-inducible factor-1 (HIF-1) expression. Then HIF-1 activates transcription of vascular endothelial growth factor (VEGF), which plays an essential role in angiogenesis.[6] Kidney injury causes tubular system damage, followed by rapid cell proliferation. The proliferation is an acute compensatory mechanism of injury, which is characterized by differentiation of tubular epithelial cell.[7] Those responses are associated with initial phase of injury. Cell proliferation can be represented by the expression of *proliferating cell nuclear antigen* (PCNA). PCNA is a monoclonal antibody which is expressed dominantly on S phase and essential for DNA replication.[8]

Imbalance between vasodilator and vasoconstrictor mediator causes reducing renal perfusion in AKI.[2] Endothelial dysfunction is responsible for reducing renal blood flow by impaired dilator capacity, which is attributed to reduce production of nitric oxide.[9] Endothelial nitric oxide synthase (eNOS) has important role in preservation of medullary blood flow in response to renal vasoconstrictor, such as angiotensin II. However, following renal injury, eNOS function is impaired which is demonstrated by a loss of responses to acetylcholine and bradykinin.[9] Endothelial cells also secrete endothelin-1 (ET-1), a potent vasoconstrictor. Through vasoconstriction effect, ET-1 induces reduction of renal blood flow and glomerular filtration rate,[2] which causes oliguria. In maladaptive response of maintenance phase, ROS can causes interstitial cells expansion and extracellular matrix production by inhibit tubular epithelial cell proliferation.[10] Fibrogenesis process and kidney interstitial fibrosis are shown in interstitial area expansion which is the main characteristic of progressive kidney disease.[11] This study was conducted to elucidate the kidney tissue response to prolonged injury, mainly about tubular injury appearance, inflammatory process, vascular changes, and myofibroblast formation.

MATERIALS AND METHODS

Animal

This was a quasi experimental study with post-test only controlled group design using 15 male Swiss-Webster mice aged 3-4 months old with 30-40 g body weights (BW). Mice were obtained from Animal Model Care Unit, the Integrated Research Testing Laboratory, Universitas Gadjah Mada, Yogyakarta and divided into three groups with five mice in each group i.e. sham operation (SO) as control group, ischemia/reperfusion day-1 (IR1) as AKI model group, and ischemia/reperfusion after 12 days (IR12) as CKD model group. Mice were maintained based on standard laboratory condition and provided diet and

water *ad libitum* before used. Protocol of this study has been approved by the Medical and Health Research Ethic Committee of the Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta.

Kidney ischemia/reperfusion injury model

The mice were administrated general anaesthesia with intraperitoneal injection of pentobarbital sodium (0.1 mg/g BW, Somnopentyl®). Kidney IRI model were performed by clamping both of renal pedicles, using non-traumatic vascular clamp (Hammacher®) for 30 minutes. Then, both clamps were released and followed by reperfusion. The incision site then closed using silk surgical thread 3/0 (One Med®).

Kidney harvesting

IR1 group was sacrificed in day-1 after operation, while IR12 groups after day 12 after operation. Prior to abdomen and thorax opened, mice were anaesthetized with intraperitoneal injection of pentobarbital sodium (60 mg/kg BW, Somnopentyl®). Perfusion of the organ was done from left ventricle, using 0.9% NaCl solution. Both perfused kidneys were harvested, one kidney was kept into RNA later® for RNA extraction and the rest was fixated into 4% PFA in PBS for 24 hours, and paraffin was used for the embedding tissue process.

Histological analysis and immunohistostaining

The kidney was embedded in paraffin block with 4 µm sections. Paraffin sections were deparaffinized and rehydrated using xylene and alcohol serial. Specimens were then stained with sirius red (SR) for measuring fibrosis interstitial fraction area and periodic acid-schiff (PAS) to determine tubular injury. For immunohistochemical staining, after deparaffinized and rehydrated, followed antigen retrieval, blocking peroxidase using H_2O_2 3% in PBS solution, and then blocking non-specific antigen using background sniper. The slides were incubated with α-SMA (1:400, Sigma, A2547), CD68 (1:400, Abcam, ab125212), and PCNA (1:200, Abcam, ab29) as 1st antibodies, TrekAvidin-HRP, 2nd antibody anti rabbit Trekkie Universal Link (Biocare Medical®), then diaminobenzidine tetrahydrochloride (DAB). α-SMA antibody immunostaining was used for measuring myofibroblast expansion, CD68 antibody for counting macrophage cells, and PCNA for assessing cell proliferation in kidney injury. Quantification was measured from 15 fields for each sample with 400x magnification, using ImageJ software.

Tubular Injury and Interstitial Fibrosis Fraction-area quantification

Tubular injury score was assessed by PAS staining, which was determined using semi quantitative scoring system in 15 fields for each specimen with 200x magnifications. The variables of scoring are tubular atrophy and dilatation, loss of brush border, accumulation of inflammatory cells, and intraluminal cast. Scale of the lesion are from 0 to 4: 0, normal; 1, mild, injury <25%; 2, moderate, injury 25-50%; 3, severe, injury 50-75%; 4, extensive damage, injury >75%. Fibrosis fraction-area was stained using Sirius red, which was quantified using ImageJ software on 15 non-overlapping fields and expressed in percentage (%).

Vascular remodeling

Lumen area and wall thickness were measured on sirius red staining of intra renal arteries. 10-15 arteries were randomly chosen,

and then assessed using ImageJ software. The arteries were measured vessel area (diameter of outer layer), lumen area (diameter of inner layer), vessel perimeter, lumen perimeter, wall area (the difference between vessel area and lumen area), and central perimeter (the average of vessel and lumen perimeter). Wall thickness calculation is from the ratio between wall area and central perimeter.

Reverse transcriptase PCR (RT-PCR)

RNA was extracted using Genezol solution (Genezol®, Cat. No. GZR100), followed by RNA concentration quantification using sphectophotometry. cDNA was synthesized using Rever Tra Ace® (Toyobo, Japan, Cat. No. TRT-101) and random primer (Toyobo, Japan, Cat. No. 3801). Reverse transcriptase PCR was done for assessing the expression of following genes: HIF-1α forward AGCTTCTGTTATGAGGCTCACCATC3', reverse AATGTCAAGATCACCAGCAC-3'), MCP-1 (forward 5'-CTTCTGGGCCTG CTGTTCA-3', reverse 5'- CTTCTGG GCCTGCTG TTCA-3'), ppET-1 (forward 5'-GCCACAGACCAGGCAGTTAGA-3', reverse 5'-ACCAGCTGCTGATA GATACACTTC-3'), GAPDH (forward 5'-TTGCTGTTGAAGTCGCAGGAG-3', reverse 5'-TGTGTCCGTCGTGGAT CTGA-3') were used as reference. The gene expressions were quantified using densitometry analysis (ImageJ software) and GAPDH gene was used to normalized the gene expressions (housekeeping gene).

Statistical analysis

Data were presented as mean ± standard error of mean (SEM) for PCNA, HIF-1α, MCP-1 levels, fibrosis fraction-area, myofibroblast expansion, vascular remodelling (wall thickness and lumen area), and ppET-1 expression. For tubular injury score and macrophage cell counting were presented as median (min-max) data. Median data were analyzed using non-parametric test, Kruskal-Wallis, and then each group was compared using post-hoc Mann-Whitney. While the rest data, which have normal data distribution, were analyzed using one-way ANOVA test, followed by post-hoc LSD test. The level of statistical significance was $p<0.05$.

RESULTS

Kidney IRI induced tubular injury, cell proliferation, and inflammation

Tubular injury in kidney IR1 model is a response to acute injury. It was shown in tubular injury score of IR1 group was increased significantly ($p<0.001$), compared to control group and IR12 (FIGURE 1.B). This result was parallel to PCNA immunostaining. PCNA positive cell was extremely increased in IR1 group, and it was decline in IR12 ($p< 0.001$, FIGURE 1.C). Paradoxically, HIF-1α was more expressed significantly in the CKD model (IR12 group) as shown in FIGURE 1.E. It was due to HIF-1α expression is associated with the maintenance phase.

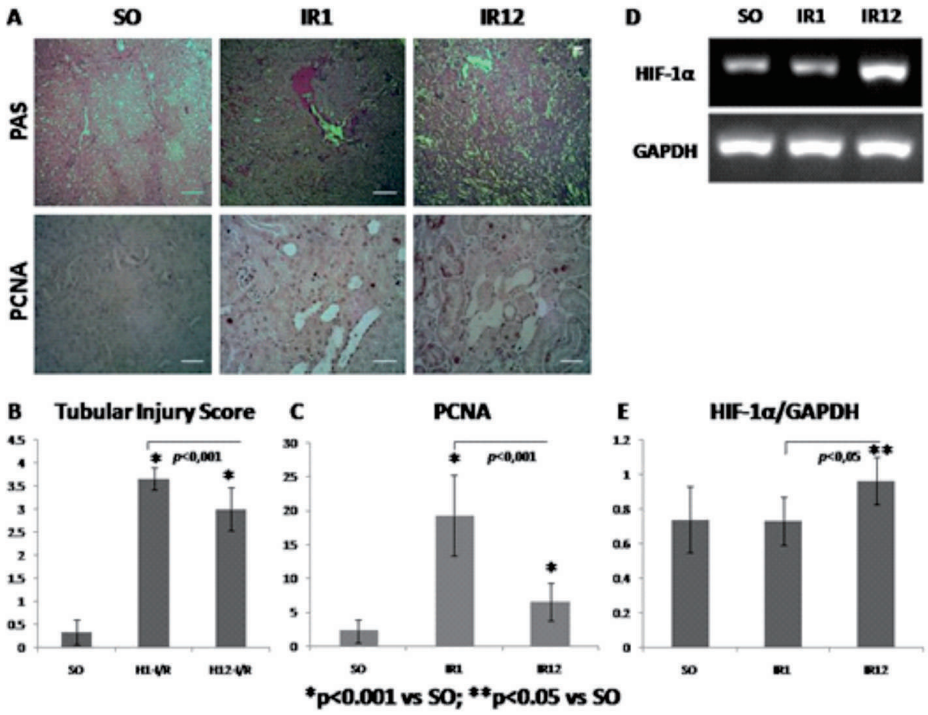

FIGURE 1. A. Microscopic picture of PAS and PCNA staining to show tubular injury and cell proliferation; B-C Quantitative analysis of tubular injury score and PCNA positive cell count; D-E Electrophoresis band and RT-PCR measurement of HIF-1α.

This study used MCP-1 and macrophage as representation of inflammation process. MCP-1 is a regulator of macrophage migration and infiltration. Therefore, increase of MCP-1expression in IR1 group was followed by increasing macrophage cell number, using CD68 immunostaining (FIGURE 2 C-D). This enhancement was persisted until chronic phase. It can be observed in IR12 group, which was still increased.

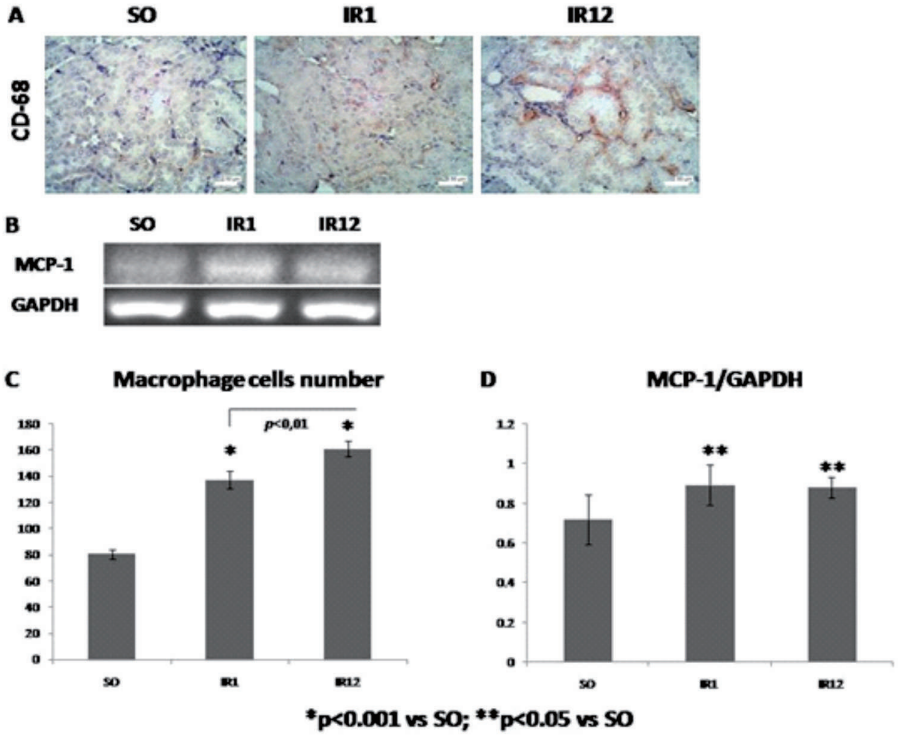

FIGURE 2. A. Representative picture of CD-68 immunostaining; B. Electrophoresis band of MCP-1 and GAPDH; C-D Quantitative analysis of macrophage cell count and RT-PCR measurement of MCP-1

Vascular remodelling of intrarenal artery

Vasoconstriction response in acute phase is related to the expression of ppET-1 gene. It was found that there were histological vascular changes of intrarenal artery (FIGURE 3.A). One day post-exposure, the vessel was constricted. It was proved by increase of wall thickness as well as decrease of vascular lumen area in IR1 group (FIGURE 3 D-E). And after 12 day post I/R, that condition was returned, showed by no significant difference between IR12 group and control group (FIGURE 3.D-E). Consistently, this condition was followed by increase of ppET-1 gene expression one day after IRI, then decline in IR12 group ($p < 0.05$, FIGURE 3.C).

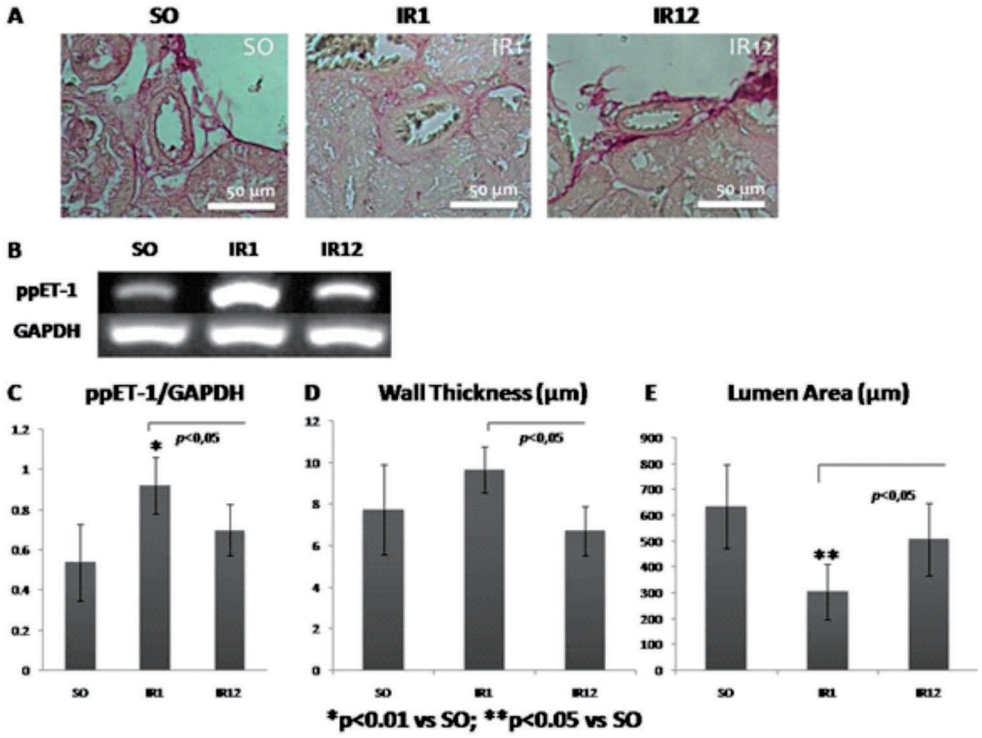

FIGURE 3. A. Histological changes of intrarenal artery are evaluated using sirius red staining; B-C. Electrophoresis band and RT-PCR measurement of ppET-1; D-E. Quantitative analysis of intrarenal artery on wall thickness and lumen area.

Fibrosis and myofibroblast expansion

Fibrosis area was stained in purplish-red by sirius red staining (FIGURE 4.A). The widest fibrosis fraction-area was shown from IR12 group (4.927%), then followed by IR1 group (3.260%), and SO group (1.021%) (FIGURE 4.B). These finding was similar to myofibroblast expansion. There were abundant of myofibroblast in IR12 group, compared to IR1 and control group. Statistical analysis showed significant difference between groups of fibrosis fraction-area and myofibroblast expansion ($p<0.01$, FIGURE 4.B-C).

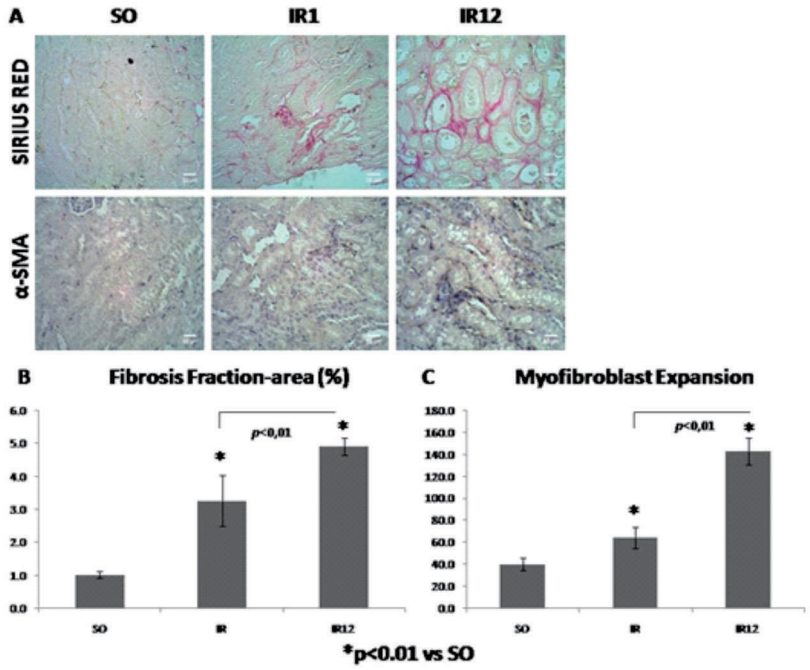

FIGURE 4. A. Sirius red and α-SMA IHC staining to show fibrosis and myofibroblas expansion; B-C. Quantitative analysis of fibrosis fraction-area and myofibroblast expansion

DISCUSSION

Kidney IRI effect on cell proliferation and inflammation response to tubular injury

The principle of kidney IRI is sudden temporary impairment of blood flow to the kidney.[1] There is injury/regeneration process in kidney injury which is characterized by impairment of cell polarity, cytoskeleton integrity, and loss of renal tubular brush border which can induce apoptosis and necrotic process.[12,13] Brush border and cell debris can cause intraluminal obstruction which form an intraluminal cast in the distal tubules. These obstructions can promote the dilatation and atrophy of proximal tubules in acute kidney injury.[14] Therefore, those characteristics were used for assessing tubular injury score. After 7 days post injury, there is a repairing process by the differentiation tubular cell and restoration of renal function and structure.[7] In this study, IR1 group, as an acute response, was impaired significantly compared to SO and IR12 group (FIGURE 1.A-B). It was correspond with study that was done by Basile *et al.*[9] reduction of tubular injury score in IR12 group was affected by repairing mechanism of tubular cells which was restored incompletely (FIGURE 1.B).

Bonventre & Duffield[15] study showed that injury in renal tubules was increased in 24 hours after IRI, followed by cell proliferation and optimized in 48 hours post IRI induction. This study used the expression of PCNA antibody for describing cell proliferation in acute and chronic phase after IRI. In normal physiologic condition, tubular epithelial cells have less ability to proliferate, it is shown by slowness of cell turnover. After injury, proliferation rate is increased significantly to

replace the necrotic/apoptotic cells.[16] PCNA positive cell of IR1 group was more expressed than in chronic period (IR12) ($p< 0.001$, FIGURE 1.C). It meant that the proliferation rate in chronic injury was reduced. Decreasing of cell proliferation is caused by repair process which is began from day-7 after IRI.[7] Furthermore, there is a maladaptive repair process that decrease cell proliferation by discontinuing G2/M phase, then PCNA, which is dominantly expressed in S phase, will be decreased.[16]

Persistent hypoxia in I/R injury can causes sustainable injury and increase of HIF-1α expression.[17] HIF is activated by low oxygen condition and induces widespread changes in gene expression.[17] Many of genes whose expression is increased by HIF are expected to improve the cellular capacity when oxygen supply is reduced.[17] Therefore, activation of HIF may improve the survival of ischemic cell and also promote adaptive changes, such as increased angiogenesis.[18] HIF appear in 10 minutes after ischemia, the most optimal period is 2 hour post-ischemia, and will be decreased in 8-24 hours after the optimum phase.[18] HIF-1α expression of IR1 group was not increased, it was estimated that the level was decline after 24 hours post-exposure, due to HIF-1α is degraded rapidly (FIGURE 1.E). Several study revealed that HIF has protective effect in acute phase of ischemia injury. Paradoxically, in prolonged period of hypoxia, HIF-1α will be increased. Higgins *et al*.[19] found that the ablation of HIF-1α gene can prevent fibrosis tubulointerstitial expansion, through mesenchyme-epithelial cell transition. Moreover, Haase[20] showed that the prolonged HIF signal activation can stimulate fibrosis and persistent destruction of tissue. When HIF is stable and not degraded, it can lead to enhancement of pro-fibrotic gene transcription, connective tissue growth factor (CTGF).[20] Hence, the highest level of HIF-1α was expressed in IR12 group significantly ($p<0.05$) (FIGURE 1.E). It is due to maladaptive response of repair process, so that in certain condition the level of HIF is associated with chronic injury.

Inflammatory response is the main role in pathogenesis of kidney injury. It can affect on acute and chronic (maladaptive amelioration) phase.[21] When kidney is exposed to ischemia injury, the epithelial cells will be change, the barrier and endothelial integrity can be damaged.[9] This process produce proinflammatory cytokine and chemotactic, such as TNF-α, MCP-1, IL-8, IL-6, TGF-β, RANTES and ephitelial neutrophil-activating protein 78 (ENA-78), which activate inflammatory cells, including macrophage.[21] Enhancement of MCP-1 is associated with the presence of macrophage.[5] Sutton *et al*.[4] reported that in initial phase (less than 24hour) cytokine and chemokine are increased, including MCP-1. MCP-1 can be produced by vascular smooth muscle cells.[22] Ischemia condition will stimulate endothelial dysfunction, followed by inflammatory cells infiltration. As a compensated mechanism, smooth muscle tone will be increased, then induces the activation of vascular smooth muscle to produce MCP-1.[22] Therefore, MCP-1 level will be increased and macrophage infiltration will be stimulated. Increase of MCP-1 in exposed group (IR1 and IR12) is significant ($p<0.05$, FIGURE 2.D). This enhancement was followed by increase of macrophage cell significantly ($p<0.05$), which is observed by CD68 immunostaining (FIGURE 2.C).

Repair phase of kidney injury consist of two conditions, complete and incomplete restoration. When injured tissue is restored completely, tubular cell will differentiate and proliferate to replacing the dead cells.[21]

Contrarily, when the repair response is incomplete, it will induce maladaptive process, such as fibroblast proliferation, excessive extracellular matrix deposition, and inflammatory response will be persistent,[21] so that proinflammatory chemokine still produced. It can be found in MCP-1 expression and macrophage level of IR12 group, which still increased (FIGURE 2.C-D).

Kidney IRI effect on vascular remodelling

Renal ischemia affects the renal vascular and tubules. There is morphological and structural changing of renal tubules post-exposure, while auto regulation disturbance and vasoconstriction are the vascular response to ischemia. Furthermore endothelial dysfunction, stimulated by ROS, also play role in vascular maladaptive response.[14] One of endothelial reaction to any type of injury, including ischemia injury, is remodelling of vascular wall.[14] This mechanism involves cell growth, cell death, cell migration and degradation or cellular matrix production.[9] These changes eventually result in intimal accumulation of smooth muscle-like cells and extracellular matrix, medial smooth muscle degeneration, and adventitial fibrosis.[23] The histopathological changes can be observed by thickening of vascular wall and narrowing of lumen area, which increase vascular resistance. Based on the result, there were enhancement of vascular thickness and narrowing of lumen area after one day exposure, then restored after day12 (FIGURE 3.A). Those alteration between IR1 and IR12 group was significant statistically ($p<0.05$, FIGURE 3.D-E). The changes that occurred in IR12 group are considered as restoration mechanism in maintenance phase of ischemia injury.[4]

Vasoconstriction response in ischemia injury is influenced by the presence of ET-1, mediated by ET_AR.[2] Endothelial cells secrete ET-1 as a response to endothelial injury, caused by ischemia.[1] ET-1 can stimulate hypertrophy, migration, and proliferation of vascular smooth muscle cell by transduction signal.[24] Therefore, ET-1 can promote the thickening and narrowing of intrarenal vessel, caused by smooth muscle cell proliferation. ET-1 expression is proportional to the vascular remodelling mechanism. It is consistent with this study, ET-1 expression was increased significantly ($p<0.05$) in IR1 group as well as enhancement of wall thickness and narrowing of lumen area (FIGURE 3.C-E). Those expressions then decrease in chronic group, it signified the presence of repairing process. It was correspond to the previous study conducted by Arfian *et al*.[2] that reduction of the remodelling level is associated with ET-1 deletion in IRI model.

Kidney IRI effect on myofibroblast formation

It has been explained that there is amelioration mechanism which is characterized by cell differentiation and restoration of kidney function. When the injury process is extended and the restoration mechanism is incomplete, it will progress to chronic injury which is observed by the presence of fibrosis in tubulointerstitial area.[12] In acute periode, tubular injury can be compensated by adaptive amelioration process, through inconsiderable fibrosis formation and tubular cell proliferation as compensation to maintain the kidney structurally.[4] Kidney tissue is intact, but the function is reduced. Reduction of kidney function is caused by replacement of fibrotic tissue which is loss of elasticity, proliferation capacity, and differentiation ability.[25] However, adaptive mechanism often continues to be maladaptive response,

supported by persistent inflammatory process.[4] This mechanism is stimulated by hypoxia environment which activate proinflamatory cytokines, profibrotic, growth factor.[13]

Tubulointerstitial fibrosis was illustrated on IR1 and IR12 group significantly ($p<0.05$, FIGURE 4.B). Purplish-red colored area in sirius red staining (FIGURE 4.A) indicates the presence of type I and II collagen.[25] The widest fibrosis area was found in IR12 group (4.927%), represented chronic injury in IRI model (FIGURE 4.B). It was correspond to study conducted by Skrypnyk *et al*.[26] and Varrier *et al*.[27] that reported the highest level of fibrosis was presence in chronic condition and there is TGF-β1 expansion which stimulate myobibroblast activation. The activated myofibroblast will produce and degrade a matrix, then stimulate connective tissue formation in tubulointerstitial area. α-SMA expression, as spesific marker, is used for indicating the presence of myofibroblast.[28] It was found enhancement of α-SMA expression in IR1 and IR12 ($p<0.05$, FIGURE 4.C). Significantly, IR12 group was the most expressed between goups ($p<0.05$) (FIGURE 4.C).

Fibrosis fraction-area and myofibroblast expansion are more progressive on IR12, it is related to ROS which is originated from inflammatory metabolism.[26] ROS can promotes death cell of tubular system and stimulates proliferation factor in interstitial tubular cell.[4] Kim *et al*.[10] showed that enhancement of interstitial cell proliferation parallel to ROS level in kidney tissue. It is proved by increasing of α-SMA, FSP1, dan protein NADPH oksidase-2 expression.[10] The excessive proliferation and expansion of extracellular matrix, followed by apoptotic tubular cell without regeneration mechanism, will accelerate the progressivity of injury.[9,10]

CONCLUSION

Prolonged IRI leads to chronic injury via persistent hypoxia and inflammatory response, signified by myofibroblast formation in tubulointerstitial area. Vascular remodelling and cell proliferation response are reduced in long-term period of injury.

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The frequency of DISC1 $^{Leu}607^{Phe}$ gene polymorphism in schizophrenia patients at Dr. Soetomo General Hospital Surabaya

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ABSTRACT

Schizophrenia is a common health problem in the world, including in Indonesia. Polymorphism of gene disrupted in schizophrenia 1 (DISC1) $^{Leu}607^{Phe}$ is allegedly related to the predisposition to schizophrenia. However, studies on the relationship between polymorphism of DISC1 $^{Leu}607^{Phe}$ and schizophrenia in various ethnics provided different results. The purpose of this study was to determine the frequency of DISC1 $^{Leu}607^{Phe}$gene polymorphism and its association with treatment response in patients with schizophrenia at Department of Psychiatry, Dr. Soetomo General Hospital Surabaya. In this study, the number of male patients with schizophrenia was more than that of the female patients. The mean age of male patients with schizophenia was lower than that of the female patients. Schizophrenia patients were primarily came from Javanese ethnic with positive and negative symptom score (PANSS) lower in male patients than that in the female patients. In conclusion, no DISC1 gene polymorphism at codon 607 is observed in schizophrenia patients at Dr. Soetomo General Hospital Surabaya, but the G nucleotide variation at the number 196.339 in intron regions is found instead.

ABSTRAK

Schizophrenia adalah masalah kesehatan umum di dunia termasuk di Indonesia. Adanya polimorfisme gen schizophrenia 1 (DISC1) $^{Leu}607^{Phe}$ diduga berkaitan dengan predisposisi schizophrenia. Namun demikian, berbagai penelitian yang menghubungkan polimorfisme gen DISC 1 $^{Leu}607^{Phe}$ dan schizophreniapada berbagai etnik memberikan hasil yang berbeda. Penelitian ini bertujuan menentukan frekuensi gen DISC 1 $^{Leu}607^{Phe}$ dan hubungannya dengan respon pengobatan pasien Schizophrenia di Departemen Psikiatri, Rumah Sakit Umum Dr. Soetomo, Surabaya. Dalam penelitian ini jumlah pasien pria lebih banyak dari pada wanita. Rerata umur pasien pria lebih rendah dari pada pasien wanita. Pasien schizophrenia umumnya dari suku Jawa dengan skor skor simptom negatif dan positif lebih rendah pada pria dibandingkan wanita. Dapat disimpulkan, tidak ditemukan polimorfisme gen DISC1 pada kodon 607 pada pasien schizophrenia di Rumah Sakit Umum Dr. Soetomo, Surabaya, namun ditemukan variasi nukleotida G pada nomor 196.339 di daerah intron.

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Keywords: Schizophrenia - DISC1 $^{Leu}607^{Phe}$ gene – polymorphism – PANSS - predisposition

INTRODUCTION

Schizophrenia is a complex psychiatric disorder,which remains a health problem worldwide,[1,2] including Indonesia.[3] According to the DSM-IV-TR (Diagnostic and Statistical Manual of Mental Disorders 4th ed. Text Revision), the annual incidence of schizophrenia is influenced by the geographic variation.[2] It is estimated that there are 24 million people with schizophrenia worldwide, 50% of whom do not get optimal treatment, and 90% of these patients are in developing countries.[4] In Indonesia, the prevalence of schizophrenia ranges from 0.3 to 1% and is especially between the age of 18 - 45 years.[5]

The mechanisms underlying schizophrenia remain unclear, but there are statements of multifactorial etiology.[6] Etiology, which allegedly plays an important role in the predisposition to schizophrenia, is a combination of genetic factors and environmental factors.[2] Forms of genetic variation which allegedly acted as a predisposing factor of schizophrenia is Single Nucleotide Polymorphism (SNP). Polymorphism generally can be analyzed by referencing to the reference sequence (rs) in the database at the National Center of Biotechnology Information (NCBI).[7] Polymorphism that has been studied in various countries is the DISC1 gene.[8-13] The method which can be used to detect polymorphism, among others, are Polymerase Chain Reaction (PCR) followed by Restriction Fragment Length Polymorphism (RFLP),[13,14] or sequencing.[11,15]

DISC1 protein resulted from DISC1 gene expression is a scaffold protein that plays a role in the regulatory process of neural progenitor cells proliferation, neurite outgrowth, neuronal migration, and c-AMP signals.[6] DISC1 protein will interact with a variety of signaling molecules and affect the occurrence of impaired cognitive function and working memory in patients with schizophrenia.[16] Signaling molecules interacting with DISC1 protein plays an important role for developing antipsychotic drugs.[2,6]

The main therapy for schizophrenia patients is an antipsychotic (typical or atypical or combination of both). Identification and analysis of polymorphisms as biological biomarkers in patients with schizophrenia are expected to be very useful for the clinician to predict the effectiveness of antipsychotic, side effects of antipsychotic drugs and to track gene hereditary disease in families of patients.[17,18] Considering that there has never been any research on the role of genetic factors implicated in schizophrenia, the study aimed to detect polymorphisms of DISC1 $^{Leu}607^{Phe}$ gene in patients with schizophrenia at the Department of Psychiatry, Dr. Soetomo General Hospital, Surabaya.

MATERIALS AND METHODS

Subjects

This was an observational descriptive study with cross-sectional design to evaluate the polymorphism of DISC1 $^{Leu}607^{Phe}$ gene in patients with schizophrenia and its association with treatment response in patients with schizophrenia at Department of Psychiatry, Dr. Soetomo General Hospital Surabaya. All patients with schizophrenia who came/were treated at the hospital for 4 months and met the inclusion-exclusion criteria were involved in this study. The study was performed after an ethical clearance was obtained from the Research Ethics Committee of Dr. Soetomo General Hospital Surabaya.

Procedure

Prior to research performed, patients's families were explained concerning the background, objectives and benefit of the study and then were given an informed consent to be signed. The diagnosis of schizophrenia of patients was based on a psychiatric history and mental status examination using Code Classification and Diagnosis of Mental Disorders in Indonesia III (PPDGJ issue 3)[19] and the criteria of Positive and Negative symptom Scale (PANSS).[20]

Blood samples of patients were taken and put into venoject 5 ml tubes with EDTA anticoagulant. Mononuclear Peripheral Blood Cells (PBMCs) were then separated and put in eppendorf tubes. Furthermore, PBMC was stored at a temperature of minus 80^0 C at the Institute of Tropical Disease, Airlangga University until laboratory examination. Deoxyribonucleic Acids (DNA) was extracted from PBMC, followed by the PCR with the primary: 5 - GAT GGC AAT GGA TTC ACC AC - 3 '(forward) and 5'-CAG ACA GTT GGG GAG AAC AG - 3' (reverse).[11] After that, electrophoresis was performed. PCR products in the form of DNA fragments with a length of 689 bp were done by sequencing using ABI 310 genetic analyzer sequencer from applied biosystems, inc. Multiple alignment with genetyx version 10 was then conducted to the result of sequencing.

Statistical analysis

Data of the characteristics of patients were presented as percentage.

RESULTS

Characteristics of patients with schizophrenia at Department of Psychiatry, Dr. Soetomo General Hospital are shown in TABLE 1. The number of patients having a history of mental illness in the family was higher than patients who did not have the history. Patients with schizophrenia receiving typical antipsychotic therapy was considerably less (28.28%) compared to those getting atypical antipsychotics (36.36%) and combination (36.36%). Female patients, who received antipsychotic therapy, had the least combination of numbers (22.22%), followed by a typical antipsychotic (33.33%) and the highest was an atypical antipsychotic (44.45%).

TABLE 1. Characteristics of patients with schizophrenia at SMF Dr. Soetomo General Hospital

Sex	Number of patients		Mean age range	Ethnic						Family History			
	N	%	Year	Javanese		Madurese		Mixed		Yes		No	
				n	%	n	%	n	%	n	%	n	%
Male	11	55	38.82 (25 – 62)	9	45	1	5	1	5	4	20	7	35
Female	9	45	43.11 (31 – 58)	8	40	1	5	0	0	7	35	2	10
Total	20	100	40.97 (25 – 62)	17	85	2	10	1	5	11	55	9	45

PCR product in this study was DNA fragment with a length of 689 bp. The electrophoresis, when compared with 100 bp DNA marker band, would appear as a band placed marker bands between 600 and 700 bp.

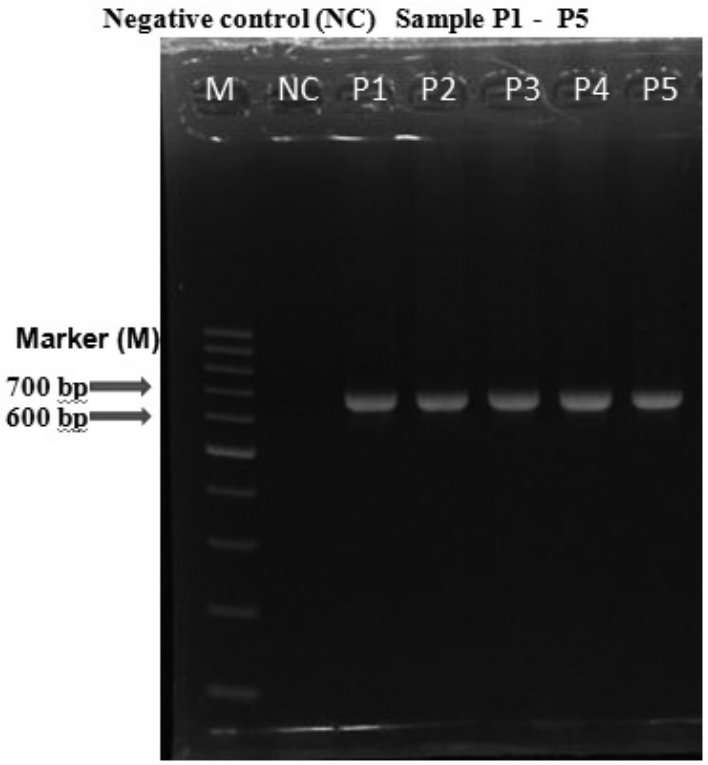

FIGURE 1. Examples of some of the electrophoresis results of PCR products DISC1 $^{Leu}607^{Phe}$ gene with positive results

If the PCR results were positive, the sequencing would be conducted by using ABI 310 sequencer and forward primary (sense). The results of sequencing were used for Multiple alignment of the nucleotide sequence of the sample compared to rs 6675281, using a computer program genetyx version.10. Results of Multiple alignment pieces rs 6675281 sequencing results of 20 samples of patients with schizophrenia at Department of Psychiatry, Dr. Soetomo General Hospital is shown in FIGURE 2.

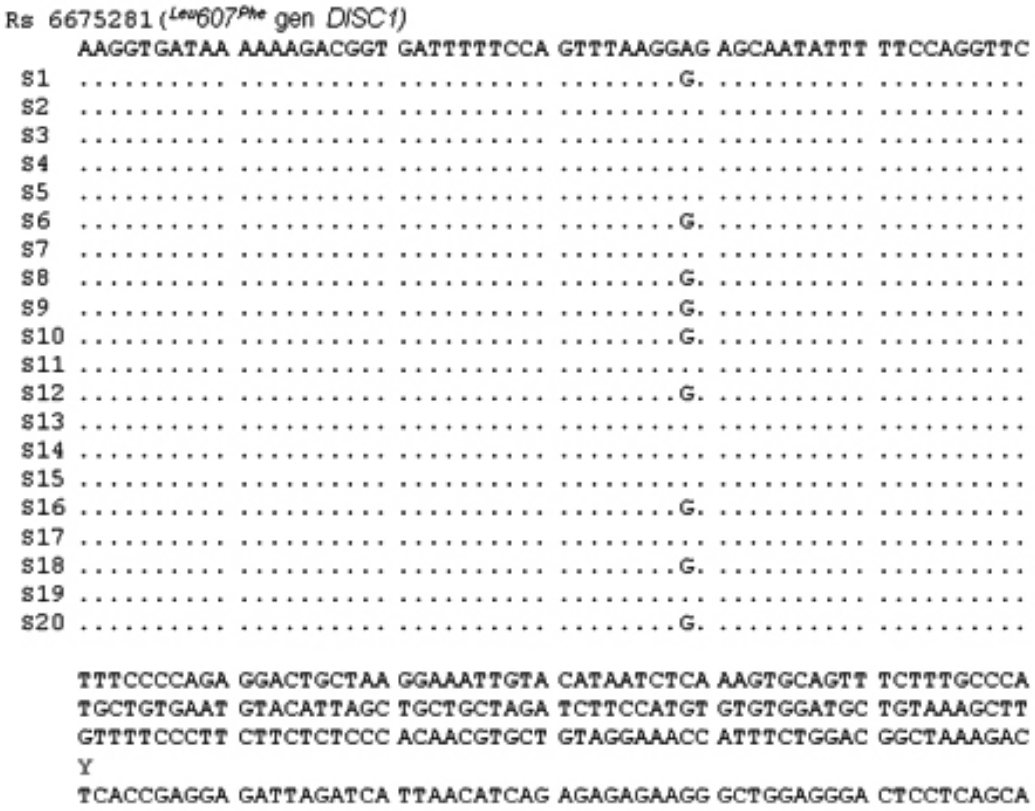

FIGURE 2. Results of multiple alignment pieces *rs* 6675281 (DISC1 SNP $^{Leu}607^{Phe}$ gene) from the sequencing result of 20 samples of schizophrenia patients at Department of Psychiatry of Dr. Soetomo General Hospital Surabaya.

In this study there was no DISC1 $^{Leu}607^{Phe}$ gene polymorphism found with the code of rs 6675281 located in exon 9 at codon number 607, because all samples show C nucleotides in the nucleotide sequence of numbers 196.541 (y), according to the basic data DISC1 gene from NCBI. On further analysis of the sequencing results, it was obtained that electropherogram of samples 1, 6, 8, 9, 10, 12, 16, 18 and 20 had the variation of nucleotide G, while at the the other sample numbers nucleotide A were obtained in accordance with the basic data of DISC1 genes from NCBI (rs 6675281). Example of electropherogram sequencing result DISC1$^{Leu}607^{Phe}$gene with the nucleotide A at number 196.339 in patients with schizophrenia is shown in FIGURE 3.196.339

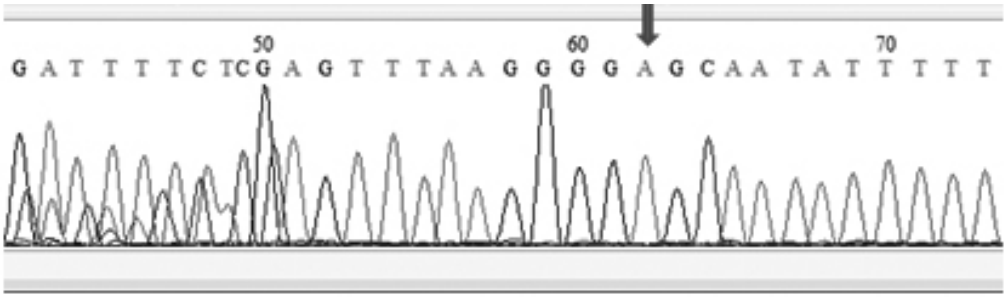

FIGURE 3. Example of electropherogram intron sequencing result DISC1 $^{Leu}607^{Phe}$ gene with the nucleotide A at number 196.339.

DISCUSSION

The mean age range of the male schizophrenia patients was younger than the average age of the female patients. It is believed to be related to the estrogen hormone thought to have a protective effect against schizophrenia, so the manifestation of schizophrenia in men tend to occur at a younger age than women.[2,21] In female schizophrenia, psychotic symptom fluctuation is obtained during the menstrual cycle. The estrogen hormone has a pleiotrophic effect to variations of brain developmental processes in adults.[21]

The data showed that genetic factors play a quite significant role as predisposition to schizophrenia. This is supported by previous research on families, twins and adopted children, which demonstrated that genetic factors play a major role for schizophrenia.[22] Genetic predisposition to schizophrenia is a complex factor for their interaction with environmental factors.[2,22] In Scottish family DISC1 gene is identified as potential genes associated with susceptibility to the onset of psychiatric disorders, because the Scottish family obtained schizophrenia and other psychiatric disorders with a high frequency.[15,22]

DISC1 gene plays a key role during the process of brain development, especially on the thickness of the cerebral cortex, which is highly heritable. This is supported by evidence from MRI examination, which shows a reduction of the thickness of the cerebral cortex in schizophrenia patients.[23] From the results of sequencing on samples of patients with schizophrenia at SMF Psychiatry of Dr. Soetomo Hospital, there was no DISC1 $^{Leu}607^{Phe}$ gene polymorphism. Study of DISC1 $^{Leu}607^{Phe}$ gene polymorphism in patients with schizophrenia in some ethnic population provides inconsistent results. The earlier study in patients with schizophrenia by Brauns in 2011 showed an interference of neural activity in the dorsolateral prefrontal cortex (DLPFC) during the process of working memory and the reduction of the thickness of the cortex cerebri in the left part of gyrus supramarginal of the carrier allele Phe from DISC1 $^{Leu}607^{Phe}$ gene when compared to the homozygote Leu/Leu.[23] Hodgkinson's study in 2004 also found an increased risk of schizophrenia in the DISC1 $^{Leu}607^{Phe}$ gene polymorphism.[24] Results of research DISC1 $^{Leu}607^{Phe}$ gene are various, because the predisposition to schizophrenia is influenced by multigenic and multifactorial factors. Thus, in different demographic areas and ethnicities we can find some different variation polymorphism from the DISC1 gene or polymorphisms in other genes that are also allegedly associated with predisposition to schizophrenia.[2,13-15]

In the group sample of patients with schizophrenia, it was found that the number of male patients who come from Javanese ethnicity are far more than any other ethnicities or the mixed ones; it also happened in the case of the female patients. This is presumably caused by the fact that the sampling was performed in Dr. Soetomo General Hospital which is a referral hospital in the east region of Java island, so that patients with schizophrenia are mainly dominated by the Javanese. Data from Indonesia Basic Health Research in 2013 reported that the prevalence of severe mental disorders in East Java was 0.22%, and the highest prevalence was found in Yogyakarta Special Region area that was equal to 0.27%, while the national prevalence was 0.17%.

The overall sample of patients with schizophrenia receiving various antipsychotic therapy. Olincy *et al*.[25] reported that the DISC1 protein is allegedly associated with the response to antipsychotic drug therapy and plays an important role to identify the lowest effective dose for the patient as well as the lowest side effects. Another previous studies conducted on rats showed that the administration of atypical antipsychotics will increase the DISC1 gene expression in the frontal cortex, while the provision of typical antipsychotic effect is not found in the DISC1 gene expression.[26]

This study did not find any DISC1 gene polymorphism at codon 607, but found variations of G nucleotide at the number 196.339 in intron regions. These nucleotide differences in this study with the SNPrs 6675281 (NCBI) could occur because schizophrenia is a disease that has a multifactorial etiology, so it is possible that the DISC1 $^{Leu}607^{Phe}$gene polymorphism is not a dominant genetic factor. The impact of the differences in the nucleotide has been known, yet.

The results of this study were not in accordance with the research conducted previously on the Finnish population[15,27] which found the presence of DISC1 $^{Leu}607^{Phe}$gene. Presumably, this is because the predisposition to schizophrenia is associated with a multifactorial and multigenetic etiology.[2,28]

CONCLUSION

It can be concluded that the male patients with schizophrenia is higher in number, with a lower average age, and lower PANSS scores than those of the female patients. Patients with schizophrenia at the Department of Psychiatry, Dr. Soetomo General Hospital mainly come from Javanese ethnic. This study does not find any DISC1 gene polymorphism at codon 607, but find variations of G nucleotide at the number 196.339 in intron regions.

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Insecticide resistance and posible mechanisms of *Aedes aegypti* (Diptera: Culicidae) in Yogyakarta

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ABSTRACT

For several decades, applications of malathion and cypermethrin insecticides have been extensively used to control *Aedes aegypti.* Hence it is important to study mosquito vector resistance status and its posible mechanisms in relation to long term use of insecticides. This study aimed to determine the resistance status and to characterize mechanisms of *Ae. aegypti* to malathion and cypermethrin. Larvae and puppae of *Ae. aegypti* were collected in the field of Plosokuning, Minomartani, Sleman, Yogyakarta Special Region, Indonesia. The biological assay was carried out using CDC Bottle Bioassay to test the resistant status. The biochemical assay was conducted using microplate assay with substrate α-naphthyl acetate to test the presence of esterase elevated activity. The molecular assay was done using PCR with primers AaSCF1 and AaSCR4 to detect of point mutation at S989P, I1011M (or V), L1014F sites, and AaSCF7 and AaSCR7 to detect of point mutation at F1534C site. The biological assay showed *Ae. aegypti* suggests the possibility of resistance to malathion with 82% mortality (246/300) and already resistant to cypermethrin with 76% mortality (228/300). The biochemical assay of *Ae. aegypti* showed the presence of non-specific esterase elevated activity. The PCR method using AaSCF1 and AaSCR4 primers did not show specific DNA bands with the size of 619bp. However using AaSCF7 and AaSCR7 primers showed specific DNA bands with the size of 748bp. Voltage gated sodium channel gene sequencing compare with Gene Bank (AB914687 and AB914688) showed that there was no changes on the 1534 site. Long term use of insecticides did not successfully eliminate the targeted dengue vector, because *Ae. aegypti* were resistant to both insecticides. The results demonstrate the importance of designing better health policies regarding insecticide usage.

ABSTRAK

Dalam beberapa dekade, penggunaan insektisida malation dan sipermetrin sangat luas untuk pengendalian nyamuk *Aedes aegypti*. Oleh sebab itu penting untuk mempelajari status resistensi dan mekanisme terjadinya resistensi dalam hubungannya dengan penggunaan insektisida secara terus menerus dalam jangka waktu yang lama. Tujuan penelitian ini adalah menentukan status resistensi *Ae. aegypti* terhadap insektisida malation dan sipermetrin berserta mekanismenya. Larva dan pupa *Ae. aegypti* dikoleksi dari daerah Plosokuning, Minomartani, Sleman, Daerah Isttimewa Yogyakarta, Indonesia. Bioasai dilakukan dengan metode CDC *Bottle Bioassay*, metode biokhemis dilakukan

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dengan *microplate assay* dengan substrat α-naphthyl asetat. Metode penetapan molekular digunakan PCR dengan primer AaSCF1 and AaSCR4 untuk deteksi mutasi pada titik S989P, I1011M (atau V) dan L1014F adapun primer AaSCF7 dan AaSCR7 untuk deteksi mutasi pada titik F1354C. Hasil bioasai menunjukkan *Ae. aegypti* sudah resisten terhadap malation dengan mortalitas 82% (246/300) dan sudah resisten terhadap sipermetrin dengan mortalitas 76% (228/300). Dengan metoda biokhemis menunjukkan terjadi peningkatan aktivitas enzim esterase non spesifik. Pada metoda PCR dengan primer AaSCF1 and AaSCR4 tidak muncul pita DNA spesifik dengan ukuran 619bp, tetapi dengan primer AaSCF7 dan AaSCR7 muncul pita spesifik dengan ukuran 748bp. Hasil sekuensing *gene voltage gated sodium chanel* dibandingkan dengan Gen Bank (AB914687 dan AB914688) menunjukkan tidak ada mutasi pada pada titik 1354. Penggunaan insektisida dalam jangka waktu yang lama tidak berhasil mengeleminasi vektor dengue oleh karena *Ae. aegypti* resistan terhadap kedua insektisida tersebut. Hasil penelitian ini menunjukkan bahwa perlu dirancang kebijakan kesehatan yang lebih baik dalam penggunaan insektisida.

Keywords: malathion – cypermethrin - biological assay - biochemical assay - molecular assay

INTRODUCTION

Dengue is a mosquito-borne infection that in recent decades has become a major international public health concern. *Aedes aegypti*, Linnaeus (Diptera: Culicidae), is the primary vector of dengue and plays the most central role in transmitting dengue as they have a high affinity to humans.[1] Dengue virus is found in tropical and sub-tropical regions around the world, predominantly in urban and semi-urban areas. The worldwide cases of dengue fever have increased substantially in recent years with more than one-third of the world's population living in areas at risk for infection.[2] One recent study documents 390 million dengue infections each year, of which 96 million manifest clinically.[3] According to data from the Health Ministry of Republic of Indonesia, from January to July 2013, 50,417 dengue fever cases recorded in Indonesia, with mortality rates reaching 0.75% amounting to 380 deaths.[4] In 2013, Yogyakarta Special Region was in the top 3 of the areas with greatest incidence rate of dengue with cases of 95.99 per 100,000 people.[5] The main vector for dengue disease in Indonesia is *Ae. aegypti*. Its close relative, *Ae. albopictus* Skuse, is also involved in dengue transmission as a secondary vector.[6]

Insecticide resistance is an inherited characteristic involving changes in one or more insect gene and one feature of the evolution of insecticide resistance in the field that recurs through all the pre-genomic and genomic studies is the rapid spread of resistance alleles after the initial outbreak.[7,8] The majority of cases of insecticide resistance are either based on increased metabolic detoxification or reduction in the sensitivity of the insecticide's target site to inhibition. The target site for organophosphate insecticides is acetylcholine esterase which can be altered to a form that is less sensitive to insecticide inhibition. The target sites for pyrethroids are the ion channels of the nerve membrane, and resistance in some species is dependent on a change in binding affinities of insecticides to the sodium channels of the nerve membrane, i.e voltage-gated sodium channel (VGSC).[9-10] Several different point mutations within the VGSC gene contribute to such resistance and occurrences of VGSC gene mutations were

reported in agricultural and health threatening insects. Several mutations in segment 6 of domain II of the VGSC were reported to play important roles in pyrethroids resistance of *Ae. aegypt*i (I1011M, I1011V, V1016G and V1016I).[10-12] Yanola *et al.*[13,14] identified a novel F1534C mutation in segment 6 of domain III in DDT or permethrin-resistant *Ae. aegypti.* Harris *et al.*[15] reported that the F1534C mutation is strongly correlated with resistanc to DDT and pyrethroid. The S989P mutation in domain II of the voltage-gated sodium channel gene, which occurs in deltamethrin-resistant *Ae. aegypti*, is another principal kdr mutation that regulates pyrethroid resistance in mosquitoes.[16]

In Yogyakarta Special Region, the organophosphate insecticides have been used since 1974, and pyrethroids has also been used since 10 years ago.[17] Previous studies showed the resistance towards organophosphate and pyrethroids insecticide in most part of Central Java and Yogyakarta Special Region.[18,19] However, long term use of insecticides can lead to development of resistance, and the mechanisms of resistance have not been studied, yet. The study aimed to evaluate the status of dengue vector resistance and its mechanisms in relation to long term used of insecticides in order to develop better health policies for disease control.

MATERIALS AND METHODS

Subjects

Larvae and puppae of *Ae. aegypti* were collected in the field from Plosokuning, one of the dengue endemic areas in Sleman District, Yogyakarta Special Region, Indonesia in December 2015. The collected larvae (263) and pupae (52) were colonized in the laboratory of Parasitology, Department of Parasitology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia to get adult stage. The adult mosquitoes were identified to confirm the presence of *Ae. aegypti*. The mosquitoes were maintained at 25 ± 2°C, 80% relative humidity, with a photoperiod of 12 h of artificial daylight and 12 h of darkness and a 10% sucrose solution as mosquito feed. Colonization of the mosquitoes was continued 2 times until there were sufficient number of *Ae. aegypti*. The same methods of colonization were also applied to mosquitoes of the laboratory of Parasitology collection as positive and negative control. The positive control was F 102, highly resistant (RR) to malathion and the mortalities were 52%. The negative control was F 1112, highly susceptible (SS) to malathion and the mortalities were 100%. Mosquitoes were sorted by sex and species, and only females 2-6 days old were used. Mosquitoes fed only with 10% sugar water solution were subjected to all series of biological, biochemical and molecular assays.

Experimental and analysis

The biological assays followed the procedures for the CDC Bottle Bioassay using adult female mosquitoes. Two insecticides were used for bioassay, malathion (DREXEL, France) and cypermethrin (CYNOFF, FMC USA), after dissolving in acetone. According to this method, the diagnostic dose of malathion was 50 µg/bottle, and cypermethrin was 10 µg/bottle, whereas diagnostic time of malathion and cypermethrin were 30 min. Sample size was 100 mosquitoes for the initial test and 25 mosquitoes for the negative control as discribed by CDC.[20] In this test, the number of the mosquitoes that had been examined was 300 for initial test and 75 for negative control. All of the assay was conducted in triplicated.

The presence of esterase elevated activity, associated with organophosphate resistance, was confirmed by biochemical tests, and it was

conducted by microplate assay as discribed by Lee[21] and also apllied by Mardihusodo.[22] In this method were used α-naphthyl acetate (Sigma) as substrate; Fast Blue B salt (Sigma) in sodium dodecyl sulphat (Sigma) solution as coupling reagent. The whole body of individual mosquito was used for all experiments. A singgle mosquito was homogenized in 0.5 mL PBS using a pellet pestle. With a micropipette, 50 µL of the homogenate was transferred to each well on microplate 96 wells. Fifty µL of substrate solution freshly prepared were then pipetted into each well and left for 60 sec and 50 µL of coupling reagent was then added. Immediately the color of solution in the wells developed which turned to blue after standing for 10 min. The reaction was stopped by the addition of 50 µL 10% acetic acid into each well. The intensity of the color of reaction indicating of esterase activity of *Ae. aegypt*i mosquitoes. The color could be differenciated by eye score and by detecting the absorbance value (AV) using microplate reader (BIORAD microplate Reader Benchmark) at λ 450 nm.[23] The number of the mosquitoes that had been examined was 48 and its were replicated 3 times. Non-specific esterase activity of positive and negative control values were used as a standard. The interpretation of the results was AV < 0.700 as highly susceptible (SS), AV = 0.700-0.900 as moderately resistant (RS) and AV>0.900 as highly resistant (RR).[24] Average AV was also analyzed based on cut off positive value, calculated from the average AV of negative control + 2 SD (standard deviation).[25]

Molecular methods were carried out to determine the VGSC by using PCR. The mosquito samples were lightly dried on a paper towel and placed in a 1.5-mL PCR reaction tube, 10 mosquitoes per tube (pooling). The sample was homogenized in a mixed solution of extraction solution (40 mL) plus tissue-preparation solution (10 mL) (REDExtract-N-Amp Tissue PCR Kit; Sigma, St. Louis, MO) for extraction of DNA. The solution was heated at 95 °C for 3 min and neutralized. Initial amplification was carried out using the primers AaSCF1 (AGACAATGTGGATCGCTTCC) and AaSCR4 (GGACGCAATCTGGCTTGTTA) to test for the present of point mutation at S989P, I1011M (or V), L1014F and V1016G (or I) all of which are located in the area of segment 6 of domain II; and primers AaSCF7 (GAGAACTCGCCGATGAACTT) and AaSCR7 (GACGACGAAATCGAACAGGT) to test for the present of point mutation at F1534C located in the area of segment 6 of domain III (Integrated Dna Technology). The PCR mixture contained 4 ml of REDExtract-N-Amp ReadyMix (Sigma), 0.5 mM of each primer, and 1 mL of the DNA template in a total volume of 10 mL. The PCR was performed under the following conditions initial denaturation at 94 °C for 3 min; 35 cycles each of 94 °C for 15 sec, 55 °C for 30 sec, and 72 °C for 30 sec and a final elongation step at 72 °C for 10 min. The amplified fragments of the expected size were purified with ExoSAP-IT (USB Corporation, Cleveland, OH) at 37 °C for 30 min, and then 80 °C for 15 min.[24] Electrophoresis was performed using 2% agarose and followed by examination under ultraviolet light. The DNA sequencing was carried out by First Base Laboratories, to indentified point mutation at S989P, I1011M (or V), L1014F and V1016G (or I) (accession No. AB914689 and AB914690) and point mutation at F1534C point mutation (accession No. AB914687 and AB914688). This study has been approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta.

RESULTS

The results of bioassay with malathion (organophosphate) and cypermethrin (pyrethroids) insecticides at the CDC diagnostic dose and its diagnostic time for *Ae. aegypti* adult mosquitoes are shown in TABLE 1. The following criteria was used for interpretation of adult susceptibility test as recommended by World Health Organization (WHO), 98–100% mortality indicates susceptibility, 80–97% mortality suggests the possibility of resistance that needs to be further confirmed and <80% mortality suggests resistance.[25] According to WHO's recommendation, *Ae. aegypt*i from Plosokuning, Minomartani, Sleman, Yogyakarta Special Region was resistant to malathion with 82% mortality but need to be further confirmed, and resistant to cypermethrin with 76% mortality.

TABLE 1. Percentage mortalities in the CDC bottle bioassay of adult female of *Ae. aegypti* for evaluating insecticide resistance to diagnostic dose and diagnostic time of malathion and cypermethrin

Variable	Malathion (50ug/bottle, 30 min)	Cypermethrin (10 ug/bottle, 30 min)	Control olive oil
Mosquitoes mortality (%)	82 (246/300)	76 (228/300)	0 (0/75)

A total of 48 adult stage specimens of *Ae. aegypti* were assayed for activity of esterase with 20 adult stage species of *Ae. albopictus* (laboratory strain) as negative control and 20 adult stage species of *A. togoi* (laboratory strain) as positive control. TABLE 2 shows interpretation on the absorbance value (AV) of *Ae. aegypt*i adult stage specimens due to esterase elevated activity in hydrolyzing α-naphtyl acetate.

TABLE 2. Potential resistance to insecticides of *Ae. aegypti* based on the average absorbance value (AV) of non-specific esterase activity

Variable	% of the resistance status		
	AV<0.700 (SS)	AV = 0.700-0.900 (RS)	AV>0.900 (RR)
The absorbance value of non-specific esterase activity	75.70 (109/144)	24.30 (35/144)	0.00 (0/144)

SS= susceptible, RS= moderatly resistant, RR= highly resistant

The potential resistance (moderatly resistant) for organophosphate insecticide due to esterase elevated activities were found among *Ae. aegypti* mosquitoes which was 24.30% of total mosquito tested (48 samples, 144 replicate). These findings implied that the population *Ae. aegypti* under the study comprised at least 2 subpopulations, i.e. susceptible and resistant.

Average absorbance values (AV) of non-specific esterase activity of *Ae. aegypti, A. togoi* as positive control and *A. albopictus* as negative control are shown TABLE 3. According to cut off positive calculations from AV average of negative control + 2 SD (0.161 + (2 x 0.089)= 0.339), the AV of mosquitoes tested can be grouped into categories of susceptible if average AV < 0.339; moderate

resistance if average AV = 0.339 – 0.498 and high resistance if average AV ≥ 0.498. Using cut off positive calculations, adult mosquitoes of *Ae. aegypti* are indicated to have been moderately resistant to organophosphate insecticide.

TABLE 3. The average absorbance value of non-specific esterase activity of *Ae. aegypti*, *Ae. togoi* and *Ae. albopictus*

Mosquitoes tested	Mean ± SD
Field mosquitoes tested[a]	0.354 ± 0.125
Positive control[b]	0.498 ± 0.164
Negative control[c]	0.161 ± 0.089

[a]*Ae. aegypti* from Plosokuning, Minomartani, Sleman; [b]*Ae. togoi* and [c]*Ae. albopictus* from Parasitology Laboratory

Molecular methods using AaSCF1 (AGACAATGTGGATCGCTTCC) and AaSCR4 (GGACGCAATCTGGCTTGTTA) primers no amplicon were obtained, while using AaSCF7 (GAG AAC AAC ATG CCG TCG TT) and AaSCR7 (GAC GAC GAA GAA CAG ATC GT) primers amplicon were obtained from *Ae. aegypti* with the size 748bp (FIGURE 1).

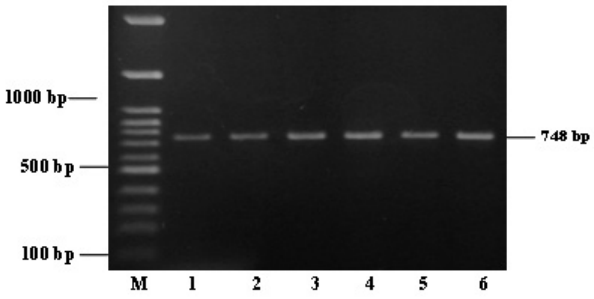

FIGURE 1. Visualization of the amplicons of approximately 748 *bp* corresponds to mutation gene F1534C. M = DNA Marker; 1-6 = *Ae. aegypti*

The results of sequencing analysis VGSC gene of *A. aegypti* with primers AaSCF7 and AaSCR7 compare with Gene Bank AB914687 and AB914688, showed that there was possible amino acid change on the 1534 site from phenylalanine (TTC) into cysteine (TGC) (FIGURE 2).

```
Mega 6.0
VGSC Gene mutation at F1534C
Gene Bank AB914687

Sample 214  GTGTTCTTCATCATCTTCGGGTCGTTCTTCACGCTGAATCTGTTCATCGGTGTC  267
            ||||||||||||||| |||||||||||||||||||||||||||||||||||
Sbjct  181  GTGTTCTTCATCATCTGCGGGTCGTTCTTCACGCTGAATCTGTTCATCGGTGTC  234
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FIGURE 2. The results of sequencing analysis VGSC gene of *Ae. aegypti* with primers AaSCF7 and AaSCR7 compare with Gene Bank Gen Bank (AB914687 and AB914688)

DISCUSSION

Resistance of vector to insecticide has continued to spread and affect disease control in many countries. *Aedes aegypti* field population showed resistance to both malathion and cypermetrin insecticide, these data may provide early evidence that malathion and cypermethrin are losing their effectiveness. These results might be due to the same mechanism operationally underlying the resistance associated with enzyme activity. It is evident that this important vector species, *Ae. aegypti*, may allow cross-resistance of malathion (organophosphate group) and cypermethrin (pyrethroid group). Cross-resistance possibly occurs in some mosquitoes, where the individual mosquito has developed resistance to two or more insecticide group.[26]

Non-specific esterase is well recognized as an important enzyme for detoxification of

related chemical insecticides and one of many insecticide resistance mechanisms known to occur in mosquitoes.[17] This result also revealed the presence of consistency that mosquito resistance to organophosphate and pyrethroid insecticides is directly associated with esterase elevated resistance mechanism.[29,30] Most of the insecticide groups contain ester linkages which are susceptible to hydrolysis by esterase. Resistant insects usually show a very high activity of estrases.[30,31] Detoxification mechanism mediated through non-specific esterases is another major mechanism of resistance in insects. These esterases detoxify organophosphate, carbamates and synthetic pyrethroid insecticides by two main ways, hydrolysis of the ester bond and binding of the pesticide to the active site of esterase.[32]

After the first description of the newly identified F1534C point mutation in *Ae. aegypti* collected in Thailand,[13,14] the same mutation was reported in Vietnam,[31] Brazil, Venezuela, Madeira Island, Portugal,[28] and Grand Cayman Island, UK.[15] In the present study, was determined a moderate frequency of this point mutation in Yangon City.[24] Additionally, the same mutation was reported in another DHF vector, *Ae. albopictus*, at high frequency (73%).[31] Elucidation of the worldwide distribution of the F1534C mutation in *Ae. aegypti* and *Ae. albopictus* will provide a valuable insight into DHF epidemiology and yield useful information for vector control programs. The development of mosquito resistance to chemical insecticides makes the control of mosquitoes and hence the diseases more difficult.[25] Continuous monitoring of insecticide susceptibility in *Aedes* populations is critical for decisions on insecticide use. Source reduction, environmental manipulation and personal protection must be emphasized in order to reduce insecticide use and to delay the further development of organophosphate resistance.

The limitations of this study on molecular testing with PCR are the isolation of mosquito DNA in each test sample derived from 10 mosquitoes (pooling method) and no positive control and negative control. Further research on the mutation of the *Ae. aegypti* VGSC gene should be performed using individual mosquito samples, positive and negative controls, so that the percentage of mutations in the VGSC gene in this *Ae. aegypti* population can be obtained.

CONCLUSION

Based on biological, biochemical and molecular assays, *Ae. aegypti* mosquitoes from the village of Plosokuning, Minomartani, Sleman, Yogyakarta Special Region are resistant to both organophosphate and pyrethroid insecticides by underlying mechanisms of esterase elevated and no changes on the 1534 site from phenylalanine into cysteine. These findings also indicate the *Ae. aegypti* are cross-resistant to insecticides, organophosphate group and pyrethroid group. The results of this study can serve to guide the development of better health policies concerning disease control.

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Comparison of Bcl-xL protein expression in placental trophoblast cells between pregnancy complicated by severe preeclampsia and normotensive pregnancy

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ABSTRACT

Preeclampsia is one of the main causes of maternal and perinatal mortality and morbidity. The pathogenesis of preeclampsia remains unclear until now. It is believed that regulation of apoptosis in trophoblast cells plays an important role in the pathophysiology of preeclampsia. Failure of spiral arteries remodeling will eventually lead to placental hypoxia lead to excessive trophoblast apoptosis. The molecular mechanism of apoptosis is very complicated involving many signaling molecules included Bcl-2 proteins. The Bcl-2 protein group consists of proapoptosis proteins (Bax) and apoptosis inhibitor proteins (Bcl-2 and Bcl-xL). The aimed of this stuty was to compare the expression of Bcl-xL protein in placental trophoblast cells of pregnancy complicated by severe preeclampsia with that normotensive pregnancy. This study was an observational study with cross sectional design involving 43 pregnancy patients with severe preeclampsia and 38 normotensive pregnancy who treated in Dr. Sardjito General Hospital, Yogyakarta from October 2011 until March 2012. Placenta samples were obtained from all subjects for Bcl-xL protein expression analysis using immunohistochemistry technique. Data were analyzed using independent t-test, chi-square test, and logistic regression. A p value <0.05 was considered significant. Significant difference in Bcl-xL protein expression in trophoblast cells of pregnancy complicated by severe preeclampsia (1.29 ± 0.12) compared to that normotensive pregnancy (1.71 ± 0.14) was reported ($p = 0.00$). In addition, logistic regression test showed that diagnosis of severe preeclampsia had a statistically significant role in Bcl-xL protein expression ($p = 0.000$). In conclusion, the expression of Bcl-xL protein is lower in pregnancy complicated by severe preeclampsia compared to normotensive pregnancy.

ABSTRAK

Preeklamsia merupakan salah satu penyebab utama mortalitas dan morbiditas maternal dan perinatal. Patogenesis preeklamsia masih belum jelas sampai saat ini. Diduga pengaturan apoptosis pada sel trofoblas memegang peranan penting dalam patofisiologi preeklamsia. Kegagalan remodeling arteri spiralis akan menyebabkan hipoksia pada plasenta dan apoptosis trofoblas yang berlebihan. Mekanisme molekuler apoptosis sangat komplek yang melibatkan banyak molekul sinyal termasuk protein Bcl-2. Kelompok protein Bcl-2 terdiri dari protrin proapoptosis (Bax) dan penghambat apoptosis (Bcl-2 dan Bcl-xL). Penelitian ini bertujuan untuk membandingkan ekspresi protein Bcl-xL sel trofoblas plasenta pada kehamilan dengan preeklamsia berat dengan kehamilan nomal. Penelitian

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observasi dengan rancangan potong lintang ini melibatkan 43 pasien wanita hamil dengan preeklamsia berat dan 38 wanita hamil normotensi yang dirawat di RSUP Dr. Sardjito, Yogyakarta antara Oktober 2011 sampai Maret 2012. Sampel plasenta diambil dari semua subjek untuk pemeriksaan ekspresi protein Bcl-xL menggunakan teknik imunohistokimia. Data yang diperoleh dianalisis denga uji t independen, uji chi square dan uji regresi logistic. Nilai $p < 0.05$ digunakan sebagai dasar menyatakan perbedaan nyata. Dijumpai perbedaan nyata ekspresi protein Bcl-xL pada sel trofoblas kehamilan dengan preeklamsia berat (1,29 ± 0,12) dibandingkan dengan kehamilan normotensi (1,71 ± 0,14) ($p = 0,00$). Selain itu, uji regresi logistik menunjukkan diagnosis preeklamsia berat mempengaruhi secara nyata terhadap ekspresi protein Bcl-xL ($p = 0,000$). Dapat disimpulkan bahwa ekspresi protein Bcl-xL lebih rendah pada kehamilan dengan preeklamsia berat dibandingkan dengan kehamilan normotensi.

Keywords: trophoblast - severe preeclampsia - Bcl-xL protein – apoptosis - normotensive

INTRODUCTION

Preeclampsia is one of the main causes of maternal and perinatal mortality and morbidity. The pathogenesis of preeclampsia remains unclear until now. However, it is believed that the failure of spiral arteries remodeling will eventually leads to placental hypoxia. This theory may not be the main cause of preeclampsia, but at least it is involved in the pathogenesis of this disease.[1, 2]

Apoptosis has an important role not only in the development of placenta but also in the pathophysiology of pregnancy complicated by preeclampsia. Apoptosis of trophoblast cells is increasing with gestational age and this increase has been studied as a complication of pregnancy with preeclampsia and intrauterine growth restriction (IUGR). Although this hypothesis is still under study, it is believed that regulation of apoptosis in trophoblast cells plays a key role in the pathophysiology of preeclampsia.[3, 4]

The molecular mechanism of apoptosis in human is very complicated involving many signaling molecules included Bcl-2 proteins. The Bcl-2 protein family consists of proapoptotic protein such as Bak and Bax, and apoptosis inhibitor protein such as Bcl-2 and Bcl-xL. During pregnancy, Bcl-2 is found in placenta since first trimester of pregnancy until third trimester and the concentration is decreasing with gestational age. In pregnancy complicated by preeclampsia, regulators of placental apoptosis are expressed differently. Several recent studies have found that expression of Bcl-2 and Bcl-xL as antiapoptotic molecules in patients with severe preeclampsia and IUGR are lower than patients with normal pregnancy.[3, 4]

The purpose of this study was to compare the expression of Bcl-xL protein in placental trophoblast cells of pregnancy complicated by severe preeclampsia with normotensive pregnancy. This study also aimed to evaluate the effect of maternal age, gestational age, and maternal mean arterial pressure (MAP) in the expression of Bcl-xL protein.

MATERIALS AND METHODS

Subjects

This was an observational study with cross sectional design. The population were patients with severe preeclampsia and normotensive patients who treated in Dr. Sardjito General Hospital, Yogyakarta, Indonesia from October 2011 until March 2012. The inclusion criteria

were patients with severe preeclampsia in 28-40 weeks of gestational age and agreed to be included in the study. The exclusion criteria were presence of comorbid diseases such as chorioamnionitis, chronic hypertension, diabetes, systemic lupus erythematosus, sickle cell disease, thyroid diseases, heart diseases, bronchial asthma, seizure which was caused by other etiologies beside preeclampsia, HIV, and fetus with major congenital disorder. Written inform consent were obtained from each patient after sufficient information was given.

Protocol of study

Samples were taken from the placenta immediately after the baby was born. Samples were then sent to Histology Laboratory, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia. Samples of placental tissue were stained using immunohistochemistry technique to measure the expression of Bcl-xL protein. The expression of Bcl-xL protein was reported to be positive if brown color was found in cytoplasm or cell membrane. The expression of Bcl-xL protein was measured using semiquantitative immunohistochemical scoring system (HSCORE). The formula was HSCORE = $\sum Pi\ (i+1)$, where *Pi* was percentage of cells which are stained positively with Bcl-xL immunostaining and *i* was intensity of staining with different grades (0=negative; 1=weakly positive; 2=moderately positive; 3=strongly positive). The HSCORE measurement was conducted by two observers with concealment of sample's identity. Inter observer agreement was tested using kappa test and the result of kappa value was 0.88 which showed that there was a strong agreement between two observers. Expression of Bcl-xL was observed with microscop using high magnification (400x) in 5 fields of view from each samples. The protocol of the study was approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta.

Statistical analysis

All numerical data were presented as mean ± standard deviation (SD). Statistical analysis was conducted using independent t test to evaluate the difference in mean between two groups. Bivariate analysis using chi-square test was used to evaluate correlation between two categorical variables. Multivariate analysis using logistic regression was applied to evaluate the relationship between independent variable (preeclamptic vs normotensive group), confounding variables (patient's age, gestational age, and mean arterial pressure), and dependent variable (Bcl-xL protein expression). A p value less than 0.05 was considered statistically significant.

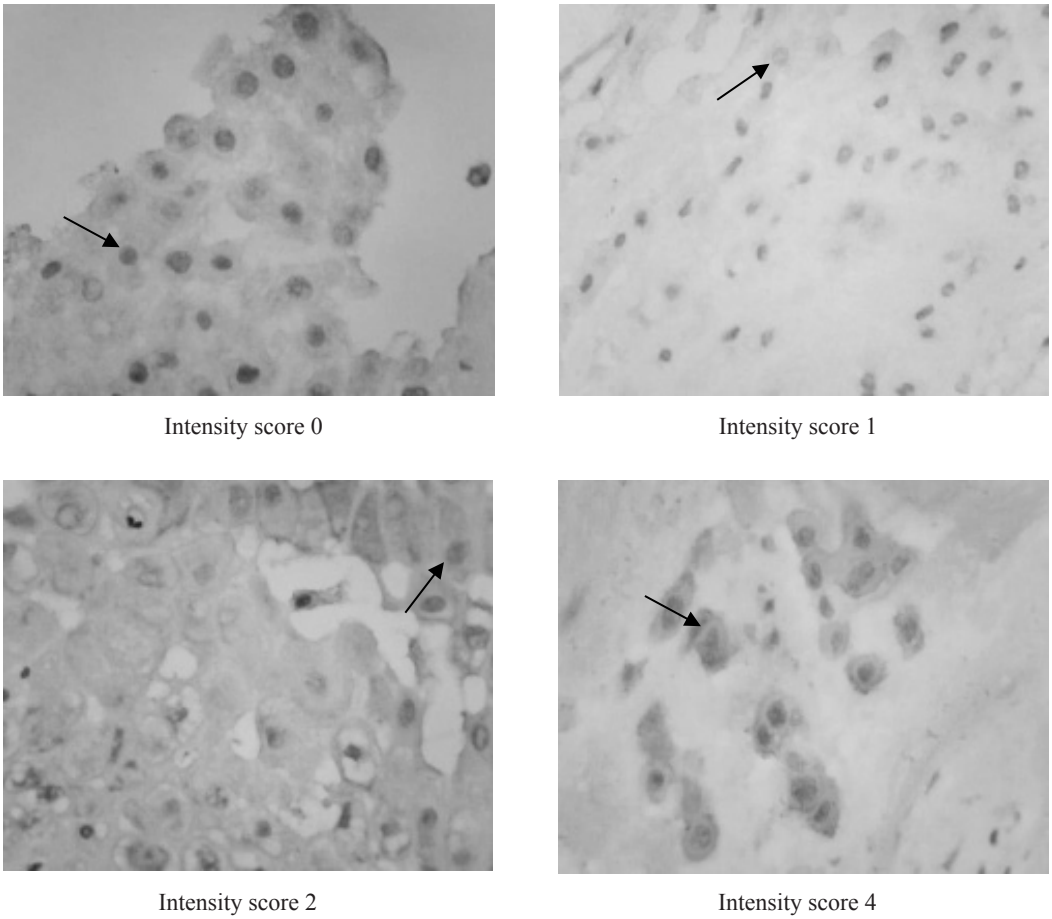
RESULTS

Placenta samples were obtained from 43 patients with pregnancy complicated by severe preeclampsia and 38 patients with normotensive pregnancy. All samples were stained using immunohistochemistry technique to evaluate the expression of Bcl-xL protein Characteristics of subjects of normotensive and severe preeclamptic groups are shown in TABLE 1. No significantly different of patient's age of severe preeclamptic compared to normotensive group was observed ($p>0.05$). Meanwhile, there were significant differences of gestational age and MAP of both groups ($p<0.05$).

TABLE 1. Characteristics of subjects (mean ± SD) of normotensive and severe preeclamptic groups

Variable	Normotensive Group (n=38)	Severe Preeclamptic Group (n=43)	Mean Difference (95% CI)	p
Patient's age (years)	28.42 ± 6.77	28.37 ± 7.08	0.5 (-3.0136 – 3.116)	0.974
Gestational age (weeks)	35.67 ± 3.01	38.39 ± 1.85	-2.72(-3.82 - -1.63)	0.000
Mean arterial pressure (mmHg)	126.29 ± 17.00	88.95 ± 6.44	37.34 (31.74-42.94)	0.000

In this study, the expression of Bcl-xL protein was observed in decidual trophoblast. In accordance with early onset preeclampsia theory which stated that the failure of spiral artery remodelling occured in decidual layer. The change in intensity of brown color in trophoblast cells of decidual layer corresponded to expression of Bcl-xL as shown in FIGURE 1.



Intensity score 0 Intensity score 1

Intensity score 2 Intensity score 4

FIGURE 1. Intensity of color in Bcl-xL protein staining

TABLE 2 shows comparison of Bcl-xL protein expression in placenta of severe preeclamptic and normotensive group. The mean expression of Bcl-xL protein in severe preeclamptic group was significantly lower than that normotensive group (p=0.00).

TABLE 2. The Bcl-xL protein expression (mean ± SD) in placenta of severe preeclamptic and normotensive groups

Variable	Severe Preeclamptic Group (n=43)	Normotensive Group (n=38)	Mean Difference (95% CI)	p
Bcl-xL Expression	1.29 ± 0.12	1.71 ± 0.14	- 0.42 (-0.47 – -0.36)	0.00

Receiver operating characteristic (ROC) analysis was conducted to determine the cutoff point of Bcl-xL protein expression. The analysis found that the area under curve (AUC) was 0.93 or 93% with cutoff point of 1.495 as shown in FIGURE 2.

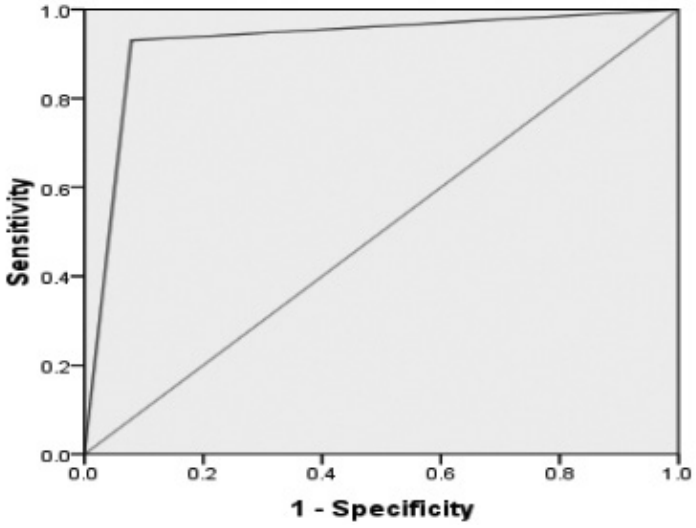


FIGURE 2. ROC curve of Bcl-xL protein expression

TABLE 3 shows bivariate analysis between independent and dependent variable. The pregnant patients with severe preeclampsia had more chances to have expression of Bcl-xL protein valued less than 1.495 compared to that normotensive patients, with relative risk of 11.78 (3.96-35.01) and p=0.000.

TABLE 3. Bivariate analysis between independent and dependent variable

Variable	Bcl-xL expression		%	RR (95% CI)	p
	<1,495	≥1,495			
Severe preeclamptic group	40	3	93.0	11.78 (3.96-35.01)	0.000
Normotensive group	3	35	7.9		

Bivariate analyses between confounding variables and the expression of Bcl-xL protein were shown in TABLE 4. Patient's age did not significantly affect the expression of Bcl-

Xl protein [RR= 0.93 (0.03-31.7); p >0.05]. In contrary, gestational age and MAP affected the expression of Bcl-Xl protein (RR =0.38 (0.25-0.58; p<0.001) and [RR= 2.67 (1.83-3.91); p<0.001], respectively.

TABLE 4. Bivariate analyses between confounding variables and expression of Bcl-xL protein

Variables	Bcl-xL expression		%	RR (95% CI)	p
	<1,495	≥1,495			
Patient's Age					
<20 and >40 years old	7	7	50	0.93	>0.05
20-40 years old	36	31	54	(0.03-31.7)	
Gestational Age					
≥37 weeks	18	35	34	0.38	<0.001
<37 weeks	25	3	89	(0.25-0.58)	
Mean Arterial Pressure (MAP)					
>123 mmHg	24	2	92.3	2.67	<0.001
≤123 mmHg	19	36	34.5	(1.83-3.91)	

Multivariate analysis using logistic regression between independent variable, dependent variable, and confounding variable is shown in TABLE 5. Diagnosis of severe preeclampsia consistently affected the expression of Bcl-xL protein (p=0.000), while gestational age and MAP did not affect the expression of Bcl-xL protein (p>0.05).

TABLE 5. Multivariate analysis using logistic regression between independent variable, dependent variable, and confounding variable

Variables	OR	95% CI	p
Diagnosis of severe preeclampsia	220,036	14.62-66.12	0.000
Patient's age	2.63	0.286-24.260	0.393
Gestational age	1.219	0.101-14.728	0.876
Mean arterial pressure	0.94	0.072-12.301	0.965

DISCUSSION

No significantly different in patient's age between severe preeclamptic group and normotensive group was found in this study. Previous studies reported that women with severe preeclampsia were older than normotensive pregnancy although it was not statistically significant.[5-7] Meanhwile, other studies found that preeclampsia were more common in women with advanced maternal age (≥35 years old) compared to younger women. Advanced maternal age is an independent risk factor for adverse outcomes in first-time mothers with preeclampsia.[8, 9]

Mean gestational age in severe preeclamptic group was significantly lower than normotensive group indicating an inhomogenous distribution in research samples. This result was in accordance with a study by Zhang *et al.*[7] which found that mean gestasional age in severe preeclamptic group was significantly lower than control group. On the contrary, Sharp *et al.*[6] and Allaire *et*

al.[10] found no difference in gestasional age between preeclamptic group and control group.

This study also found that MAP in severe preeclamptic group was higher than in normotensive group. This could be understood clearly because in preeclampsia there will be an increase in blood pressure.

This study found that expression of Bcl-xL protein as antiapoptotic molecule in patients with preeclampsia was lower than normotensive patients. This was in accordance with a study by Shu *et al.*[11] which found that Bcl-xL expression was down-regulated in preterm preeclampsia, but not in term preeclampsia and controls. Allaire *et al.*[10] concluded that the presence of apoptotic marker could be used as a sign of intrauterine hypoxia. Hung *et al.*[12] found that hypoxia and prolonged hypoxia-reoxigenation seemed to cause more reduction in the levels of Bcl-xL, although the difference was not statistically significant. Meanwhile, a study by Zhang *et al.*[7] found that Bcl-xL mRNA expression levels was unchanged in severe preeclamptic placentas when compared to control. In contrast, Whitehead *et al.*[13] found significantly increased placental RNA expression of Bcl-xL in early onset FGR (fetal growth restriction), PE complicated by FGR, and PE without FGR compared with preterm controls. This perhaps reflects a disordered regulation of apoptosis in placental dysfunction that is as yet not clearly understood.

No correlation between maternal age and expression of Bcl-xL protein as antiapoptotic molecule was observed in this stuudy. In contrast, Kavathia *et al.*[14] reported that there was a positive linear correlation between apoptosis and person's age. The discrepancy could be caused by group arrangement in this study which was based on risk factor of preeclampsia, where patient with age<20 years old and >40 years old were considered in high risk, and patient between 20-40 years old were in low risk.

This study also found that there was a correlation between gestational age and expression of Bcl-xL protein. In normal condition, apoptotic activity is increasing with gestational age. Previous study by Smith *et al.*[15] which compared apoptosis in normotensive pregnant women from first trimester and third trimester found that there was an increase in apoptosis index in third trimester. In normal condition, antiapoptotic expression is decreasing with gestational age as shown in a study by Kim *et al.*[16] which found that that there was a decrease in expression of Bcl-2 protein, an antiapoptic molecule, in third trimester.

Abnormality in apoptosis stimulation in patients with essential hypertension showed that antiapoptotic factors concentration in those patients were decreased and it could be caused by ischemia.[17] This was in accordance with this study where bivariate analysis between MAP and expression of Bcl-xL found that patients with MAP≥123 mmHg had 2.67 increases in probability to have expression of Bcl-xL <1.50 compared to patients with MAP<123 mmHg.

CONCLUSIONS

In conclusion, this study found that expression of Bcl-xL protein is lower in pregnancy complicated by severe preeclampsia compared to normotensive pregnancy. In addition, diagnosis of severe preeclampsia consistently affect in the expression of Bcl-xL protein.

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Correlation between CD4 cell counts with mucocutaneous manifestations: study of HIV patients in Dr. Sardjito General Hospital, Yogyakarta

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ABSTRACT

Mucocutaneous disorders often seen in HIV patients with varying morbidity. The HIV progression is characterized by the declining of CD4 cell counts and emergence of mucocutaneous manifestations. The aim of the study was to evaluate the relationship between CD4 cell counts with mucocutaneous manifestations in HIV patients. This was a cross-sectional study based on medical records at Dr. Sardjito General Hospital during the period January 2011-December 2015. Data of patient's age, sex, risk factors of transmission, most mucocutaneous manifestations and CD4 cell count were gathered. The correlation between CD4 cell counts with muscocutaneous manifestations were analyzed using chi-square test. A total 928 patients were involved in the study. More than half of the patients were male (65.4%) and mostly, the patients aged 20-29 years (38.69%). The main risk factors for HIV transmission were unsafe sex (75%). The highest CD4 cell counts was 1094 cells/mm^3 and the lowest was 1 cell/mm^3. We found 306 cases of mucocutaneous manifestations. The most mucocutaneous manifestations was a fungal infection (40.4%) with the highest infection type was oral candidiasis(33.8%); then non-infection (28%) with the highest type was drug eruption(35.9%); and tumors(0.5%) that was only Kaposi sarcoma cases. We also found sexually transmitted infections (STIs) (18.85%) with the highest cases was condyloma acuminata (49.3%). Statistical analysis showed a significantly relationship between CD4 cell counts with a fungal infection ($p < 0.0001$; OR= 3.8; 95% CI: 2.29 - 6.30), viral infection ($p = 0.0031$; OR= 0.4; 95% CI: 0.24-0.74) and parasitic infection ($p = 0.043$; OR= 0.2; 95% CI: 0.06-0.61). In conclusion, alteration in CD4 cell counts affects opportunistic infections occurence in HIV patients. Lower CD4 cell counts (< 200 cells/mm^3) increases the risk of fungal infection as much as 3.8 times. Higher CD4 cell counts (> 200 cells/mm^3) increases the risk of viral infection by about 2.5 times and parasitic infections as much as 5 times.

ABSTRAK

Kelainan mukokutan sering dialami pasien HIV dengan morbiditas yang bervariasi. Progresitas HIV ditandai dengan penurunan angka CD4 dan munculnya manifestasi mukokutan. Penelitian ini bertujuan untuk mengkaji hubungan antara angka CD4 dengan manifestasi mukokutan pada pasien HIV. Penelitian dengan rancangan potong lintang ini dilakukan dengan mengambil data dari rekam medis di RSUP Dr. Sardjito, Yogyakarta selama periode Januari 2011-Desember 2015. Data umur pasien, jenis kelamin, faktor risiko penularan, manifestasi mukokutan paling sering terjadi dan angka CD4 dikumpulkan.

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Hubungan antara angka CD4 dan manifestasi mukokutan dianalisis dengan uji chi square. Total 928 pasien terlibat dalam penelitian. Lebih setengah pasien adalah pria (65,4%) dan sebagian besar berumur antara 20-29 tahun (38,69%). Faktor risiko utama penularan HIV adalah perilaku seksual tidak sehat (75%). Angka CD4 tertinggi adalah 1094 sel/mm^3 dan terendah adalah 1 sel/mm^3. Ditemukan 306 kasus manifestasi mukokutan dengan paling banyak berupa infeksi jamur (40.4%) sebagian besar disebabkan kandidiasis oral (33,8%); selanjutnya bukan Karena infeksi (28%) sebagian besar disebabkan erupsi obat (35,9%) dan tumor (0,5%) yaitu Kaposi sarcoma. Infeksi penularan seksual ditemukan sebanyak 18,5% dengan kasus paling banyak condyloma acuminate (49,3%). Hasil analisis statistic menunjukkan hubungan nyata antara angka CD4 dengan infeksi jamur ($p < 0,0001$; OR = 3,8; 95% CI: 2,29 – 6,30), infeksi virus ($p = 0,0031$; OR = 0,4; 95% CI: 0,24-0,74) dan infeksi parasit ($p = 0,043$; OR = 0,2; 95% CI: 0,06-0,61). Dapat disimpulkan, perubahan angka CD4 menyebabkan terjadinya infeksi oportunitis pasien HIV. Angka CD4 lebih rendah (< 200 sel/mm^3) meningkatkan risiko infeksi jamur sebesar 3,8 kali. Angka CD4 lebih tinggi (> 200 sel/mm^3) meningkatkan risiko infeksi virus sebesar 2,5 kali dan infeksi parasit sebesar 5 kali.

Keywords: CD4 cell count- mucocutaneous manifestations – HIV – opportunistic infection – fungal infection

INTRODUCTION

Human immunodeficiency virus (HIV) is still remain a main global health problem.[1] At the end of 2016, 36.7 million people were living with HIV and one million people died of AIDS-related causes around the world. An estimated 1.8 million people became newly infected with HIV in 2016. The burden of the HIV/AIDS worldwide vary based on countries and regions. Sub-Saharan Africa remains most severely affected, with nearly 1 in every 25 adults (4.2%) living with HIV or nearly two-thirds of the people living with HIV worldwide.[2] Indonesia is one country with rapid growing of incidence of HIV/AIDS than other Asian countries. In Indonesia in 2014, about 22.869 new cases have been reported by Ministry of Health and Yogyakarta Special Region ranked 4th from the 33 provinces.[3,4]

Human immunodeficiency virus infection is characterized by a progressive decline of CD4 cell count which associated with impairment of cellular immune system and increasing susceptibility to opportunistic infections. Mucocutaneous manifestations are the common opportunistic infection found in the HIV patients. The mucocutaneous manifestations have been associated with the CD4 cell counts and used in the initial of HIV infection and in determining the clinical stage of the disease.[5-8] This study aimed to evaluate the relationship between CD4 cell counts and mucocutaneous manifestations in HIV patients in the Dr. Sardjito General Hospital, Yogyakarta.

MATERIALS AND METHODS

Subjects

This was an observational study with a cross-sectional design conducted in Department of Dermatology and Venereology, Faculty of Medicine, Universitas Gadjah Mada/Dr. Sardjito General Hospital, Yogyakarta. Data were obtained from medical records of HIV patients during the period January 2011-Desember 2015.

Protocol of study

The protocol of the study was conducted after ethical approval from the Medical and

Health Research Ethics Committee, Faculty of Medicine, Universitas Gadjah Mada/ Dr. Sardjito General Hospital was obtained. Data of the HIV patients included age, sex, risk factors of transmission, mucocutaneous manifestations and CD4 cell counts were gathered from the Medical Records Department (MRD), Dr. Sardjito General Hospital, Yogyakarta and evaluated.

Statistical analysis

Data were presented as mean ± standard deviation (SD) or percentage. The relationship between CD4 cell counts and mucocutaneous manifestations were analysed using chi-square test or Fisher exact test. A p value < 0.05 was considered significant.

RESULTS

Demography

As much as 1,241 data of HIV patients were obtained, however only 928 data could be selected and only 313 data could be analysed due to incomplete data. The highest CD4 cell counta was 1094 cells/mm^3 and the lowest was 1 cell/mm^3. More than half of the subjects were 607 male (65.4%) and 321 were female (34.5%). The ratio of male:female was about 1.89: 1. The mean of the patients age was 39 ± 14.2 years, wit the youngest patients was 5 months and the oldest was 79 years. The highest HIV cases found on the age group 20-29 years (38.69%) (FIGURE 1). The main risk factors for HIV transmission was unsafe sex (75%) (FIGURE 2).

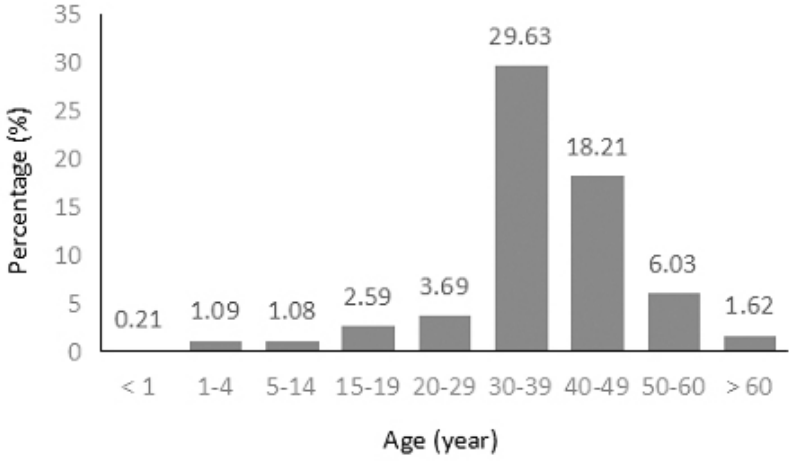

FIGURE 1. Number of HIV patients (%) according to age range

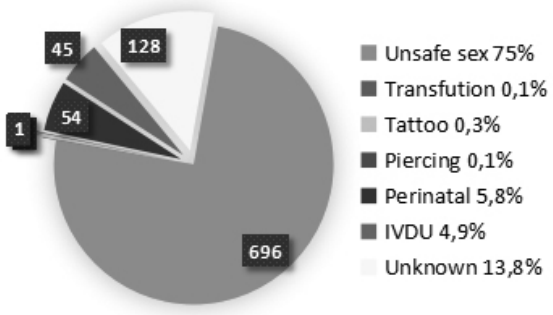

FIGURE 2. Number of HIV patients (n) accordinng to transmission risk factors

Mucocutaneous manifestations in HIV patients

Of the 205 HIV patients, 366 cases of mucocutaneous manifestations consisting of 261 cases of infection, 103 cases of non-infection and 2 cases of tumor were observed. The most type of infection found in this study was a fungal infection with 38.3% of them was oral candidiasis. The most cases of non-infection types were drug eruption (35.9%)

and we found only Kaposi's Sarcoma for tumor cases (0.5%) (FIGURE 3).

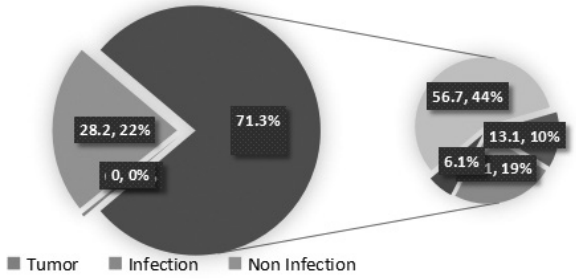

FIGURE 3. Mucocutaneous manifestations in patients with HIV and mucocutaneous manifestations by infection

If we divided the infection by the types, STIs and non-STIs, we found more non-STIs cases (74%) (FIGURE 4). Meanwhile for STIs cases, the highest type was genital wart cases (49.3%) (FIGURE 5)

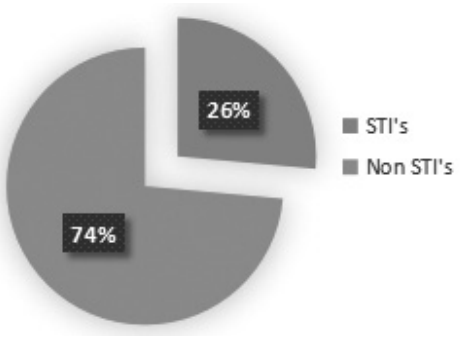

FIGURE 4. Percentage of cases of STIs and non-STIs in patients with HIV

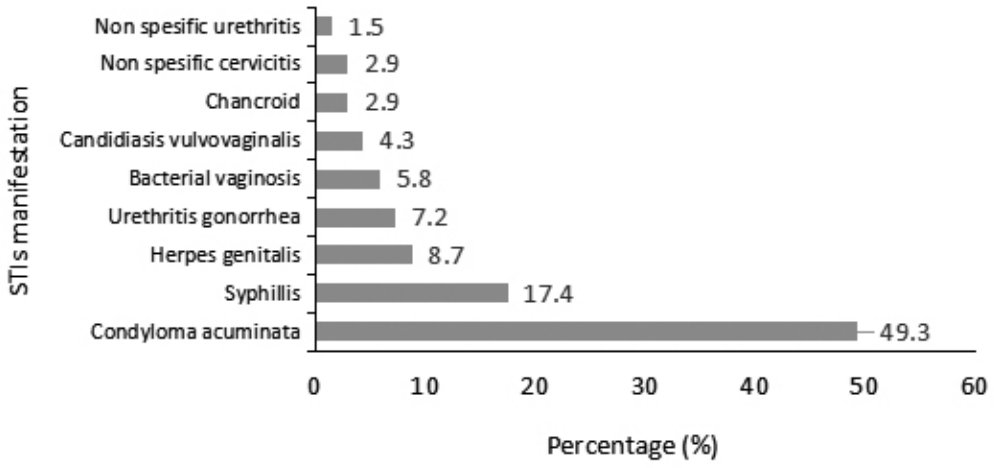

FIGURE 5. Percentage of STIs clinical manifestations in HIV patients

Relationship between CD4 cell counts and mucocutaneous manifestations

Significantly association between fungal infection (p <0.0001; OR 3.8; 95% CI 2:29 to 6:30), viral infections (p = 0.0031; OR 0.4; 95% CI 0:24 - 0.74) and parasites infection (p =0.043; OR 0.2; 95% CI 0:06 - 0.61) were observed in this study (TABLE 1). Strong relationships with CD4 cell counts <200 cells/mm^3 only found in fungal infections with a risk of 3.8 times compared with CD4 cell counts ≥200 cells / mm^3. In contrast, the number of CD4 cell counts ≥200 cells/ mm^3 was associated with increased risk of parasite infection and virus 5 and 2.5 times, respectively.

TABLE 1. The correlation between CD4 cell count and mucocutaneous manifestation

Mucocutaneous manifestation	Total n=366 (%)	Case with CD4 <200 n=246 (%)	Case with CD4 ≥200 n=120 (%)	p	OR (95% CI)
Fungal infection	40.4	50	20.8	<0.0001	3.8 (2.29 – 6.37)
Viral infection	17.3	13	25.9	0.0031	0.4 (0.24 – 0.74)
Bacterial infection	9.3	7.3	13.3	0.083	0.5 (0.25 – 1.04)
Parasite infection	4.4	2.0	9.2	0.0043	0.2 (0.06 – 0.61)
Non infection	28.2	27.3	30	0.6210	0.8 (0.53 – 1.41)
Tumor	0.5	0.4	0.8	0.5488	0.4 (0.03 – 7.8)

DISCUSSION

The majority of HIV patients in this study occurred in the age group 20-29 years old. This is consistent with general age of HIV patient reported by the Ministry of Health, Republic of Indonesia in 2014.[3] The HIV patients predominantly were males, with a ratio of male to female was 1.89: 1. A prevous study conducted in India by Singh *et al.*[6] reported HIV patients also was dominated by male than female with a ratio 1.5: 1. Based on the AIDS data center of Ministry of Health, since 1987 to 2014, cases of HIV infection had been dominated by males with a ratio of 2: 1. It can be caused by the rising number of men sex with men (MSM) who practicing unsafe sex and intravenous drug users (IVDU) which is dominated by male.[4,9]

Unsafe sex was the highest risk factor of HIV transmission in this study. Aydin *et al.*[5] reported the main risk of HIV transmission among Turkish HIV/AIDS patients in Istanbul was unsafe sex. In the mid 1990s, IVDU was the highest risk factor for HIV transmission in Indonesia. This shifting of risk factors begins with the increasing prevalence of sex workers since 1995-1996, followed by increasing cases in MSM and housewives.[10] Until now, patients with HIV highly observed among housewives.[4]

The introduction of safe sex to teenagers should start at early age. Lack of strategies to approach adolescent for sex education can be a risk factor because of lack of knowledge about the risks of early sexual intercourse and condom use. The high numbers of adolescent who run away from home make them easily exposed to alcohol and drugs that will increase the risk of HIV transmission.[9] The use of alcohol and drugs can increase the desire to perform unsafe sex.[9,10]

Human immunodeficiency virus infection impairs the cellular immune system causing people infected with HIV will show susceptibility to opportunistic infections which is the leading cause of mortality and morbidity. The impairment of the cellular immune system can be monitored by measuring the number of CD4 lymphocytes in the blood. The decline of the CD4 lymphocytes number is a sign of disease progression of HIV/AIDS. Mucocutaneous manifestations can be increased with the development of HIV and CD4 decline.[5]

Mucocutaneous manifestations in HIV patients can be found as infection, non-infection and tumor. In developed countries, non-infection cases in HIV patients commonly encountered due to the differences in their skin pigmentation, climate, hygiene, genetic,

environment, demographic and behavioral patterns. In contrast in developing countries such as Indonesia, infection cases in HIV patients commonly encountered.[11] The different in mucocutaneous manifestations leads to different clinical manifestations and epidemiological patterns. In this study, the infection cases were higher (71.3%) than that reported in the previous studies.[5,6] The main infection cases was a fungal infection (56.7%), which is dominated by oral candidiasis. This can be caused by tropical climate in Indonesia with relatively high levels of air humidity make various types of germs, including fungal infections growing easily.[12]

A significant association between CD4 cell counts and a fungal infection ($p < 0.0001$), viral infection ($p = 0.002$) and parasitic infection ($p = 0.0017$) was reported in this study. We reported strong relationships with CD4 cell counts <200 cells/mm^3 found in fungal infections with a risk 3.8 times compared with CD4 cell counts ≥200 cells/mm^3. In contrast, the number of CD4 cell counts ≥200 cells/mm^3 increase the risk of parasite infection and virus 5 and 2.5 times, respectively. The HIV patients with high CD4 cell counts still had good immune responses that can good respond to specific pathogens. In contrast, the HIV patients with a low CD4 cell counts had lower immune response which cause opportunistic infections such as viral and parasitic infections hardly recognized.[13] Unlike viral and parasitic infections, fungal infections were more common in low CD4 cell counts. The HIV infection can alter the course of fungal diseases, so the lower the CD4 cell counts leading to higher fungal infections.[14]

Sexually transmitted infections (STIs) may increase the risk of HIV transmission. The sexual behavior like not using condoms, multiple partners increases the risk of STIs. Inflammation due to STIS can cause skin and mucosa not intact, so can facilitate the entry of HIV virus.[15] In this study STIs cases found only 26% of all cases of HIV infection. This can happen because some the HIV patient in the Dr Sardjito General Hospital might perform safe sex like a condom use, do not change partners, abstinence, or their STIs cases have been cared in primary care. The highest STIs cases found in this study was condyloma acuminata cases (46.3%). Condyloma acuminata is the most common manifestation of STIs found in HIV patients. The difficulty to eliminate HPV virus with various therapeutic modalities make condyloma acuminata has high resistance.[16]

Patients with HIV/AIDS have high risk to malignancy, such as Kaposi's sarcoma, non-Hodgkin lymphoma and cervix carcinoma. This three malignancy is often called AIDS-defining condition, i.e. when it is found one of this three malignancies, can show the course of HIV infection has reached the stage of AIDS. The relationship between HIV infection with a particular type of malignancy is still unexplained, possibly related to a decrease in immune system.[17] Kaposi's sarcoma was first found on Jewish descent, young men in Africa and organ transplant recipients. Currently Kaposi sarcoma is the most common malignancy in homosexual HIV patients and related to human herpes virus (HHV).[17,18] In this study only found one case of Kaposi sarcoma among 205 HIV patients. This can be caused by other malignancies in patients at Dr. Sardjito General Hospital not performed HIV screening.

CONCLUSION

Alteration in CD4 cell counts affects the opportunistic infections occurence in HIV patients. Lower CD4 cell counts (<200 cells/mm^3) increases the risk of fungal infection as much as 3.8 times. Higher CD4 cell counts

(>200 cells/mm^3) increases the risk of viral infection by about 2.5 times and parasitic infections as much as 5 times.

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The *SLCO1B1**15 haplotype associated with lower clinical outcome in Indonesian tuberculosis patients

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ABSTRACT

Rifampin is one of first-line drugs for the treatment of tuberculosis. In Indonesia nearly all tuberculosis patients show lower rifampin plasma concentration's possibly due to genetics. Rifampin is a substrate of the organic anion-transporting polypeptide 1B1 (OATP 1B1) encoded by the solute carrier organic anion transporter family member 1B1 (*SLCO1B1*). This study aimed to identify haplotype polymorphisms of tuberculosis drug transporters with an impact on clinical outcome in tuberculosis patients. Thirty-six patients from I.A Moeis District Hospital, Samarinda, East Kalimantan were involved in the study. Buffy coat from patient blood samples were tested for *SLCO1B1* and *SLCO1B3* polymorphisms by RFLP and ARMS PCR, whereas the clinical outcome was examined based on the sputum conversion histopathology residuals. The frequency of patients with *SLCO1B1**15 haplotype was 63.9%. The *SLCO1B1**15 haplotype was associated with susceptibility to failure of clinical outcome ($p = 0.005$; RR = 4.52; 95% CI: 1.22-16.64). The OATP1B1*15 haplotype revealed that the failure of clinical outcome was markedly increased compared to the three other haplotypes. These results suggest that the SLCO1B1*15 haplotype is an important predisposing factor for lower clinical outcome. Our data indicate that individualized treatment should be considered for Indonesian tuberculosis patients based on genetics characteristics of patients.

ABSTRAK

Rifampin merupakan salah satu obat utama untuk pengobatan tuberkulosis. Di Indonesia hampir semua penderita tuberkulosis menunjukkan konsentrasi rifampisin yang rendah yang kemungkinan karena faktor genetik. Rifampin merupakan substrat dari Organic anion-transporting polypeptide 1B1 yang dikode oleh gen Solute carrier organic anion transporter family member 1B1 (*SLCO1B1*). Penelitian ini bertujuan mengidentifikasi polimorfisme haplotipe transporter obat tuberkulosis dan hubungannya dengan luaran klinis penderita tuberkulosis. Sebanyak 36 penderita tuberkulosis dari RSUD I.A. Moeis, Samarinda, Kalimantan Timur terlibat dalam penelitian. Buffy coat sampel darah pasien diperiksa polimorfisme *SLCO1B1* dan *SLCO1B3* menggunakan PCR RFLP dan ARMS, sedangkan luaran klinis ditentukan berdasarkan konversi sputum dan hasil histopatologi. Frekuensi penderita dengan *SLCO1B1**15 haplotipe sebanyak 63,9%. *SLCO1B1**15

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haplotipe berhubungan dengan risiko kegagalan pengobatan hasil klinis (p = 0,005; RR = 4,52; 95% CI: 1,22-16,64). OATP1B1*15 haplotipe mengungkapkan bahwa kegagalan hasil klinis lebih tinggi dibandingkan ketiga haplotipe lainnya. Hasil penelitian ini terbukti *SLCO1B1**15 haplotipe merupakan faktor predisposisi penting untuk memprediksi rendahnya luaran klinis. Disarankan agar pengobatan penderita tuberkulosis secara individu harus dipertimbangkan berdasarkan karakteristik genetik pasien.

Keyword: *SLCO1B1**15 haplotipe - tuberculosis - clinical outcome

INTRODUCTION

Tuberculosis (TB) is one of the major mortality risks in the world and is caused by *Mycobacterium tuberculosis,* which infects over 2 billion people, or almost one third of the world's 7 billion population.[1] Indonesia has the second ranked largest number of cases in the world. Rifampin (RIF) is a key component in the treatment regimen and due to its efficacy is used as a primary anti-TB drug.[1,2] The development of resistance to RIF is found to be related to RIF concentrations.[3] Despite this concern, there is limited RIF dosing information to ensure optimal outcome. A number of studies have shown interindividual RIF pharmacokinetic variability[4-6] and this genetic diversity may support the need to increase RIF dosage in Indonesian TB patients.[7] Recent research has explored a link between genetics and treatment efficacy[4,8] and the present study is the first to assess genetic diversity in Indonesian patients with confirmed diagnosis of pulmonary TB (PTB) and extrapulmonary TB (EPTB). In evaluating clinical outcomes, we examined major single-nucleotide polymorphisms (SNPs) of *SLCO1B1* and *SLCO1B3*. Considering genetic aspects potentially influential on the reduced levels of RIF concentration's.

As a substrate of organic anion transporting polypeptides OATP1B1 (coded by *SLCO1B1*) and OATP1B3 (coded by *SLCO1B3*),[9,10] SNP research has associated the *SLCO1B1* SNP C463A[4] and rs4149032[8] with reduced RIF exposure. Other recent studies showed that it did not influence the exposure and involves genetic variability[11,12] while presently there are no studies of the major SNPs in Indonesian patients. This study aimed to determine the major SNPs which may influence efficacy of treatment among Indonesian PTB and EPTB patients and the second objective of the study was to evaluate their potential impact on the clinical outcome. Applying our assessment of genetic variability to clinical outcomes, the data may assist in therapeutic-drug treatment schedules and monitoring in PTB and EPTB patients in Indonesia.

MATERIALS AND METHODS

Participants

This study used a cohort study, where we analyzed 36 patients with PTB and EPTB who had received an oral anti-TB regimen at I.A. Moeis District Hospital, Samarinda, Indonesia in 2014. The study participants had sputum smear-positive pulmonary TB or extrapulmonary TB from tissue biopsy which was definite if histopathology results showed the typical necrotizing granuloma containing macrophages, lymphocytes and Langhans giant cells. Caseous necrosis can be sometimes found in the central part of the granuloma, including: lymph node TB, gastrointestinal TB, skin and soft tissue TB. Directly observed daily administration of treatment involved

fixed-dose combination tablets (4FDC) with 150 mg rifampin, 75 mg isoniazid, 400 mg pyrazinamide and 275 mg ethambutol. Following Indonesian standard tuberculosis treatment guidelines, patients < 38 kg received 2 tablets daily, those weighing 38 to 54 kg received 3 tablets, 55 to 70 kg received 4 tablets daily and patients > 71 kg received 5 tablets once daily.[13] All patients received TB drugs from the same manufacturer. The following variables were available: sex, age, BMI, ethnic and clinical outcome, including sputum smear and post-treatment biopsy. The study has been approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health and Nursing Universitas Gadjah Mada, Yogyakarta.

Genotyping

We extracted blood sample DNA from the buffy coat by using the GeneJET Genomic DNA Purification Kit #K0722 (Thermo Fisher Scientific). We used the restriction fragment length polymorphism (RFLP) PCR for allelic discrimination in genotyping [*SLCO1B1* rs2306283 (c.388 A>G), *SLCO1B1* rs11045818 (c.411 G>A), *SLCO1B3* rs4149117 (c.334 T>G) and rs7311358 (c.699 G>A)] and the amplification refractory mutation system (ARMS) PCR [*SLCO1B1* rs4149056 (c.521T>C)]. Primers were sourced from Integrated DNA Technologies (Coralville, Iowa, USA). Two samples of each genotype were directly sequenced to confirm and validate our genotyping results with Applied Biosystems 3730 DNA Analyzer. The protocol to prepare the PCR involved an initial denaturation step at 94°C for 5 to 7 min, followed by 35 to 40 cycles of denaturation at 94°C for 30 to 60 s and then annealing temperature for 30 to 60 s and extension at 72°C for 30 to 60 s (TABLE 1). All PCR amplifications were conducted in a PCR-engine apparatus T100™ Thermal Cycler (Bio-Rad Life Science, USA). Restriction enzymes were sourced from Thermo Fisher Scientific. TABLE 1 below identifies the following SNPs based on allele frequencies previously shown to be involved in drug transport from the blood to the liver.

TABLE 1. Primers and PCR conditions used for genotyping of gene polymorphisms

Polymorphism	Primer sequence (5' ➡3')[a]	Fragment size (bp)	Annealing temperature (°C)	Amplification cycles	Restriction enzyme
SLCO1B1 rs2306283 and rs11045818	F: GCAAATAAAGGGGAATATTTCTC R: AGAGATGTAATTAAATGTATAC	274	46	37	TaqI
SLCO1B1 rs4149056	F:AAGTAGTTAAATTTGTAATAGAAATGC WT: GGGTCATACATGTGGATATAAGT MT: AAGCATATTACCCATGAACG R: GTAGACAAAGGGAAAGTGATCATA	260	48	35	-
SLCO1B3 rs4149117	F: GAAGGTACAATGTCTTGGGC R: CTCTCAAAAGGTAACTGCCC	339	58	35	AluI
SLCO1B3 rs7311358	F: ATGATTACATTCCCTGGATC R: ACTATCATGGTACCTTGTTC	303	56.3	40	RsaI

[a]F: Forward primer; R: reverse primer; WT: wild-type; MT: mutant-type

For *SLCO1B1* rs2306283 and rs11045818, the PCR product was digested using 5U of restriction enzyme at 65°C overnight. The products of restriction were placed in 3% agarose gel for electrophoresis and visualized by ethidium bromide staining. The restriction pattern consist of four distinct zones (154, 142, 132 and 119 bp), each representative for 388A>G and 411G>A wild-type and mutant alleles. This pattern in descending order stands for the two polymorphisms as follows: 154 bp for 411A, 142 bp for 388A, 132 bp for 411G and 119 bp for 388G.[14]

For *SLCO1B1* rs4149056, the tetra primer used in ARMS-PCR with slight modifications.[15] Because the restriction endonuclease was unnecessary, the PCR products were detected by means of 2% agarose gel with electrophoresis and by ethidium bromide staining. This pattern stands for the polymorphisms as follows: wild-type (260 and 179 bp); heterozygote (260, 123 and 179 bp) and mutant (260 and 123 bp). For *SLCO1B3* rs4149117, the PCR product was digested using 5U of restriction enzyme at 37°C overnight. The products of restriction were placed in 2% agarose gel for electrophoresis and visualized by ethidium bromide staining. The restriction pattern consists of two distinct zones (253 and 213 bp). This pattern stands for the polymorphisms as follows: wild-type (253 bp); heterozygote (253 and 213 bp) and mutant (213 bp).[16]

For *SLCO1B3* rs7311358, the PCR product was digested using 5U of restriction enzyme. The restriction pattern consists of two distinct zones (242 and 275 bp). This pattern stands for the polymorphisms as follows: wild-type (242 bp); heterozygote (242 and 275 bp) and mutant (275 bp).[16] In follow-up we assessed the clinical outcome with sputum conversion (PTB) or tissues through fine needle aspiration biopsy (EPTB). We compared the pre and post-treatment results. We categorized success if sputum conversion or lesions typically disappeared and improved histopathologically after the initiation of treatment. The failure of treatment outcome meant sputum conversion or lesions did not disappear and did not improve histopathologically.

Statistical analysis

Data were presented as median or percentage. The association between genetics polymorphisms of *SLCO* and clinical outcome was analysed using Fisher's exact test. A p-value <0.05 was considered as significant.

RESULTS

Thirty-six tuberculosis patients including 24 (66.7%) PTB and 12 (33.3%) EPTB were involved in this study. The demographics and genetics of the patients are presented in TABLE 2.

TABLE 2. Demographics and genotype description of cohort

Demographics		**Median (range) or *n* (%)**	
		Male 22 (61.11%)	**Female 14 (38.89%)**
Age (years)		42 (23-60)	39 (16-65)
Body weight (kg)		48 (37-65)	46.5 (35-98)
Height (m)		1.56 (1.42-1.72)	1.51 (1.4-1.63)
BMI (kg/m^2)		20.1 (16-27.7)	21.4 (15.4-38.3)
Genetics	**Homozygous wild type, *n* (%)**	**Heterozygous mutant type, *n* (%)**	**Homozygous mutant type, *n* (%)**
SLCO1B1 rs11045818	31 (86.11)	5 (13.89)	0 (0)
SLCO1B1 rs2306283	0 (0)	10 (27.78)	26 (72.22)
SLCO1B1 rs4149056	3 (8.33)	32 (88.89)	1 (2.78)
SLCO1B3 rs4149117	2 (5.55)	14 (38.89)	20 (55.56)
SLCO1B3 rs7311358	2 (5.55)	14 (38.89)	20 (55.56)
Haplotype	***n* (%)**	**Male, *n* (%)**	**Female, *n* (%)**
SLCO1B1 *1a	9 (25)	3 (13.6)	6 (42.8)
SLCO1B1 *1b	35 (97.2)	22 (100)	13 (92.3)
SLCO1B1 *5	8 (22.2)	3 (13.6)	5 (35.7)
SLCO1B1 *15	23 (63.9)	11 (50)	12 (85.7)

BMI: body mass index

The two most common *SLCO1B1* SNPs (c.521T>C and c.388A>G) form four functionally distinct haplotypes: *SLCO1B1**1a (c.388A-c.521T, as reference haplotype), *1b (c.388G-c.521T), *5 (c.388A-c.521C), and *15 (c.388G-c.521C).[17-19] All polymorphisms were in Hardy-Weinberg equilibrium. The patients carrying at least one *15 haplotype had a significantly increased risk for failure of treatment (p=0.005), and subjects carrying the *15 haplotype had a 4.52-fold significantly elevated risk (95% CI: 1.22-16.64) for failure of treatment. The *SLCO1B1* rs11045818 was not analyzed due to its insignificance. The *SLCO1B3* rs4149117 and rs7311358 polymorphisms did not have a statistically significant association with clinical outcome. We evaluated the differences in the haplotype-associated effects on clinical outcome, which also were associated with differences in overall drug exposures (TABLE 3).

TABLE 3. Response treatment based on genotype of TB patients (n = 36)

Genetics	Success 18 (50%)	Failure 18 (50%)	p^a	RR (CI)[b]
SLCO1B1 rs11045818				
GG	15 (41.7)	16 (44.4)	1	0.775 (0.25-2.39)
GA	3 (8.3)	2 (5.6)		
AA	0 (0)	0 (0)		
SLCO1B1 rs2306283				
AA	0 (0)	0 (0)	0.711	-
AG	6 (16.7)	4 (11.1)		
GG	12 (33.3)	14 (38.9)		
SLCO1B1 rs4149056				
TT	2 (5.5)	1 (2.8)	0.603	1.54 (0.30-7.92)
TC	15 (41.7)	17 (47.2)		
CC	1 (2.8)	0 (0)		
SLCO1B3 rs4149117				
TT	1 (2.8)	1 (2.8)	0.389	1 (0.24-4.16)
TG	9 (25)	5 (13.9)		
GG	8 (22.2)	12 (33.3)		
SLCO1B3 rs7311358				
GG	1 (2.8)	1 (2.8)	0.389	1 (0.24-4.16)
GA	9 (25)	5 (13.9)		
AA	8 (22.2)	12 (33.3)		
Haplotype				
[c]*1a (388A521T)	5	4	1	0.85 (0.38-1.93)
Other *1a	13	14		
[d]*1b (388G521T)	17	18	1	-
Other *1b	1	0		
[e]*5 (388A521C)	5	3	0.691	0.7 (0.27-1.82)
Other *5	13	15		
Haplotype groups				
-/-[f]	11	2	0.005	4.52 (1.22-16.64)
*15/-[g]	7	16		

[a]Data are calculated using Fisher's exact test, [b]Risk ratio (Confidence Interval), [c]*1a including (*1a/*1a, *1a/*1b, *1a/*15), [d]*1b including (*1b/*1b, *1a/*1b, *1b/*5, *1b/*15), [e]*5 including (*1b/*5, *1a/*5, *5/*5, *5/*15), [f]-/- including (*1a/*1a, *1a/*1b, *1b/*1b), [g]*15/- indicates at least one *15 allele (*1b/*15, *1a/*15, *5/*15, *15/*15).

DISCUSSION

Our study is the first in an Indonesian population to demonstrate that the *SLCO1B1**15 haplotype can be associated with clinical outcome in patients with PTB and EPTB. Among the TB patients with lower RIF concentrations,[20] we found this haplotype in 63.9%, which is consistent with other studies of Indonesian patients.[7] The lower RIF concentrations observed for Indonesians could be partially accounted for by the *SLCO1B1**15 haplotype. The high frequency of the *SLCO1B1**15 haplotype suggests that the haplotype can help to predict the clinical outcome for certain Indonesian populations. We investigated the effect of major haplotypes

of drug transporters and related demographics on clinical outcome. We analyzed factors that could influence outcome of treatment in Indonesian PTB and EPTB patients. The variable - *SLCO1B1**15 haplotype -was found to significantly affect the clinical outcome. We identified a significant link between *SLCO1B1**15 haplotype and treatment,[21-23] which could be explained by decreased hepatocellular uptake due to reduced drug transporter activity mediated by OATP1B1.[24]

Some studies examining both peak plasma concentration (C_{max}) and area under the curve (AUC) of RIF exposure have demonstrated definite correlation with clinical outcomes.[25,26] Related to genetic diversity, advances in genotyping of *SLCO1B1* rs11045818 have identified a synonymous variant (411G>A) (https://www.ncbi.nlm.nih.gov/clinvar/variation/307936/). For *SLCO1B3*, we did not observe any significant pharmacogenetic effects due to its limited impact. Our findings confirmed there is some effect of the *SLCO1B1**15 haplotype on RIF exposure and treatment outcome, but presently it is not well understood. Although c.521T>C and c.388A>G SNPs appear to be insignificant, the *SLCO1B1**15 haplotype (which includes both SNPs) has been consistently associated with reduced OATP1B1 transport activity.[21]

There were also some limitations in our study. For example, all of our investigated patients were coadministered isoniazid, ethambutol and pyrazinamide with RIF, and thus any confounding effects of medicines could not be fully eliminated in this study. Nonetheless, the present results indicate that OATP1B1 variants may be involved in the failure of clinical outcome and provide a new potential failure risk for patients with this disease.

CONCLUSION

Our final results show the *SLCO1B1**15 haplotype exists in the Indonesian population and is associated with the effect of reducing the clinical outcome. This link was examined in an Indonesian population with PTB and EPTB. Because of high frequency of the *SLCO1B1**15 haplotype compared to non-*15 haplotype, our data suggests that this genotype may provide some prediction for clinical outcome and therefore, specialized diagnosis and treatment should be considered for select Indonesian patients discriminatively based on individual genetic assessment. Actual clinical studies are needed to evaluate and confirm the variability of genotypes for the general population.

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The influence of acetylation status of tuberculosis patients on the isoniazid serum concentrations and sputum conversion after intensive phase therapy

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ABSTRACT

Isoniazid (INH), one of the major antituberculosis drugs, is metabolized by acetylation. Previously study proved the significant differences of serum INH concentration between subject with fast and slow acetylation status. However, the correlation of acetylation status with treatment outcome after fixed-dose combination antituberculosis therapy (FDC-ATT) was not explained. The aim of this study was to evaluate the influence of acetylation status on the treatment outcome and the serum INH concentrations in the adult tuberculosis patients underwent FDC-ATT. A cross sectional study was carried out on 31 tuberculosis patients. Acetylation status was measured by spectrophotometer and serum INH concentration was measured by high performance liquid chromatography (HPLC). Sputum conversion assay was conducted by Ziehl Nelsen method. t-Test, chi square, Mann-Whitney, and Fisherman were used to analyze the data. The proportion of the fast acetylator was 61.3%, whereas the slow acetylator was 38.7%. The proportion of success and failure sputum conversion were 83.9% and 16.1%, respectively. The mean serum INH concentration in the fast acetylator groups (1.52 ± 0.15 μg/mL) was significantly lower than that in the slow acetylator groups (3.84 ± 0.35 μg/mL). The failure conversion risk of the fast acetylator group was about two folds higher than the slow acetylator group, although it was not significantly different (RR = 2.53; 95% CI = 0.32-20.00; $p > 0.05$). Moreover, the mean serum INH concentration in success (2.46 ± 0.31 μg/mL) and failure (1.89 ± 0.20 μg/mL) sputum conversion was not significantly different ($p > 0.05$). In conclusion, the acetylation status does not influence the sputum conversion in adult tuberculosis patients after FDC-ATT although the serum INH concentration on slow acetylation status is higher than that fast acetylation status.

ABSTRAK

Isoniazid (INH) merupakan salah satu obat antituberkulosis utama yang metabolismenya melalui asetilasi. Pada penelitian sebelumnya dilaporkan ada perbedaan kadar INH serum pada orang dengan asetilator cepat dan lambat. Namun demikian hubungan antara status asetilasi dengan keberhasilan terapi penderita tuberkulosis (TB) yang mendapat obat antituberkulosis kombinasi dosis tetap (OAT-KDT) belum dijelaskan secara rinci. Tujuan

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penelitian ini adalah untuk mengkaji pengaruh status asetilasi terhadap keberhasilan terapi dan kadar INH serum penderita TB paru dewasa. Rancangan penelitian adalah potong lintang dengan subjek penelitian sebanyak 31 orang penderita TB. Status asetilasi ditetapkan dengan spektrofotometer dan kadar INH serum dengan ***high performance liquid chromatography*** *(*HPLC). Pemeriksaan sputum dilakukan dengan metode Ziehl Nelsen. Analisis statistik dengan uji t, Chi-square, Mann-Whitney, dan Fisherman. Proporsi status asetilasi cepat sebesar 61,3% sedangkan asetilator lambat 38,7%. Proporsi keberhasilan konversi sputum 83,9% dan gagal konversi 16,1%. Rerata kadar INH serum pada asetilator lambat (3,84 $\pm$ 0,34 μg/mL) lebih tinggi secara nyata dibandingkan pada asetilator cepat (1,52 $\pm$ 0,15 μg/mL) ($p < 0,05$). Risiko gagal konversi pada kelompok asetilator cepat sekitar 2,53 kali dibandingkan pada asetilator lambat, meskipun tidak bermakna secara nyata (RR = 2,53; 95%CI = 0,32-20,00; $p > 0,05$). Rerata kadar INH serum pada kelompok berhasil konversi yaitu 2,46 $\pm$ 0,31 μg/mL tidak berbeda nyata dengan kelompok gagal konversi yaitu 1,89 $\pm$ 0,20 μg/mL ($p > 0,05$). Dapat disimpulkan, status asetilasi tidak mempengaruhi konversi sputum pada penderita TB dewasa yang mendapat OAT-KDT meskipun kadar serum INH pada status asetilasi lambat lebih tinggi dari pada status asetilasi cepat.

Keywords: acetylation status – isoniazid level - success of therapy - conversion sputum - intensive phase treatment - tuberculosis

INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (*M. tuberculosis*). The spread of *M. tuberculosis* occurs from person to person primarily through droplet transmission in air.[1,2] Tuberculosis is a major health problem in the world, especially in developing countries. Approximately one third of the world's population has been infected with *M. tuberculosis* with the main source of transmission from TB patients basil hold acid (BTA) positif.[3]

In 2007, World Health Organization (WHO) reported that approximately 9.27 million new cases of TB were reported. This incidence increased from the previous year of 9.24 million new cases in 2006 and 8.3 million new cases in 2000. Mortality due to TB is also reported to be very high. In 2007, 1.3 million deaths from TB or 20 deaths per 100,000 population were reported. Approximately 90% of the total deaths occur in developing countries. According to WHO, 22 countries are classified as high-burden countries for TB, included Indonesia.[4]

Indonesia ranks third on the list of high-burden tuberculosis countries in the world after India and China with the number of TB patients about 10% of the total number of TB patients in the world. In 2004 there were 539,000 new cases per year and 101,000 deaths occur people from TB in Indonesia. The prevalence rate of smear positive TB in the National was 110 per 100,000 population.[3] The prevalence of TB in the Yogyakarta and Bali reported the lowest at 68 per 100,000 people. Pulmonary TB disease morbidity in Yogyakarta was 3.18 per 100,000 penduduk.[5]

Tuberculosis become more serious health problem with the increasing of Human Immunodeficiency Virus (HIV)/Acquired Immunodeficiency Syndrome (AIDS) cases.[6] Another issue that exacerbates tuberculosis is the emergence of multiple drug resistance TB (MDR-TB), which can lead to cases of TB more difficult to treat.[7] Resistance to antituberculosis mainly due to the inadequateuse use of

drugs . Inadequate therapy can be caused by inappropriate management therapy.[8] Since 1995 TB treatment has been carried out using Directly Observed Treatment Shortcourse (DOTS) which is given in two stages, intensive and continuation phases. At the end of the intensive phase and continuation phase, a patient sputum examination is conducted and evaluated. Successful treatment is considered if the patient sputum smear negatif in a microscopic examination is observed.[3]

Isoniazid (INH) is one of the main TB drugs which is generally given in the form of oral antituberculosis (OAT) drug in combination with others. Isoniazid has bacteriostatic and bactericidal activities. The metabolism of INH is affected by a drug metabolizing enzymes in the liver that is N-acetyltransferase 2 (NAT2) through acetylation reaction. Based on the reaction of NAT2 acetylation by this person can be categorized as fast acetylators or lambat.[9,10]

Individuals with rapid acetylator phenotypes must take INH doses greater than those with slow acetylator phenotypes.[11] Meanwhile another study proved that low serum INH concentrations was observed in individuals with rapid acetylator phenotypes.[10] The mean blood INH concentration 2 hours after INH administration in individuals with slow acetylator phenotypes was two times higher than those with rapid acetylator phenotypes.[12] Other studies also reported an association between INH acetylators status with outcome of TB treatment with once-weekly INH and rifapentine.[13]

NAT2 gene polymorphisms that determine the acetylator phenotypes of INH has been known to vary at different race or ethnicity. In the Indonesian population known to 65.4% of people classified as fast acetylators, and 35.6% are slow acetylators.[14] This study was conducted to evaluate the influence of acetylator phenotypes of tuberculosis patients on the serum INH concentration and sputum conversion after intensive phase therapy.

MATERIALS AND METHODS

This was an observational study using cross sectional design involving 31 subjects who met the inclusion and exclusion criteria. The inclusion criteria were adult patients with pulmonary TB (aged over 18 years) smear positive TB new cases, received TB treatment category 1 (2HRZE / 4H3R3), and willing to participate in the study. The exclusion criteria were subjects with diabetes mellitus, suffer from liver dysfunction, had a history of allergy to sulfa drugs, could not communicate well, not willing to follow the course of TB treatment with DOTS strategy. Protocol of the study was approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta.

Protocol of Study

Acetylation status determination

The acetylators phenotypes were determined using sulphadimidine according to the method developed by Rao *et al*.[15] Patients were given sulphadimidine 500 mg orally early in the morning on empty stomach. They were not allowed to take anything for the next two hours. Urine sample were collected before and 6 hours after sulphadimidine administration. Free and total sulphadimidine in the urine were determined by using spectrophotometer (Spectronic 20D + Thermo Fiher Scientific) at a λ of 540 nm. Acetylation status was determined based on the ability to produce the acetylated sulphadimidine (total minus free sulphadimidine) and expressed as a percentage of the total sulphadimidine. Patient was considered as rapid acetylator if

the acetylated sulphadimidine was >70% and as slow acetylator if it was <70%.

Measurement of serum INH concentration

Blood sample was taken before (day 0) and at the end of the intensive of treatment (day 56 of treatment). Three mL blood sample was taken from the median cubital vein two hours after OAT administration. The blood sample was then centrifuged at 3000 rpm, serum was taken and stored at -80 ^{o}C until analysed. Serum INH concentration was determined by using High Performance Liquid Chromatography (HPLC) in the Integrated Research and Testing Laboratory, Universitas Gadjah Mada, Yogyakarta. A simple extraction was conducted by protein precipitation with 150 µL of trichloroacetic acid 15% in 300 µL serum. The mixture was then vortexed for two min and centrifuged at 3000 rpm for 10 min. The supernatant was collected and 20 µL was injected into the HPLC system. The stationary phase was Novapak® C18 column (150 x 3.9 mm, 3 microns). The mobile phase consisted of 0.05 M sodium dihydrogen phosphate and acetonitrile (97: 3) and set at a flow rate of 1 mL/min at room temperature. The detection was performed at λ of 280 nm.

Sputum examination

Sputum was spread evenly over the central area of the slide which was air dried for about 30 min and heat fixed. The sputum slide was then flooded with Ziehl Neelsen (ZN) stain which was then heated to dry, kept for 5 min and washed with aquadest. The slide was then flooded with 3 mL of concentrated hydrochloric acid and 97 mL of ethanol 95% to decolourise the ZN stain and washed with aquadest. Thereafter, the slide was flooded with 0.3 g of methylene blue and 100 mL of aquadest for 2 min. The slide was air dried at room temperature and viewed on a microscope under oil immersion for presence of acid-fast bacilli. The bacilli were stained red and the background material was stained blue.

Statistical analysis

Data were presented as mean ± standard deviation (SD) or percentage and analyzed using SPSS software version 16. Numerical data were compared by using t-test or Mann-Whitney U test, whereas categorical data were compared by using Chi-square test or Fisher exact test. To determine the relative risk (RR) conversion failures in the rapid and slow acetylators group, 2 x 2 table relative risk analysis (Epi Info Program) at the 95% confidence interval was performed.

RESULTS

Among 34 subjects who met the inclusion criteria, only 31 patients completed the study. One subject can not be included until the end of the study period due to suffer from liver dysfunction as characterized by high levels of serum transaminases in early therapy and two subjects do not abide taking the drugs. The characteristics of subjects are presented in TABLE 1.

TABLE 1. Patients characteristic (n=31)

Characteristic	Value
Age (mean ± SD year)	44.2 ± 13.7
Sex [n (%)]	
Male	24 (77.4)
Female	7 (22.6)
Weight (mean ± SD kg)	49.5 ± 5.8
Height (mean ± SD cm)	164 ± 6
Body Mass Index (mean ± SD)	18.4 ± 2.0
Nutritional Status (%)	
Normal	13 (41.9)
Low	18 (58.1)
Smoker [n (%)]	
Yes	20 (64.5)
No	11 (35.5)
INH dose [n (%)]	
225 (3 FDC-ATT)	24 (77.4)
300 (4 (FDC-ATT)	7 (22.6)

The proportion of subjects with fast acetylators phenotype status was 61.3%, and 38.7% was people with slow acetylators status. Characteristics of patients in the fast or slow acetylators did not show any statistically significant difference (TABLE 2).

TABLE 2. Patient characteristics based acetylation status (n=31)

Characteristics	Acetylation status		p
	Slow (n=12)	Fast (n=19)	
Age (mean ± SD year)	42.1 ± 13.2	45.5 ± 14.3	0.518
Sex (%)			
Male	9 (75.0)	15 (78.9)	1.00
Female	3 (25.0)	4 (21.1)	
Weight (mean ± SD kg)	49.3 ± 4.9	49.6 ± 6.5	0.884
Height (mean ± SD cm)	165 ± 7	163 ± 6	0.251
Body Mass Index (mean ± SD)	18.0 ± 2.1	18.7 ± 1.9	0.224
Nutritional Status (%)			
Normal	3 (25)	9 (47.4)	0.129
Low	9 (75)	10 (52.6)	
Smoker (%)			
Yes	8 (66.7)	12 (63.2)	0.842
No	4 (33.3)	7 (36.8)	

Most patients had successful sputum conversion (83.9%), whereas only 16.1% had failure sputum conversion. Characteristics of patients based on the success and failure of sputum conversion are presented in TABLE 3.

TABLE 3. Patient characteristics based on sputum conversion (n=31)

Characteristics	Acetylation status		p
	Success (n=26)	Failure (n=5)	
Age (mean ± SD year)	42.3 ± 13.1	54.0 ± 14.0	0.083
Sex [n (%)]			
Male	21 (80.8)	3 (60.2)	0.562
Female	5 (19.2)	2 (40.0)	
Weight (mean ± SD kg)	49.9 ± 6.0	47.2 ± 5.2	0.646
Height (mean ± SD cm)	164 ± 6	161 ± 6	0.333
Body Mass Index (mean ± SD)	17.7 ± 2.0	18.2 ± 2.1	0.774
Nutritional Status [n (%)]			
Normal	11 (42.3)	3 (60.0)	1.000
Low	15 (57.7)	2 (40.0)	
Smoker [n (%)]			
Yes	18 (69.2)	2 (40.0)	0.317
No	8 (30.8)	3 (60.0)	
Acetylation status [n (%)]			
Fast	15 (57.7)	4 (80)	0.624
Slow	11 (42.3)	1 (20)	
INH concentration (mean±SEM µg/mL)	22.88±0.51	1.89±0.26	0.519
INH dose [n (%)]			
225 mg (3 FDC-ATT)	19 (73.1)	5 (100)	0.562
300 mg (4 FDC-ATT)	7 (26.9)	0 (0)	

Zero in serum INH concentration was observed before treatment begin. After complete treatment (day 56), the mean serum INH concentration was 2.72 µg/mL (ranged from 0.53 to 13.32 µg/mL). Most individuals who failed sputum conversions (80%) had a fast acetylators phenotype, whereas in the group succeeded only 57.7% sputum conversion. But this was not statistically significant (Fisher's exact test, $p > 0.05$). Based on the analysis of the 2x2 table (Epi Info Program) obtained RR = 2.53 (95% CI: 0.32 to 20.00). Although the risk of conversion failure in fast acetylators group seen 2 times greater than the slow acetylators, but not statistically significant (TABLE 4).

TABLE 4. Conversion failure risk based on acetylation status (n=31)

Acetylation status	Sputum conversion		Total	x^2, p	RR (95% CI)
	Success	Failure			
Fast	4	15	12	0.342	0.32-20.00
Slow	1	11	19		
Total	5	26	31		

The mean serum INH concentration in the slow acetylators patients (4.63 µg/mL) was much higher than fast acetylators (1.52 µg/mL). But in the group of slow acetylators, one subject had extreme INH concentration until 13.32 µg/mL. After extreme data excluded, the mean INH concentration of the slow acetylators (3.84 ± 0.34 µg/mL) was two times higher than that the rapid acetylators (1.52 ± 0.15 µg /mL) ($p < 0.05$) as shown on TABLE 5.

TABLE 5. Serum INH concentration in the fast and slow acetylation status (n=30)

Acetylation status	n	Serum INH concentration (µg/mL)				
		Mean	SEM	95% CI	Min-Max	p
Fast	19	1.52	0.15	1.20-1.83	0.53-2.54	0.000
Slow	11	3.84	0.34	3.07-4.61	2.29-5.01	

The mean INH concentration in the subjects with failed in sputum conversions (1.89 ± 0.20 µg/mL) was significantly lower than the subjects with success in sputum conversions (2.88 ± 0.51 µg/mL) ($p<0.05$). One subject with extreme INH concentration was found in the subjects with success in sputum conversion. After subjects with extreme INH concentration excluded, the mean INH concentration between the subjects with success in sputum conversion (2.46 ± 0.31 µg/mL) was similar to those with failed in sputum conversion (1.89 ± 0.20 µg/mL) ($p>0.05$).

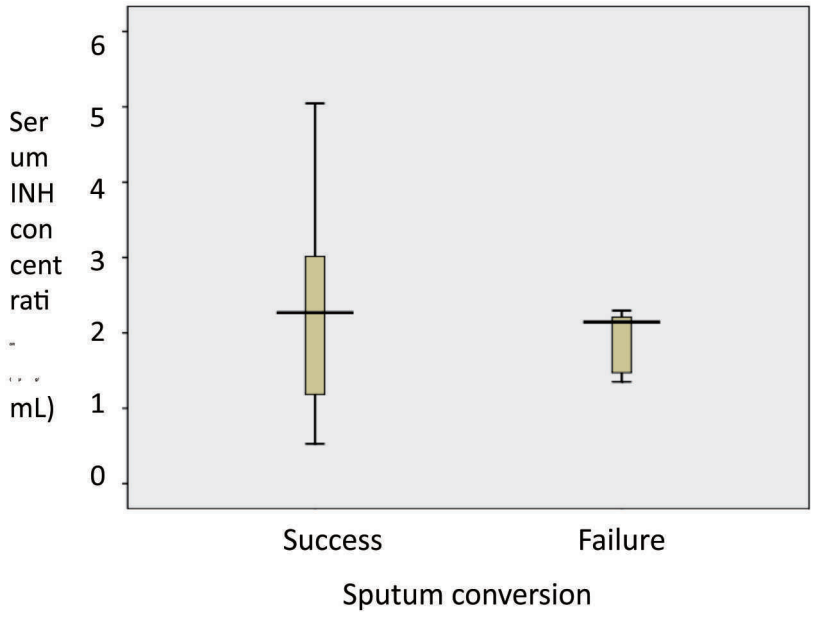

FIGURE 1. Serum INH concentration based on sputum conversion (n=30)

Most subjects (77.4%) received 3 FDC-ATT tablets (225 mg INH), and 22.6% received 4 FDC-ATT tablets (300 mg). The mean serum INH concentration in the group receiving 225 mg or 3 tablets FDC-ATT was no higher than those receiving 300 mg or 4 tablets FDC-ATT (2.55 ± 0.30 µg/mL vs. 1.63 ± 0.31 µg/mL; Mann-Whitney test $p > 0.05$) (TABLE 6).

TABLE 6. Serum INH concentration based on INH dose (n=30)

Dose of INH	n	Mean	SEM	95% CI	Min-Max	p
225 mg	24	2.55	0.30	1.92-3.18	0.59-5.05	0.147
300 mg	6	1.63	0.31	0.83-2.44	0.53-2.67	

DISCUSSION

The proportion of rapid acetylation status (61.3%) was higher than that slow acetylation status (38.7%) in this study. This proportion was similar with the results reported in the previous study conducted in the Community Health Care and Center for Pulmonary Disease Treatment (BP4) in Yogyakarta which found 64% of patients were rapid acetylation status and 36% of patients were slow acetylation status.[16] Furthermore, it was estimated that approximately 2/3 (66.67%) of the Indonesian people classified as rapid acetylation status and 1/3 (33.33%) as slow acetylation status.[14]

The mean serum INH concentration on 31 patients two hours after drug administration was 2.72 ± 0.43 µg/mL ranged from 0.53 to 13.32 µg/mL and the median was 2.21 µg/

mL in this study. Serum INH concentration on TB patients after treatment has been reported in the previous studies. A therapeutic drug monitoring study on TB patients conducted in Virginia, USA reported that the median of serum INH concentration two hours after drugs administration was 1.9 µg/mL (interquartile range 1.1-3.5 µg/mL).[17] Another study conducted on TB patients in Turkey found the mean serum INH concentration two hours after OAT treatment was 3.83 ±2.09 µg/mL.[18] Furthermore, study conducted on TB patients in Alberta, Canada found the mean serum INH concentration was 4.13 ± 3.9 µg/mL.[19]

The success of TB treatment is proven by the sputum conversion from smear positive to smear negative at the end of the intensive phase of treatment. In this study TB treatment success rate achieved 83.9%, while the failure rate was 16.1%. The TB treatment success rate has been reported from various regions. The Province Health Office in Yogyakarta Special Region reported that the TB treatment success rate achieved 87%.[20] A study conducted in UK demonstrated the TB treatment success rate was 87.%.[21] However, in 2013 in America and European regions the TB treatment success rate was 75%, whereas in Malaysia was 67% less than the 85% success target set by the WHO. Globally, the TB treatment success rate for people newly diagnosed with TB was 86% in 2013.[22, 23]

The TB treatment failure on the rapid acetylator group (80% or 4 people) was higher compared to on the slow acetylation status group (20% or 1 people). The RR of failure in the sputum conversion of fast acetylation status group was two times higher than the slow acetylation status group although it was not significantly different. This results is different from the previous study that showed a relationship between acetylation status and outcome of TB treatment with once-weekly INH and rifapentine.[13] The different of dosage regimen and patient compliance may cause the different of these results.

The mean serum INH concentration of slow acetylation status group (3.84 ± 0.34 µg/mL) was higher than that rapid acetylation status (1.52 ± 0.15 µg/mL). The higher serum INH concentration of slow acetylation status compared to that rapid acetylation status was also reported in the previous studies. Schaaf *et al.*[12] reported the serum INH concentration of slow acetylation status (8.6 µg/mL) was two-old higher than that rapid acetylation status (3.94 µg/mL). Furthermore, Conte *et al*.[24] also reported the serum INH concentration of slow acetylation status (1.1 ± 0.8 µg/mL) was higher than that rapid acetylation status (0.5 ± 0.6 µg/mL).

The mean serum INH concentration in the patients with failed in sputum conversions (1.89 ± 0.20 µg/mL) was significantly lower than the subjects with success in sputum conversions (2.46 ± 0.31 µg/mL). However, it was not significantly different ($p>0.05$). It was reported that effective of tuberculostatic minimal concentration of INH range from 0.025 to 0.05 µg/mL, whereas the therapeutic INH concentration range from 0.5 to 2.0 µg/mL.[25-27] In this study, the mean serum INH concentration in the both patients with filed and success in sputum conversions met the therapeutic INH concentration that indicated serum INH concentration did not influence the sputum conversion. No different in serum INH concentration in the patients with failed and success in sputum conversions nor in the patients with delays and avoid delay in sputum conversions were also reported in the previous study.[25]

CONCLUSIONS

Adult pulmonary TB patient with rapid acetylation status has lower serum INH concentrations than slow acetylation status. However, these serum INH concentrations are still within the therapeutic INH concentration therefore the acetylation status does not affect the sputum conversion after the intensive phase of TB treatment.

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Cinnamomum burmannii improves insulin serum level in the normal obese subjects: preliminary study

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ABSTRACT

Obesity is characterized with excessive accumulation of the body fat which occurs when the energy intake exceeds the expenditure. It is routinely associated with insulin resistance and hyperinsulinemia. Additionally, suppressing insulin level protects female mice from weight gaining. Cinnamon [Cinnamomum burmannii (Ness) Bl. Cortex] suppresses hyperinsulinemia condition in the type 2 diabetic rat suggesting the possible beneficial its role in the obesity. We aimed to investigate the effect of Cinnamon extract in the normal obese subjects. In this preliminary cross-over clinical trial, 24 normal obese subjects were recruited and divided randomly into two groups i.e. treatment and placebo. Two grams of the cinnamon extract were given twice daily for 56 days in the treatment group. Normal obese subjects given placebo were allocated as the placebo group. After the treatment, each of the group ran a one month run-in period, then the groups were cross-overed for the next 56 days. Body mass index (BMI), insulin serum level, cholesterol and triglyceride plasma levels were measured at the beginning and at the end of the study. No diet restriction nor exercise intervention was given during the study. At the end of the study, BMI in the treatment group (58%) were slightly reduced when compared to the placebo group (33%), however, it was not significantly different ($p>0.05$). Moreover, significantly reduction in the insulin serum level was observed in 63% subject in the treatment group compared to 33% subject in the placebo group ($p < 0.05$). Additionally, there were no significant differences of cholesterol and triglyceride plasma level observed in the both group. In conclusion, cinnamon extract may give beneficial role in the normal obese subjects by suppressing the serum insulin level. Further studies are required to elucidate the specific role of cinnamon in preventing weight gain.

ABSTRAK

Kegemukan ditandai dengan akumulasi berlebhan lemak tubuh yang terjadi ketika pemasukan energi melebihi pengeluaran. Kegemukan sering dikaitkan dengan resistensi insulin dan hiperinsulinemia. Selain itu terbukti penurunan kadar insulin mencegah kegemukan pada mencit. Kayu manis [Cinnamomun burmannii (Ness) Bl. Cortex] dapat menekan kondisi hyperinsulinemia pada tikus diabetes tipe 2 dan diduga bermanfaat dalam mencegah kegemukan. Penelitian ini bertujuan untuk mengkaji efek ekstrak kayu putih pada sukarelawan sehat. Pada uji klinik awal dengan rancangan silang ini, 24 sukarelawan dengan kegemukan normal yang dilibatkan penelitian dibagi acak menjadi dua kelompok yaitu kelompok perlakuan dan kontrol. Dua gram ekstrak kayu putih diberikan

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dua kali sehari selama 56 hari pada kelompok perlakuan, sedangkan kelompok kontrol diberi plasebo. Setelah perlakuan, masing-masing kelompok diberi latihan (lari) setiap hari selama satu bulan, kemudian masing-masing kelompok disilang untuk mendapatkan perlakuan atau kontrol selama 56 hari berikutnya. Indeks massa tubuh (IMT), kadar insulin serum, kadar kolesterol dan trigliserit plasma diukur di awal dan akhir penelitian. Tidak ada pembatasan dan juga latihan diberikan selama penelitian. Di akhir penelitian, IMT kelompok perlakuan (58%) sedikit turun dibandingkan kelompok plasebo (33%). Namun demikian perbedaan ini tidak bermakna ($p > 0,05$). Selanjutnya, penurunan kadar insulin serum secara nyata terjadi pada 63% sukarelawan pada kelompok perlakuan dibandingkan 33% pada kelompok plasebo ($p < 0,05$). Tidak ada perbedaan nyata kadar kolesterol dan trigliserida plasma antara kedua kelompok. Dapat disimpulkan, ekstrak kayu manis bermanfaat bagi subjek dengan kegemukan normal melalui perannya dalam menurunkan kadar insulin serum. Penelitian lanjutan diperlukan untuk mengkaji peran spesifik kayu manis dalam mencegah kegemukan.

Keywords : Cinnamomum burmannii – obesity – insulin - body mass index - lipid profile

INTRODUCTION

Obesity is a metabolic disorder characterized with excessive expansion of adipose tissue due to imbalances between nutrient intake and energetic activity.[1] Obesity has remarkably increased worldwide and leads to significant morbidity and mortality related to cardiovascular disease, metabolic syndrome, type 2 diabetes, hypertension, degenerative joint disease and some kinds of cancer. It is affecting 33% of adults in the United States and becomes the most common public health problems.[2] Some strategies have been proposed to reduce enormous body weight in the obese state by inhibiting fat absorption in the gut or supressing appetite in the brain. Recently, it is found that insulin resistance and hyperinsulinemia are the key characteristics of obesity which contributes to its further complication on health.[3-5] In the obese state, compensatory rise of insulin due to hyperglycemia, may lead to insulin resistance.[6] Furthermore, hyperinsulinemia may promote obesity, resulting in a vicious cycle between obesity, insulin resistance and hyperinsulinemia.[6-11] It has been reported that attenuating hyperinsulinemia in the experimental young female mice provides protection against obesity and reducing insulin secretion may promote weight loss in obese adults with insulin hypersecretion.[6,12,13] Conclusively, reducing insulin secretion may have beneficial role in the strategy of obesity treatment.

Cinnamomum burmannii (Ness) BI. Cortex, widely known in Indonesia as cinnamon or *kayu manis*, *cassia* in Padang and Batavia, is an endogenous plant that has been traditionally used as spices, herb and medicine.[14] It is currently marketed as a supplemental herbal for diabetes mellitus, dyslipidaemia and glucose intolerance since experimental. Clinical evidence showed that cinnamon has a role as insulin sensitizing agent. In 3T3L1 adipocyte tissue, cinnamon extract stimulates glucose uptake and glycogen synthesis and further activates glycogen synthase.[15] Additionally, cinnamon bioactive component stimulates enzymatic reaction of phosphorylation and dephosphorylation, confirming its role as an insulin mimetic.[16] Furthermore, cinnamon extract may decrease the blood glucose level and stimulate glucose uptake in the experimental type 1 diabetic

rat or type 2 diabetic mice.[17-20] Recently, evidences have been reported that cinnamon bioactive components, proanthocyanidin and cinnamaldehyde, could improve the formation of pancreatic islet polypeptide and suppress hyperinsulinemia condition in the type 2 diabetic rat.[21-23]

In the clinical setting, daily consumption of 1, 3 or 6 g cinnamon supplement reduced the blood glucose level up to 29% in the type 2 diabetic patients[24] and routine consumption of 3 g cinnamon supplement in eight weeks reduced body weight and body mass index (BMI) in type 2 diabetic patients.[25] These mechanisms may be mediated through the role of cinnamon in improving the body composition and attenuating lipogenic processes in the liver and adipose tissue.[26] Conversely, another study reported that 4 month treatment with a dietary supplement containing cinnamon, chromium and carnosine decreased fasting plasma glucose (FPG) and increased fat-free mass. However, there was no different with placebo with respect to body weight and BMI in overweight or obese pre-diabetic subjects.[27] Despite our significant understanding of the role of cinnamon in the improvement of diabetes, the roles of cinnamon in obesity and its insulin regulation are largely unknown. In the present study, we investigated the effect of cinnamon extract in the normal obese subjects.

MATERIALS AND METHODS

Cinnamon burmannii extract

Cinnamon extract was prepared in a capsules preparation by UD Rachma Sari and certified by the National Agency of Drug and Food Control, Republic of Indonesia (TR 123365801). Each cinnamon capsules contained two g of cinnamon extract. Placebo capsules were packaged as the same as the cinnamon capsule. Both the cinnamon and placebo capsules were packaged in plastic bag containing 14 capsules (two capsules of two g for 7 days) and prepared for distribution of the subjects. Subjects received one capsule twice daily (with the total dose of 4 g/day) for 56 days. The dose were decided based on two previous studies using dose range from 1 – 6 g/day.[24,25] Subjects were evaluated every 7 days for supplement compliance until the end of the study. Compliance was monitored by capsule count, subjects interview and daily diary analysis.

Design

The design of this preliminary study was randomized cross-over clinical trial study. The study was divided in two phase, in each of the phase every subject received 56 days of capsules (treatment or placebo). Subjects were recruited and allocated randomly into two groups i.e. treatment and placebo. Cinnamon capsules were given for 56 days in normal obese subjects in the treatment group. Another group of normal obese subjects were given placebo as the placebo group. After finishing 56 days treatment, each of the group ran a one month run-in period, and then the groups were cross-overed for the next 56 days. Subjects were not informed which treatment they have received until the end of the study.

Study population

This study was conducted in the Faculty of Medicine and Health Science, Islamic State University and was approved by the Ethics Committee and Human Studies Review Board of Faculty of Medicine and Health Science, Islamic State University. Selection criteria for the study were adult normal obese subjects with BMI $\geq$ 23 kg/m^2 and age ranged from 18 to 70 year old. Subjects were excluded from the study if they have: degenerative

disease (hypertension, diabetes mellitus, coronary artery disease, atherosclerosis), cancer, scheduled diet process, pregnancy, breastfeeding, long term pharmacotherapy (chemotherapy, corticosteroid, insuling sensitizing agent, anti-hypertension and anti-cholesterol).

Follow up and outcome measures

Body mass index were measured by dividing body weight (kg) with squared of body height (m). The serum insulin level was measured by ELISA technique using ELISA insulin kit (Calbiotech, Spring Valley, CA, USA). In brief, subjects sera were incubated for 60 minutes with insulin enzyme conjugate, washed, added with TMB substrat for 15 min. After the addition of stop solution, sera were analyzed by ELISA reader. The plasma cholesterol level was measured by cholesterol esterase/cholesterol oxidase technique using cholesterol kit (Sclavo Diagnostics, Siena, Italy). The plasma triglyceride level was measured by triglyceride kit (Diasys Diagnostic System, Holzheim, Germany). Subjects were followed up every 7 days, however, outcome measures were conducted only on day 1 and day 56. At each visits, the occurrence of study outcomes was ascertained according to the intention-to-treat principle.

Data analysis

The variable of this study were the BMI, the serum insulin level, the plasma cholesterol and tryglyceride levels. All variable data from the recruited patients were included in the analyses. Comparison among groups was performed using one-way analysis of variance (ANOVA) or student's t-test, wherever applicable. A $p < 0.05$ was considered as statistically significant.

RESULTS

Baseline characteristics

The characteristics of subjects were summarized in TABLE 1. Twenty four normal obese subjects were recruited and followed up every 7 days until the end of the study. None of them were dropped out during the study. No significant differences of BMI, insulin serum level, cholesterol plasma level and triglyceride plasma level were observed among the groups ($p > 0.05$).

TABLE 1. Baseline characteristics (mean ± SD) between placebo and treatment group

Variablle	Placebo group n=24	Treatment group n=24	p
Male	12	12	
Female	12	12	
Body Mass Index (kg/m^2)	28.9 ± 4.8	28.9 ± 5.0	0.9
Insulin (mg/dL)	23.6 ± 22.2	19.4 ± 14.3	0.4
Cholesterol (mg/dL)	164 ± 41	171 ± 27	0.5
Triglyceride (mg/dL)	121 ± 70	111 ± 66	0.6

Effect of cinnamon on BMI and insulin serum level

The effect of cinnamon extract on the BMI and the serum insulin level were summarized in TABLE 2. There was no significantly different on the BMI observed between the placebo and the treatment group, however, slight decrease of 0.1 point was observed.

The serum insulin level increased 8.2 point, however, it was not significantly different. The serum insulin was decreased in 8 subjects received placebo (33%) and increased in the rest of the subjects. Conversely, cinnamon extract decreased the serum insulin level in 15 subjects (63%). Briefly, the serum insulin level was significantly higher on day 1 (19.4 ± 14.3 mg/dL) than on day 56 (16.6 ± 16.4 mg/dL) in the treatment group ($p<0.05$). No significantly different in plasma cholesterol and triglyceride levels of the both group on day 1 and on day 56 were observed ($p > 0.05$).

TABLE 2. Effect of cinnamon extract on BMI, serum insulin level, plasma cholesterol and triglyceride levels after 56 days of treatment

Variable	Placebo		Treatment		p
	Day 1	Day 56	Day 1	Day 56	
Body Mass Index (kg/m^2)	28.9 ± 4.8	28.9 ± 4.9	28.9 ± 5.0	28.8 ± 4.7	0.94
Insulin (mg/dL)	23.6 ± 22.2	31.8 ± 27.3	19.4 ± 14.3	16.6 ± 16.4	0.02
Cholesterol (mg/dL)	164 ± 41	169 ± 18	171 ± 27	165 ± 14	0.47
Triglyceride (mg/dL)	121 ± 70	135 ± 84	111 ± 66	109 ± 76	0.26

DISCUSSION

The salient finding of our research are: (1) cinnamon extract decreased the level of insulin serum in 63% subjects and (2) cinnamon extract decreased significantly the level of insulin serum in 56 days compared to the placebo. In the obese state, insulin may elevate as a compensatory mechanism due to chronic hyperglycemia. Additionally, the chronic elevation of insulin serum level may decrease the insulin responsiveness in the tissues and lead to insulin resistance.[6] Chronic hyperglycemia and hyperinsulinemia work reciprocally and worsen the obese state, resulting in a vicious cycle between obesity, insulin resistance and hyperinsulinemia.[6-11] Clinical and experimental evidence have shown that attenuating hyperinsulinemia not only promote weight loss but also provide protection against obesity.[6,12,13]

Cinnamon has been widely used as spices, herb and traditional medicine. It has been reported that cinnamon exerts potent anti-diabetic effects through its role as insulin mimetic and insulin sensitisizing agent.[15-20] Moreover, cinnamon gives beneficial role in the type 2 diabetic patient by reducing significantly plasma blood glucose level.[24,25] We have shown in this study that cinnamon extract decreased the level of insulin serum in 63% normal obese subjects and it decreased significantly the level of insulin serum in 56 days of study. Our results were consistent with the previous finding that cinnamon component of proanthocyanidin improves the formation of pancreatic islet polypeptide and its cinnamaldehyde suppresses hyperinsulinemia condition.[21-23]

Additionally, cinnamon extract decreased the BMI of the subjects, however, it was not significantly different. Previous study has shown that routine consumption of 3 g cinnamon supplement in eight weeks reduced body weight and BMI in type 2 diabetic patient.[25] These mechanisms may be mediated through the role of cinnamon in improving the body composition and attenuating lipogenic processes in the liver and adipose tissue.[26]

Another study reported that 4 month treatment with a dietary supplement containing cinnamon, chromium and carnosine decreased fasting plasma glucose (FPG) and increased fat-free mass, however, there was no difference versus placebo with respect to body weight and BMI in overweight or obese pre-diabetic subjects.[27] We have concluded that negative result of cinnamon on the BMI in our study may be due to differences in the dose regiment and duration. We should give the proper regiment with longer treatment to evaluate the long-term effect of cinnamon in preventing the weight gain.

Some strategies have been proposed to reduce excessive body weight in the obese state by inhibiting fat absorption in the gut or suppressing appetite in the brain.[2] Recently, it is found that insulin resistance and hyperinsulinemia are the key characteristics of obesity which contributes to its further complication on health.[3-5] Consistent with the previous finding, we have shown that cinnamon may give beneficial role in obesity not only by increasing insulin sensitivity but also by attenuating hyperinsulinemia condition.

CONCLUSIONS

Cinnamon extract may give beneficial role in the normal obese subjects by suppressing the serum insulin level. Further studies are required to elucidate the specific role of cinnamon in preventing weight gain.

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Correlation between *Toxoplasma gondii* and *Cytomegalovirus* infections and somatic symptom in community

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ABSTRACT

The prevalence of *Toxoplasma gondii* and *Cytomegalovirus* (CMV) infections are high in the world. Indonesia is one of the countries with high prevalence of these infections varied from 20 to 63%. The *T. gondii* and CMV infections infections can be chronic and cause maternal and fetal deatht as well as infant defects. Previous clinical study reported that chronic infections can cause somatic symptoms indicating psychological stress. The aim of this study was to evaluate the correlation between *T. gondii* and CMV infections with somatic symptoms. This was an observational study with a cross sectional design involving 103 eligible patients with seropositive IgG *T. gondii* or and CMV from six cities in Java, Indonesia. The presence of somatic symptoms was detected by using somatic symptoms inventory (SSI) questionnaire. Logistic regression analysis was used to evaluate the correlation. The percentage of patients with somatic symptoms (SSI score >48) in seropositive groups of IgG anti-*T. gondii,* anti-CMV, anti-*T. gondii* and CMV were 70.0; 62.2 and 36.2%, respectively. In addition, the prevalence ratio (PR) for each group were 1.333, 1.178, and 0.954, respectively. No significantly different in PR was observed in this study ($p>0.05$). In conclusion, the *T. gondii* and CMV infections are not correlted with the somatic symptoms.

ABSTRAK

Prevalensi infeksi *Toxoplasma gondii* dan *Cytomegalovirus* (CMV) tinggi di dunia. Indonesia merupakan salah satu negara di dunia dengan prevalensi yang tinggi akibat infeksi *T. gondii* dan CMV ini dengan prevalensi bervariasi antara 20 sampai 60%. Infeksi *T. gondii* dan CMV dapat menjadi penyakit kronik dan menyebabkan kematian ibu dan anak serta kecacatan pada anak yang dilahirkan. Pada penelitian klinik yag dilakukan sebelumnya dilaporkan infeksi kronik dapat menyebabkan gejala somatik yang mengindikasikan adanya stres psikologi. Penelitian ini bertujuan untuk mengkaji hubungan antara infeksi *T. gondii* dan CMV dengan gejala somatik. Penelitian ini merupakan penelitian observasional dengan rancangan potong lintang yang melibatkan 103 penderita dengan infeksi *T. gondii* dan CMV dari enam kota di Jawa, Indonesia. Adanya gejala somatik dideteksi menggunakan kuesionair *somatic symptoms inventory* (SSI). Analisis regresi logistik digunakan untuk mengkaji adanya hubungan infeksi *T. gondii* dan CMV dengan gejala somatik. Persentase pasien dengan gejala somatik (skor SSI >48) pada kelompok seropositif IgG anti-*T. gondii*, anti-CMV, anti-*T. gondii* dan anti-CMV berturut-turut adalah 70,0; 62.2 dan 36,2%. Selanjutnya rasio prevalensi (RP) untuk masing-masing kelompok berturut-turut adalah

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1,333; 1,178 dan 0,954. Tidak ada perbedaan bermakna dalam RP dari hasil penelitian ini ($p > 0.05$). Dapat disimpulkan infeksi *T. gondii* dan CMV tidak berhubungan dengan gejala somatik.

Keywords: *Toxoplasma gondii* - cytomegalovirus – chronic infections - somatic symptom - stress

INTRODUCTION

Toxoplasma gondii is one of the most parasites found in human that cause the disease known as toxoplasmosis. Toxoplasmoxis is present in all countrie in the world with serelogical positive rate varies between less than 10 to over 90%.[1-3] In Indonesia the toxoplasmosis prevalence also varies between 20 to 60%. Yogyakarta Special Region is the second city with highest toxoplasmosis prevalence (51%) after Surabaya, East Java (61%).[4] Toxoplasma gondii can cause birth defect. In a pregnant women with toxoplasmosis, the *T. gondii* can cross the placenta from mother to the baby with sometimes catastrophic consequences. Children born with congenital toxoplasmosis can have classical symptoms of hydrocephalus, retinochoroiditis and encephalitis.[1]

The *Cytomegalovirus* (CMV) is one of the most common opportunistic pathogens found in immunocompromised patients.[5] Smith and Rowe in 1956 and Weller in 1957 independently isolated virus strains in human blood and suggested the term *"Cytomegalovirus"* for the virus that was also found in infant urine.[6] The prevalence of CMV infection in the world in 2009 reached ≥70% for the countries with bad infection management and 50-70% for those with some blood infection management. The prevalence of CMV infection in Indonesia in 2004 reached 87.8%.[7]The chronic infection such as *T. gondii* and CMV can cause psychological stress such as depression. Markovitz *et al*.[8] reported that individuals with seropositive of *T. gondii* had more than twice as high risk of depression compared to those with seronegative. In addition Goebel *et al.*[9] reported that patients with seropositive IgG anti-CMV have higher risk of depression. The depression is associated with somatic symptoms. Somatic symptoms including anxiousness and fatigue are a common feature in patients with depression.[10] This study was conducted to evaluate the correlation between *T. gondii* and CMV infections with somatic symptom in community setting.

MATERIALS AND METHODS

Subjects

This was an observational study with cros sectional design involving people who suspected *T. gondii* and CMV infections from six cities in Java including Bogor and Bandung West Java, Yogyakarta Special Region, Semarang Central Java, Malang and Surabaya East Java. The study was conducted over a period of one month (February 6th to March 5th) in collaboration with Indonesia Aquatreat Therapy Foundation. The *T. gondii* infection was diagnosed based on IgG anti-*T. gondii* examination, whereas the CMV infection based on IgG anti-CMV examination. Protocol of the study was approved by the Medical and Health Research Ethics Committee of the Faculty of Medicine of Universitas Gadjah Mada, Yogyakarta (Number KE/FK/156/EC/2016).

Protocol of study

On the day that has been agreed, patients with *T. gondii* and CMV infetions in each city were gathered to be selected. Data of the patients were obtained from the Indonesia Aquatreat Therapy Foundation. An explanation concerning background, objectives and benefit of the study was given. Patients who met the inclusion and exclusion criteria were given an informed consent to be signed. The inclusion criteria were patients with the seropositive IgG anti *T. gondii,* and IgG anti-CMV in just examination. The exclusion criteria were the patients with more than one IgG anti *T. gondii* and IgG anti-CMV examinations or underwent treatment and the IgG anti *T. gondii* and IgG anti-CMV examinations were not standard. Selected patients were then grouped into three groups i.e. patients with just the seropositive IgG anti *T. gondii* (Group 1), patients with just the seropositive IgG anti-CMV (Group 2) and patients with both the seropositive IgG anti *T. gondii,* and IgG anti-CMV (Group 3). All selected patients were given somatic symptoms inventory-24 (SSI-24) questionnaire to be filled under supervison of the research assistants.[11] Patients without the somatic symptoms were considered if the SSI score ≤ 48 and patients with the somatic symptoms if the SSI score > 48.

Statistical analysis

Data were presented as frequency or percentage or mean ± standard deviation (SD). Logistic regression analysis was applied to calculate the prevalence ration (PR) which was used to evaluate the correlation between the *T. gondii* and CMV infections with the level of the somatic symptom. A p value less than 0.05 was considered significant.

RESULTS

The study was conducted over a period of one month (February 6th to March 5th, 2016) with 177 selected subjects and 103 eligible subjects. The characteristics of subjects were presented on TABLE 1. The mean age of subjects ranged between 32 and 33 years with most of them were females (82%). All most participants were married (98%) and graduated from university (56%). The most occupations of subjects were housewives (41%), whereas the most of subjects had income between IDR 1-3 millions. The majority of subjects came from Central Java (46%) and most of them experienced somatic symptoms at medium to high levels (SSI score > 48).

TABLE 1. Characteristics of subject in each group

Characteristics	Group 1 (n=30)	Group 2 (n=37)	Group 3 (n=36)
Age (mean ± SD years)	32.5 ± 6.0	30.5 ± 6.3	33.3 ± 7.4
Sex [n (/%)]			
• Female	27 (90)	28 (76)	28 (80)
• Male	3 (10)	9 (24)	7 (20)
Marital status [n (/%)]			
• Married	29 (97)	36 (97)	36 (100)
• Non-married	1 (3)	1 (3)	0
Education [n (/%)]			
• Postgraduate	0	0	1 (3)
• Graduate	17 (57)	22 (60)	19 (52)
• Undergraduate	4 (13)	0	1 (3)
• Senior high school	7 (23)	13 (34)	15 (42)
• Junior high school	2 (7)	1 (3)	0
• Elementary school	0	1 (3)	0
Occupation [n (/%)]			
• Government employee	7 (23)	16 (43)	13 (37)
• Entrepreneurs	2 (7)	7 (19)	7 (18)
• Housewives	16 (54)	13 (35)	13 (37)
• Educators	4 (13)	1 (3)	2 (6)
• Health professionals	1 (3)	0	1 (3)
Income (IDR) [n (/%)]			
• > 12 million	2 (7)	1 (3)	0
• 8-12 million	1 (3)	2 (5)	2 (5)
• 5->8 million	2 (7)	1 (3)	2 (5)
• 3->5 million	6 (20)	12 (32)	10 (28)
• 1->3 million	15 (50)	18 (49)	19 (53)
• <1 million	4 (15)	3 (8)	3 (9)
Domicile [n (/%)]			
• West Java	14 (47)	14 (38)	10 (30)
• Central Java	8 (27)	17 (46)	23 (64)
• East Java	8 (27)	6 (16)	2 (6)
Somatic symptoms factor [n(/%)]			
• SSI score ≤ 48	9 (30)	14 (38)	23 (64)
• SSI score > 48	21 (70)	23 (62)	13 (36)

The PR of somatic symptoms in each group was presented in TABLE 2. No significantly different in PR of somatic symptoms was observed in each group ($p>0.05$). It was indicated that the IgG anti-*T. gondii* levels (Group 1) and IgG anti-CMV levels (Group 2) or both (Group 3) were not correlated with somatic symptoms.

TABLE 2. The PR of somatic symptoms in each group

Characteristic	Group 1		Group 2		Group 3	
	Prevalence Ratio (95% CI)	p	Prevalence Ratio (95% CI)	p	Prevalence Ratio (95% CI)	p
IgG Level						
• Medium to High	1.333 (0.410)	0.625	1.178 (0.419)	0.748	0.954 (0.411)	0.916
• Low	1.000 (Reference)	-	1.000 (Reference)	-	1.00 (Reference)	-
Sex						
• Female	0.888 (0.161)	0.894	1.928 (0.528)	0.266	0.868 (0.503)	0.643
• Male	1.000 (Reference)	-	1.000 (Reference)	-	1.000 (Reference)	-
Age						
• Elderly	-	-	-	-	-	-
• Adult	0.592 (0.131)	0.495	0.937 (0.293)	0.913	-	-
• Adolescent	-	-	-	-	-	-
Domicile						
• East Java	0.437 (0.585)	0.058	1.921 (0.606)	0.267	1.024 (0.598)	0.929
• Central Java	1.750 (0.594)	0.594	3.111 (0.983)	0.053	0.785 (0.183)	0.745
• West Java	1.000 (Reference)	-	1.000 (Reference)	-	1.000 (Reference)	-
Marital status						
• Married	-	-	0.361 (0.233)	0.193	-	-
• Non-married	-	-	-	-	-	-
Education						
• High	0.857 (0.272)	0.558	0.511 (0.222)	0.108	0.928 (0.568)	0.525
• Low to medium	1.000 (Reference)	-	1.000 (Reference)	-	1.000 (Reference)	-
Occupation						
• Working	1.428 (0.474)	0.522	0.722 (0.319)	0.442	1.291 (0.729)	0.345
• Not Working	1.000 (Reference)	-	1.000 (Reference)	-	-	-
Income						
• High	2.5 (0.920)	-	0.108 (0.110)	0.575	0.761 (0.277)	0.539
• Low to Medium	1.000 (Reference)	-	1.000 (Reference)	-	1.000 (Reference)	-

*Significant at p value < 0.05

DISCUSSION

Sociodemographic factors

The mean age of the sujects of the three groups was similar in the range of 30-33 years old. The age of the sujects did not affect the emergence of somatic symptoms in all subjects. The effect of age in emotional responses to stress remains unclear. Previous studies showed that the age of subjects affect the emotional response to stress. The older subjects were more susceptible to stress compared to younger subjects.[9,10] However, another study reported that younger people (20-44 years old) experienced higher stres9 and depression compared to older people (>44 years old).[12]

In contrast to the age, the sex was significantly correlated with the emergence of somatic symptoms in this study. Females and males were more likely to express different reactions to stress both psychologically and biologically.[13] Furthermmore, it was reported that female were more likely to suffer under depression compared to male.[12,14,15] The

marital status did not affect the somatic symptoms in this study. This was due to 98% of the subjects involved in this study were married. Previous studies reported that married individuals were at higher risk of somatic symptoms compared to those who have not married on the basis of risk ratio (RR).[12,16-18]The education, occupation, and income did not affect the somatic symptoms in this study. Previous studies showed that subjects with higher education level havehigher depression risk.[12] In contrast, another study reported that subjects with lower education levels experienced somatic symptoms 1.36 higher than those with higher education levels.[18]

The domicile also did not affect the somatic symptoms in this study. Domicile represents a place for people to permanently live for a certain period of time. It describes the environment and way of thinking of the people where they live that affect their response to stress. The depression was more likely experienced by people who live in a metropolitan. The more modern of socialcultural of people, they were more susceptible to the emergence of somatic symptoms due to the presence of psychological stress and depression.[14]

The correlation of seropositive IgG level and somatic symptom level

No correlation between the IgG anti-*T. gondii* and anti-CMV and somatic symptoms was observed in this study. The correlation between chronic diseases and somatic symptoms or stress has been investigated previously with different results. Leavens *et al.*[19] reported that there is no different of somatic symptoms among patients with systemic sclerosis comapared to healthy people. In contrast, Glise *et al*.[18] reported that there is correlation between patients with exhaustion disorders and somatic symptoms. In addition, the prevalence of somatic symptoms increased in headache patients or cancer patients.[20,21]

Recent study showed that the sychological factors are associated to individuals' immune system. Stressful conditions could cause inflammation and activation of hypothalamic-pituitary-adrenal (HPA) that induce adreno-cortico-tropic hormone (ACTH) to release stress hormone cortisol.[18] The cortisol could inhibit the circulation of leukocyte cells in the blood from locations of inflammation.[19] In addition, the cortisol hormone could block immunoglobulin or antibody synthesis, which are necessary in humoral immunity response. The cortisol could also trigger lymphocyte network atrophy in the thymus, spleen, and spleen glands.[22-24] In addition to immunological effects, it also could influence human behavior and emotion such as being easily irritated and depressed.[19]

Stress could decrease the number and the function of lymphocyte T cells (CD4+, CD8+) and cytokine IL-2 that cause the lymphocyte T cells more tolerant to infection in inflammation reactions.[23,25]

CONCLUSION

In conclusion, the percentage of the patients with *T. gondii* and CMV infections who suffer somatic symptoms in six big cities in Java is high (54.4%) with the PR vary from 0.954 to 1.333. However, it is not correlated with the *T. gondii* and CMV infections. The different laboratory in the IgG anti-*T. gondii* and anti-CMV examinations may contribute in the variation of results of the IgG examination.

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Sepsis risk factor in mount Merapi eruption victims with 2nd or 3rd degree of burn injury

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ABSTRACT

Sepsis is one of the fatal complications of burns. It is the most common cause of death from burns. Burn sepsis usually occurs after a burn injury develops into infection. This study was conducted to determine correlation between age, burn area, inhalation trauma, enteral nutrition start time, escarotomy time, and albumin level of sepsis in patients with 2nd or 3rd degree burn injury. This was a cross sectional study during May to June 2012. The subjects in this study were 39 victims of mount Merapi eruption in 2010 who suffered 2nd or 3rd degree of burns injury. The result showed the correlation between widespread burns, inhalation trauma, time of escarotomy, and albumin levels with sepsis ($p < 0.05$). In conclusion, the risk factor for sepsis in patients with 2nd or 3rd degree of burns injury are the area of burns (> 50%), inhalation trauma, time of escarotomy (> 72 hours), and albumin levels (<3.5g/dL).

ABSTRAK

Sepsis merupakan salah satu komplikasi fatal dari luka bakar. Sepsis menjadi penyebab kematian tersering dari luka bakar yang muncul setelah pasien mengalami infeksi. Peneitian ini dilakukan untuk mengkaji hubungan antara usia, luas luka bakar, trauma inhalasi, waktu memulai pemberian nutrisi enteral, waktu melakukan eskarotomi, dan kadar albumin dengan sepsis pada pasien luka bakar derajat 2 dan 3. Penelitian ini merupakan penelitian potong intang yang dilakukan pada bulan Mei sampai Juni 2012. Subjek penelitian adalah 39 korban erupsi Gunung Merapi tahun 2010 yang mengalami luka bakar derajat 2 atau 3. Hasil penelitian menunjukkan adanya hubungan antara luas luka bakar, trauma inhalasi, waktu melakukan eskarotomi, dan kadar albumin dengan sepsis ($p<0.05$). Dapat disimpulkan faktor risiko sepsis pasien luka bakar derajat 2 atau 3 adalah luas luka bakar (>50%), trauma inhalasi, waktu melakukan eskarotomi (>72 jam), dan kadar albumin (<3.5g/dL).

Keywords: sepsis - risk factor - merapi eruption - 2nd degree burn injury - 3rd degree burn injury

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INTRODUCTION

Burns are a serious cause of trauma, which causes severe morbidity and severe mortality, and has considerable economic impact. The cost of treatment of burns is very expensive due to length of stay and rehabilitation. In addition, the treatment of wounds or scars due to the burns require special handling from the beginning to the end.[1,2]

In the United States, more than 1.2 million people suffer burn injury each year, most cases are minor burn injury which only requires outpatient, however, almost 100,000 are moderate to severe and requires hospitalization, and more than 5000 die due to burn injury complications.[1] In developing countries, the incidence of burn injury is of 4-5 times the incidence in the United States. Women are considered the high-risk group for burns due to household activities such as cooking while wearing traditional flammable clothing.[3] In Indonesia, comprehensive studies on the incidence of burn injury have been conducted, yet.

Progress in the treatment of burn injury in the last three decades have led to success in reducing mortality. It also has changed the most common cause of death in burn injury. Shock due to burn injury typically occurs in burn injury of more than 20%. It was the most common cause of death between the years of 1930-1940. Experience and knowledge of resuscitation have been able to overcome these complications and reduce mortality.[4] Many deaths in burn injury can be prevented with proper airway management and adequate fluid management, however, in severe burn injury, the main cause of mortality that continue to be faced is sepsis.[5]

The number of deaths caused by sepsis in Indonesia has not much been published, yet. Based on the data in the Burn Unit of Dr. Cipto Mangunkusumo General Hospital, Jakarta during 1999-2000, burn injury with systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) caused 90% of death.[2] Although the pathophysiology of burn injury and clinical factors that predispose severe sepsis and septic shock on burn injury patients have been well understood, accurate prediction of the infection complications risk on burn injury patients has not been available, yet.[6] Several factors are reported as cause of infection and sepsis in burn injury such as age, percentage of burn injury, inhalation trauma, delay in the escharotomy and wound closing, delay in enteral feeding, diabetes mellitus, and albumin levels.[1-4,7-14]

Volcanic eruptions can cause burn injury due to the volcanic gases ejection containing harmful gas to human, such as carbon monoxide (CO), carbon dioxide (CO_2), hydrogen sulfide (H_2S), sulfur dioxide (SO_2), and nitrogen (NO_2). Most deaths in the volcanic eruptions are caused by pyroclastic flows and surges (burning clouds/*nuees ardentes*) and wet debris flows (*lahars*). Thermal injury may be the cause of asphyxia following inhalation trauma. The high temperature of the gases and entrained particles cause severe burn injury to the skin and the air passage. The presence of both types of injury in a victim may increase the delayed mortality risk from respiratory complications or from infection of burn injury.[15]

Factors that cause sepsis in burn injury due to volcanic eruptions may be similar to those in burn injury in general. However, these factors have not much been investigated, yet. This study was conducted to investigate the sepsis risk factors in mount Merapi eruption victims with 2nd or 3rd degree burns injury.

MATERIAL AND METHODS

Subjects

This was a cross-sectional study to investigate the risk factors of sepsis in patients with 2nd and 3rd degree burn injury due to mount Merapi eruption in 2010. The data of the patients were collected from the Medical Records Department (IRM), Dr. Sardjito General Hospital, Yogyakarta within the period of May to June 2012. Population in this study were all patients with 2nd or 3rd degree burn injury. This study used a total sampling method where all population was taken as samples except those who did not meet the inclusion and exclusion criteria.

Procedure

Data of the factors of sepsis such as age, burn percentage, inhalation trauma, time for starting enteral feeding, time of escharotomy being performed, albumin level were collected from medical record. Data of patients with sepsis and the outcome were also obtained from the medical record. The data collection was conducted after ethical clearance obtained from the Medical and Health Research Ethics Committee, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta.

Statistical analysis

Data of risk factors of sepsis were presented as frequency or percentage. The correlation between the risk factors and the occurrence of sepsis was analyzed using Chi-square test. A p value < 0.05 was considered as significant.

RESULTS

Over a period of mount Merapi eruption in 2010, 39 patients with burn injury were treated at the Department of Surgery, Faculty of Medicine, Universitas Gadjah Mada /Dr. Sardjito General Hospital, Yogyakarta. Most patients were male (69.2%) and between the ages of 19-60 years old (74.4%). They had <50% (38.5%) of burn percentage and inhalation trauma (87.2%). They were given enteral feeding <24 hours (71.8%) and performed escharotomy >24 hours (82.1%). They had albumin level of <3.5g/dL (89.7%). Thirty three patients had sepsis (82.1%), and 29 patients died (74.4%). The characteristics of patients are presented in TABLE 1.

TABLE 1. Baseline characteristics of subjects

Characteristics	Frequency	Percentage (%)
Age (years)		
< 5	0	0
5 – 18	7	17.9
19– 60	27	69.2
> 60	5	12.8
Sex		
Male	27	69.2
Female	12	30.8
Burn percentage		
< 50%	15	38.5
> 50%	24	61.5
Inhalation trauma		
Yes	34	87.2
No	5	12.8
Time starting enteral feeding (hours)		
< 24	28	71.8
> 24	11	28.2
Time of escharotomy being performed (hours)		
< 72	7	17.9
> 72	32	82.1
Albumin level		
< 3.5g/dL	35	89.7
> 3.5g/dL	4	10.3
Sepsis		
Yes	32	82.1
No	7	17.9
Outcome		
Death	10	25.6
Alive	29	74.4

The correlation between the risk factors and the occurrence of sepsis is presented in TABLE 2. No correlation between age in general and sepsis (p=0.388) as well as between high risk age group (>60 years) and sepsis (p=0.169; PR=0.259; CI 95%=0.034-1.960) were observed. However, a significant correlation between burn percentage (>50%) and sepsis, as well as between inhalation trauma and sepsis (p=0.000; PR=41.33; CI 95%=3.423-499.146) were observed. A significant correlation between time of escharotomy performed and sepsis (p=0.003; PR=12.889; CI 95%=1.906-87.170), between time for starting enteral feeding and sepsis (p=0.067; PR=1.333; CI 95%=1.077-1.651), as well as between albumin level and sepsis (p=0.002; PR=23.250; CI 95%=1.925-280.770) were also oberved.

TABLE 2. Correlation between risk factors of sepsis and the occurrence of sepsis

Characteristics	Sepsis		P	PR (CI 95%)
	Yes	No		
Age (years)				
< 5	0	0	0.388	1.7268 (-0.0769–0.9259)
5 – 18	6	1		
19 – 60	23	4		
> 60	3	2		
Age group (years)				
< 60	3	2	0.169	0.259 (0.034-1960)
> 60	29	5		
Burn percentage (%)				
< 50	10	5	0.048	5.500 (0.907-33.345)
>50	22	2		
Inhalation trauma				
Yes	31	3	0.000	41.333 (3.423-499.146)
No	1	4		
Time of escharotomy being performed (hours)				
< 72	3	4	0.003	12.889 (1.906-87.170)
>72	29	3		
Time for starting enteral feeding (hours)				
< 24	21	7	0.037	1.333 (1.077-1.651)
>24	11	0		
Albumin level				
< 3.5 g/dl	31	4	0.002	23.250 (1.925-280.770)
>3/5	1	3		

DISCUSSION

This study showed that the average age of the patients was 39 years, with the most came from the age group of 19-60 years (69.7%), while there were only 5 patients came from the age group of >60 years (12.8%). The age group of >60 years is considered as a high-risk age group associated with the decline in cellular and humoral immunity.[7,16] Results of this study is different from other studies which

showed that age is not correlated with sepsis (p=0.338), whereas other studies revealed that age is correlated significantly correlated with sepsis, where the age group that has the higher risk is the age group >60 years.[8,17] This difference is probably caused by the limited number of samples that could be examined in this study.

This study also showed that most patients had >50% burn percentage (61.5%) that was caused by smoke and hot lava from Merapi eruption. We demonstrated that burn percentage was correlated with sepsis, therefore burn percentage is a risk factor in burn sepsis. Previous studies reported a significant correlation between higher burn percentage and sepsis and severe sepsis.[8,18] Therefore, this study is consistent with the findings of other studies.

The presence of inhalation trauma has a strong correlation with infections, especially pneumonia. It is generally agreed that inhalation trauma increases the risk of pneumonia. The inflammatory process due to inhalation trauma causes damage to the ciliary mucosal and airway epithelium. This result in a disruption of the cleaning process, the breakdown of the defense system in the respiratory tract, resulting in an increased risk of infection by bacteria in a couple of days and weeks.[1,4,7] The incidence of inhalation trauma reported at 0.3-43% in severe burn injury and 13-18% in elderly patients with burn injury.[17]

A study in Egypt reported 46.3% cases of inhalation trauma in all burn injury patients during 2008-2010.[19] In this study, the number of patient who suffered from inhalation trauma was 87.2%, in accordance with the possible cause of inhalation trauma which were the smoke and hot lava from Merapi eruption that contained harmful elements. From all patients who suffered from inhalation trauma, 91.17% developed sepsis. We demonstrated the correlation between inhalation trauma and sepsis (p<0.05), consequently, inhalation trauma is a risk factor for sepsis. A research that was conducted in 2009 found that from 47% death cases due to burn sepsis, 79% of them had inhalation trauma. Another study demonstrated a strong correlation between inhalation trauma and burn sepsis.[8]

Early enteral feeding (<24 hours) in patients with burn injury, lower the risk of sepsis. Early enteral feeding will maintain gastroduodenal mucosal villous function and prevent the translocation of microorganisms (bacteria, fungi) from the gastrointestinal tract.[20,21] In this study, the majority of the patients (71.8%) got early enteral feeding (<24 hours), which is adapted to the condition of the patient. However, in this study, we revealed no correlation between early enteral feeding (<24 hours) and sepsis. This finding is difference with previous studies which demonstrated significant correlation between early enteral feeding and sepsis. Those studies show that late enteral feeding is a risk factor for sepsis.[12,20] This difference is likely due to small sample size who received early enteral feeding (<24 hours) in this study, so it did not provide meaningful correlation.

Delay in performing escharotomy or debridement of the necrotic tissue lead to development of microorganism wich than result in sepsis. Skin layers demage become the entry site of the microorganisms into the deeper skin layers and systemic circulation.[1,7] In this study, there were 32 (82.1%) patients who underwent escharotomy in >72 hours and 29 (90.6%) of them developed sepsis. We analyzed the correlation between the delay in performing escharotomy and sepsis, in which we conclude that the delay in performing escharotomy correlates significantly with sepsis (p=0.03). However, other studies show different results. A study conducted by Xiao

et al shows significant correlation between the delay in performing escharotomy and sepsis, while the opposite result shows in a study conducted by Ong.[10,11]

Albumin is needed in the body's defense system, especially in the humoral response. In patients with burn injury, low albumin levels (hypoalbuminemia) increase the risk of further infection that could develop into sepsis.[14] In this study, there were 89.7% patients had albumin level <3.5g/dl, and 90.6% of them developed sepsis. We analyzed the correlation between low albumin level and sepsis and we found that low albumin level correlated with sepsis. It can be concluded that low albumin level is a risk factor for burn sepsis.

The mortality rate in the victims of mount Merapi eruption in this study reached 74.4%, while the mortality rate in patients who developed sepsis was 81.3%. This result is higher than a study by William in 2009, where the study reported the mortality rate of burn sepsis is 47%. Other study reported the mortality rate of burn sepsis is 2-14%.[17,22]

CONCLUSION

Burn percentage (>50%), inhalation trauma, time of escharotomy being performed (>72 hours), and albumin level (<3.5g/dL) are the risk factors of burn sepsis in patients with 2nd or 3rd degree burn injury.

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Effect of exercise on lipid peroxidation in student soccer players

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ABSTRACT

Training is conducted to improve physiological functions that can support improvement of cardio-respiratory function ($VO2$max). However, intensive training can lead to oxidative stress, which can contribute to health problems. The purpose of this study was to evaluate the effect of training on serum lipid peroxidation levels in student soccer players. The study was pre-experimental study with a one-shot case design conducted in April 2014. Twelve student soccer players from UGM who chosen by purposive sampling and met the inclusion and exclusion criteria were involved in the study. Each subject received training in the form of $VO2$max measurements using the yo-yo intermittent recovery test. Plasma malondialdehyde (MDA) levels were measured using the thiobarbituric acid method 30 min after $VO2$max measurement. Pearson correlation was used to analyze the correlation between $VO2$max and plasma MDA levels. The mean age of subject was 19.25 ± 1.06 years old. Subjects had normal nutritional status (body mass index 20.99 ± 1.65) with mean body weight of 58.13 ± 3.76 kg and mean height of 166.2 ± 3.40 cm. The mean $VO2$max score was 49.56 ± 0.61 mL/kg/min. The mean plasma MDA level was 4.32 ± 2.09 μmol/L. There was no significant correlation between $VO2$max and plasma MDA levels (p = 0.7717). In conclusion, training does not negatively impact oxidative stress conditions in student soccer players.

ABSTRAK

Latihan dilakukan untuk meningkatkan fungsi fisiologi yang dapat memperbaiki funsi kardio-respirasi ($VO2$max). Namun demikian, latihan yang berlebihan dapat menyebabkan stres oksidatif yang dapat menimbulkan gangguan kesehatan. Penelitian ini bertujuan untuk mengkaji efek latihan terhadap peroksidasi lipid serum pada pemain sepak bola mahasiswa. Penelitian ini adalah penelitian pendahuluan dengan rancangan kasul bentuk tunggal dilakukan pada April 2014. Dua belas pemain sepak bola mahasiswa dari UGM dipilih dengan teknik purposive sampling sesuai kriteria inklusi dan eksklusi dilibatkan dalam penelitian. Setiap subjek menjalani latihan untuk ditetapkan $VO2$max nya dengan uji yo-yo intermittent recovery. Kadar malondialdehid (MDA) plasma diukur dengan metode asam tiobarbiturat 30 menit setelah pengukuran $VO2$max. Uji korelasi Pearson digunakan untuk menganalisis hubungan antara $VO2$max dan kadar MDA plasma. Rerata umur subjek adalah 19.25 ± 1.06 tahun. Subjek mempunyai status gizi normal (indeks masa tubuh 20.99 ± 1.65) dengan rerata berat badan 58.13 ± 3.76 kg dan tinggi badan 166.20

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± 3.40 cm. Rerata skor *VO2*max adalah 49.56 ± 0.61 mL/kg/menit. Rerata kadar MDA plasma adalah 4.32 ± 2.09 μmol/L. Tidak ada korelasi nyata antara *VO*2max dan kadar MDA plasma. Dapat disimpulkan, pelatihan tidak menyebabkan stress oksidatif yang merugikan pada pemaian sepak bola mahasiswa.

Keywords: soccer – exercise - *VO*2max - lipid peroxidation – malondialdehyde

INTRODUCTION

Physical exercise is beneficial to the body, particularly for physical and psychological health and social wellbeing. Additionally, physical exercise can delay the aging process. There is an increase in the sympathetic nerve response at the beginning of physical exercise. Next, the cardiovascular system adaptation is adjusted, resulting in an increase in cardiac rate, cardiac force, and arterial pressure. There is also an increase in metabolism, blood glucose concentrations, and glycolysis in the liver and muscles. Furthermore, the respiratory system adapts. These factors contribute to good athletic performance.[1] For example, study conducted on a male athlete who did a solo ultra-endurance open-water swim showed that heart rate increased by 41.8% and salivary alpha-amylase (sAA) levels increased by 102.6% after athlete completed swim 78.1 km in 23 h 44 min. It confirmed that the autonomic drives depend upon exercise efforts. Indeed, exercise by doing ultra-endurance swim differently influenced cardiac function by both adaptive autonomic and non-autonomic patterns.[2] An additional study in seven healthy adult male sport-parachutists demonstrated that exercise done in the form of parachute jumping led to a strong response of salivary cortisol, α-amylase, and heart rate. There was an increase of salivary cortisol and α-amylase levels and heart rate at 12 h before jumping (basal) and within 60 s (jump).[3]

Soccer is a sport that requires strength and cardiorespiratory endurance, because the distances covered during a game range from 9,800 to 11,500 m.[4-5] Soccer is accompanied by strong and explosive activities, such as jogging, sprinting, jumping, tackling, and heading and kicking the ball.[4,6] Therefore, exercise is important to increasing the cardiorespiratory response of soccer players. One study indicated that to significantly improve achievement, athletes must increase their performance, because performance is one of the primary determinants of a win. Athletes receive training and exercise to improve their performance. Not only do athletes receive training focused on techniques and tactics but also to improve physical fitness.[4]

Cardio-respiratory function (*V*O2max) measurements can represent physical fitness. *V*O2max is an upper limit measurement of the ability of the body to consume, distribute, and use oxygen. *V*O2max is defined as the integrated physiological function of the lungs, heart, blood, and muscles.[7] The average endurance score of soccer players belonging to Gelora Remaja Football Club, Division I Football of Football Association of Pasuruan District (Persekabas) illustrated by VO_2max measurement using the multistage shuttle run test was 38.13 mL/kg/minute.[8] The average endurance score of soccer players belonging to Football Association of Kudus District, Central Java, Indonesia measured by *V*O2max using the cooper test was 50 mL/kg/minute.[9]

Study conducted by Luhtanen *et al.*[10] reported that the cardiorespiratory function of young soccer player measured by *V*O2max test on a treadmill starting at a speed of 9 km/h and increasing speed by 1 km/h after every three minutes was 53.5 mL/kg/min. Another study reported that mean values for an oxygen consumption of non-elite university-level Gaelic footballers ranged from 54.77 to 65.31 mL/kg/min for soloing with the ball and 52 to 63.63 mL/kg/minute for running without the ball.[11] Hence, training and exercise are needed to increase the cardiorespiratory response (*V*O2max) from soccer players. Previous study demonstrated that weekly 30 min session of intermittent high-intensity drills could increase physical performance of high-level soccer players in the competitive season measured by *V*O2max using an incremental treadmill test. The *V*O2max after training and exercise period (62.2 mL/kg/min) was higher than *V*O2max before training and exercise period (59 mL/kg/min).[12]

Although exercise confers numerous benefits, rigorous physical exercise can cause oxidative stress.[1,13-15] Oxidative stress is a condition in which there is an imbalance between free radicals and antioxidants.[16] Study conducted on nine futsal athletes showed that significantly 15 min after exercise there was an increasing of MDA levels—an indicator of oxidative stress.[1] Study conducted by Souza *et al.*[14] reported that exercise with treadmill running increased plasmatic MDA concentration during and after its performance. Another study in 24 physical education students indicated that concentration of MDA as oxidative stress indices significantly increased at immediately and 24 h after aerobic exercise.[15] One study determined that aerobic exercise with moderate intensity caused an increase in oxygen consumption that triggered free radical formation by mitochondria. Free radical formation can harm cells, proteins, DNA, and other cellular components through the oxidation process.[17]

Exercise can produce an imbalance between reactive oxygen species (ROS) and antioxidants, commonly known as oxidative stress. Physical exercise increases free radical production through multiple pathways. Of the oxygen used in the mitochondria, 2–5% produces free radicals. Increased oxidative phosphorylation results in increased free radical production. If the rise in free radicals is greater than the ability of the body to neutralize it, the free radicals will attack cell components and particularly body fat. An attack on body fat is commonly known as lipid peroxidation, which can further increase free radicals.[16]

A frequently used indicator of lipid peroxidation is MDA.[13,16] Serum MDA levels can significantly increase under high oxidative stress conditions.[16] Thus, it is critical to better understand the correlation between exercise, as assessed by *V*O2max measurements, and MDA levels. Several studies investigating professional athletes have demonstrated that rigorous physical exercise can cause oxidative stress, although these findings are controversial. In this study, we assessed the effect of exercise, using yo-yo intermittent recovery test, on serum lipid peroxidation levels in student soccer players.

MATERIALS AND METHODS

Subjects

This study was pre-experimental study with a one-shot case design or a one-group post only design. This study examined the effect of exercise, as assessed by *V*O2max measurements, on plasma MDA levels in student soccer players conducted in April 2014. Twelve student soccer players from

UGM who lived in Yogyakarta Special Region and chosen by purposive sampling as well as met the inclusion and exclusion criteria were involved in the study. The inclusion criteria for subjects were willingness to participate in the study, male, age ≥ 18 years, non-smokers, not taking supplements during the study, and having lived in the study area for at least 6 months. Exclusion criteria included injury, fracture, or treatment during the study.

Protocol of the study

Prior to the study subjects received an explanation concerning the background, objectives and benefit of the study. Subjects were given an informed consent to be signed. Characteristics of subjects including age, weight, height, body mass index (BMI), educational level, and position on the team were recorded. Each subject then received training in the form of *V*O2max measurements at the Stadium of Universitas Negeri Yogyakarta (UNY) using the yo-yo intermittent recovery test followed by exercise until the subject reached 80% of their maximum heart rate. In yo-yo intermittent recovery test, subjects must start on or behind the line, and began running 20 m when instructed by the recorder. Then, subject turned and returned to the starting point when signaled by the recorded beep. A warning was given when the subject did not complete a successful out and back shuttle in the allocated time. Subject was removed the next time when subject did not complete a successful shuttle. The type of exercise performed by the subjects was anaerobic endurance with fartlek training, which included walking, jogging, and sprinting. Subjects then cooled down for five min. The equation used to calculate the *V*O2max.[18] *V*O2max (mL/min/kg) = [yo-yo intermittent recovery test distance (m) × 0.0136] + 45.3.

Thirty min after *V*O2max measurements followed by exercise (anaerobic endurance with fartlek type), blood samples were collected and plasma MDA levels were measure spectrophotometrically using the thiobarbituric acid method at Laboratory of Biochemistry, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta. The day prior to analysis, study subjects received instructions through a short messages service. Specifically, the subjects were instructed not to do heavy activities, such as running or other exercise, to sleep at least 6–8 h, and to not drink caffeinated drinks, alcohol, or vitamin-mineral drinks. A 24-h food recall form, food record form, and questionnaire of sleep quality index were used to correct for the subject's condition and the subject's restraint. The protocol of the study has been approved by the Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta (KE/FK/257/EC).

Statistical analysis

Data were analyzed using the StataIC 12 statistical program, and the Shapiro-Wilk test was used to analyze data distribution. Pearson correlation coefficient was used to analyze the correlation between exercise (*V*O2max) and plasma MDA levels as well as between subject's restraint focused on food consumption (energy, fat, and carbohydrate intake) and plasma MDA levels. Spearman correlation was used to analyze the correlation between protein, vitamin A, carotene, vitamin E, and vitamin C intake and plasma MDA levels. A p-value ≤ 0.05 was considered significant.

RESULTS

The characteristics of subjects are presented in TABLE 1, whereas the *V*O2max scores and plasma MDA levels are presented in TABLE 2.

TABLE 1. Characteristics of subjects

Characteristics	Mean ± SD/ N (%)
Age (years)	19.25 ± 1.06
Weight (kg)	58.13 ± 3.76
Height (cm)	166.52 ± 3.40
BMI (kg/m^2)	20.99 ± 1.65
Educational level	
Diploma degree	5 (41.67%)
Bachelor degree	7 (58.33%)
Position on the team	
Backs	6 (50%)
Midfielders	5 (41.67%)
Goal Keeper	1 (8.33%)

The backs and the midfielders showed comparable *V*O2max scores and plasma MDA levels, while the goalkeeper showed the lowest *V*O2max score and plasma MDA level (TABLE 2). Moreover, the mean *V*O2max score was 49.56 mL/kg/min, and the mean plsma MDA level was 4.32 µmol/L. No significant correlation between *V*O2max and plasma MDA levels was observed (p=0.7717) (TABLE 2).

Other variables can also affect plasma MDA levels in student soccer players. Based on the subject questionnaires, seven subjects performed strenuous exercise, four subjects consumed caffeinated drinks, one subject consumed alcoholic drinks, and three subjects consumed supplements the day prior to the study. Additionally, four subjects did not sleep at least 6–8 hours. The 24-h food recall and food record forms were used to correct for food and drink consumption one day prior to the study. Statistical analysis of subject restraint demonstrated that there was no correlation between food consumption and plasma MDA levels (TABLE 3).

TABLE 2. *V*O2max and plasma MDA levels (mean ± SD) by team position and its correlation

Team position	N (%)	*V*O2max (mL/kg/min)	MDA (µmol/L)	Correlation	p*
Backs	6 (50%)	49.83 ± 0.44	4.50 ± 1.93	-0.0939	0.7717
Midfielders	5 (41.67%)	49.43 ± 0.62	4.60 ± 2.37		
Goalkeeper	1 (8.33%)	48.56 ± 0.00	1.77 ± 0.00		
Total	12 (100%)	49.56 ± 0.61	4.32 ± 2.09		

*V*O2max: Cardio-respiratory function; MDA:Malondialdehyde; *Pearson correlation

TABLE 3. The subject restraint

Subject restraint	N (%)	MDA (mean ± SD µmol/L)	p
Physical activity			
Active	7 (58.33%)	4.15 ± 2.11	0.769
Non-active	5 (41.67%)	4.54 ± 2.29	
Caffeinated drink consumption			
Yes	4 (33.33%)	3.11 ± 1.17	0.167
No	8 (66.67%)	4.92 ± 2.25	
Alcohol consumption			
Yes	1 (8.33%)	7 ± 0.00	0.192
No	11 (91.67%)	4.07 ± 2.01	
Supplement consumption			
Yes	3 (25%)	3.98 ± 2.99	0.763
No	9 (75%)	4.43 ± 1.93	
Sleep quality			
Good	1 (8.33%)	7.42 ± 0.00	0.111
Poor	11 (91.67%)	4.03 ± 1.94	
Food consumption			
Energy (kcal)	2197.97 ± 453.96[a]	4.32 ± 2.09	0.326[c]
Protein (g)	45.55 (36.2 – 85.9)[b]		0.430[d]
Fat (g)	53.43 ± 17.78[a]		0.568[c]
Carbohydrate (g)	380.63 ± 88.98[a]		0.270[c]
Vitamin A (µg)	31.5 (0 – 210)[b]		0.197[d]
Carotene (mg)	763.75 (62.4–28680)[b]		0.713[d]
Vitamin E (mg)	0 (0 – 0.9)[b]		0.187[d]
Vitamin C (mg)	21.15 (0 – 643)[b]		0.527[d]

MDA: malondialdehyde; [a]mean ± SD; [b]median (min-max); [c]Pearson correlation; [d]Spearman correlation

DISCUSSION

We demonstrated that the backs and the midfielders had comparable *V*O2max scores and MDA levels, while the goalkeeper had the lowest *V*O2max score and MDA level (TABLE 2). Previous studies reported the little difference between positions in the distances covered by soccer players. However, it was reported that fullbacks sprinted more than twice as much as central-defenders (2.5 times longer), while midfielders and attackers sprinted significantly more than central-defenders (1.6–1.7 times longer). Moreover, fullbacks and attackers sprinted significantly longer than central-backs and midfielders.[4] Different distances covered by athletes result in different *V*O2max scores and plasma MDA levels. This study investigated *V*O2max score and plasma MDA level differences in the backs as a group; however, an investigation of more specific positions, such as full back or central-back, may reveal additional differences.

No significant correlation between *V*O2max scores and plasma MDA levels was observed in this study (TABLE 3). This results differed from those of a previous study that reported the increase of plasma MDA levels 15 min after exercise in nine futsal athletes significantly.[1] Previous study reported that exercise with treadmill running

increase plasma MDA levels during and after its performance.[14] Another study conducted in 24 physical education students reported that plasma MDA levels increase immediately and 24 h after aerobic exercise.[15] Athletes receive training not only to improve performance of technical skills, tactical skills, and athletic experience, but also to improve physiological functions, psychological functions, and social wellbeing.[6,19] Moreover, physical exercise can delay the aging process.[1]

Although exercise increases athletic performance, rigorous physical exercise can cause oxidative stress, which is associated with fatigue and cell damage.[1,13-15,20] Oxidative stress is a condition in which there is an imbalance between free radicals and antioxidants.[16] There is an alteration in heart function and blood volume during aerobic exercise, which results in higher oxygen consumption. Exercise causes blood capillary proliferation and increased mitochondrial enzymes, alternating muscle metabolism. This results in increased lipid peroxidation.[6]

One study demonstrated that aerobic exercise with moderate intensity leads to increased oxygen consumption, impacting free radical formation by the mitochondria. Free radical formation can endanger lipid cells, proteins, DNA, and other cells through the oxidation process.[17] During physical exercise, oxygen plays an important role. It is necessary during physical exercise, however it can also be a dangerous substance due to it can increase oxidative stress. Increased oxygen volume will increase skeletal muscle metabolism, which can increase the production of ROS, a free radical with negative consequences. Additionally, NADPH oxidase activity, PLA2-dependent processes, xanthine oxidase activity, and phagocyte cells can increase ROS production.[21]

Malondihaldehyde is an indirect measurement of free radical activity.[16] Some studies reported that oxidative stress from lipid peroxidation can be measured by MDA levels.[1,14] Malondihaldehyde ($C_3H_4O_2$) is a compound with a three carbon chain and results from lipid oxidation, particularly from arachidonate, eicosapentaenoate, and docosahexaenoate fatty acids.[14] Several studies have used MDA as an indicator of oxidative stress caused by exercise. When a free radical is formed, it can attack polyunsaturated fatty acids in the cell membrane, causing the formation of other chemical reactions, commonly called lipid peroxidation. The fatty acids are then broken down and produce carbon gases (ethane or pentane) as well as aldehyde formation.[16]

This results differed from previous study indicating that excessive physical activity can increase oxidative stress, characterized by elevated plasma MDA levels. There are several possible explanations as to why this study differed from others. Under high oxidative stress conditions, plasma MDA levels significantly increase. When oxidative stress is resolved, plasma MDA levels normally decrease.[14] Athletes may be able to better compensate for the free radical elevation, which can cause lipid peroxidation and consequently increased plasma MDA levels.

Several factors contribute to overcoming oxidative stress. Subjects with increased physical activity displayed lower MDA levels than subjects with non-active physical activity, although it was not statistically significant (TABLE 4). Metin *et al*.[13] suggested that regular exercise may be beneficial in cases of oxidative stress by reducing lipid peroxidation and increasing the activity of the antioxidant enzyme superoxide dismutase. Another

variable that may contribute to plasma MDA levels was alcohol consumption. A previous study by Deshpande *et al*.[22] demonstrated a correlation between plasma MDA levels and alcohol consumption. In this study, a subject who consumed alcohol displayed higher plasma MDA levels than those of subjects who did not. Additionally, subjects taking vitamin supplements displayed lower plasma MDA levels than those of subjects who did not. Based on subject interviews, the most common supplement consumed was vitamin C. Free radicals, as part of the oxidative stress process, can be neutralized by the antioxidant defense system. The antioxidant defense system consists of enzymatic antioxidants, such as catalase, superoxide dismutase, glutathione peroxidase, and non-enzymatic antioxidants, including vitamin A, E, C, glutathione, ubiquinone, and flavonoids.[16] Antioxidant supplementation can neutralize ROS and reactive nitrogen species and reduce the risk of muscle damage. Supplementation with 1000 mg vitamin C and 300 mg α-tocopherol acetate can prevent exercise-induced lipid peroxidation.[23]

Vitamin C serves as an antioxidant by reacting with free radicals, thus reducing the ability of free radicals to attack polyunsaturated fatty acids and improving plasma MDA levels. One study demonstrated that vitamin C supplementation prevented endurance exercise-induced lipid peroxidation.[24] Additionally, vitamin C can overcome ROS, including superoxide or hydroxyl radicals.[25] Supplementation with vitamin C and vitamin E helps to reduce markers of exercise-induced muscle damage. This was illustrated by the significant reduction in oxidative stress markers (creatine kinase and malondialdehyde) in a group receiving 250 mg of vitamin C or both vitamin C and vitamin E supplementation.[26]

Vitamin E also plays a role as an antioxidant. Vitamin E supplementation for two weeks can significantly decrease MDA levels before and after an ergometric test, which measures performance.[27] A study investigating β carotene, vitamin E, A, C, zinc, and selenium supplementation demonstrated that serum MDA levels were remarkably reduced after two weeks of supplementation. Furthermore, hemoglobin levels, packed cell volume (PCV), and red blood cells (RBC) in Indian athletes were also improved.[28]

One weakness of this study is that we did not measure other variables that can be used as oxidative stress indicators, such as lipid hydroperoxide, creatine kinase, superoxide radicals, and other radicals. Additionally, this study did not measure the antioxidant capacity of the body, including measurements of catalase, superoxide dismutase, and glutathione peroxidase levels. This study was pre-experimental with a one-shot case study design or a one-group post only design. Thus, the researcher did not know the differences before and after exercise or training. Another weakness is that the subjects only received restraint instructions through a short message service. The results should be validated following subject quarantine prior to the study to control the restraints.

CONCLUSION

There is no correlation between *V*O2max scores and plasma MDA levels indicating that training or exercise do not negatively impact oxidative stress conditions in student soccer players following *V*O2max measurement using the yo-yo intermittent recovery test. Further research will be performed to investigate other variables that can be used as oxidative stress indicators, such as lipid hydroperoxide, creatine kinase, superoxide radicals, and

other radicals. In addition, further research is also needed to investigate the antioxidant capacity of the body, including measurements of catalase, superoxide dismutase, and glutathione peroxidase levels.

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Severity and treatment level of acute gastroentritis with rotavirus in children under 5 years in Indonesia

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ABSTRACT

Rotavirus diarrhea causing gastroenteritis in children under five years is an important issue that urgently needs to be addressed globally. Delay in management of rotavirus diarrhea can be fatal. Diagnostic tool for detecting rotavirus is, therefore, needed. However, until now the gold standard diagnostic tools are expensive, often not available and affordable in health care settings. The aim of the study was to compare the Vesikari clinical severity score of rotavirus-positive with rotavirus-negative in hospitalized children with acute gastroenteritis. Furthermore, the difference of the level of treatment between rotavirus-positive with rotavirus-negative was also evaluated. This was a cross sectional study that using secondary data from medical records of five general teaching hospital in Indonesia. Subjects were children aged <5 years with acute watery diarrhea admitted to the hospital. Statistical analysis used was chi square test, U-Mann Whitney, and Kruskal Wallis. The results showed that the patient with rotavirus positive have higher dehydration (80.2%) compared to rotavirus negative (70%). The severity level of clinical feature was higher in diarrhea due to rotavirus positive than non rotavirus (11.47± 2.89 vs 10.41 ± 2.70; p<0.000). The level of treatment was higher in rotavirus positive. The majority had treatment plan C (47.7%) higher than plan B and A (45.6% and 30.9%; p<0.050). This was opposite with patient with rotavirus negative that majority had treatment in plan A (69.1%) higher than plan B and C (54.4% and 52.3%) (p<0.001). In conclusion, the severity of gastroentrities in children under 5 years using vesikari score are higher in diarrhea due to rotavirus positive than non rotavirus. The treatment level plan C is higher than plan B and A in diarrhea due to rotavirus. This is opposite with non rotavirus majority have treatment in plan A higher than plan B and C.

ABSTRAK

Diare rotavirus yang menyebabkan gastroenteritis pada anak usia di bawah lima tahun merupakan masalah penting yang sangat perlu ditangani terutama di negara berkembang. Keterlambatan penanganan diare rotavirus dapat berakibat fatal secara klinis. Oleh karena itu, suatu alat diagnostik untuk mendeteksi rotavirus sangat diperlukan. Namun, hingga saat ini standar emas alat diagnostik tersebut masih mahal dan sering tidak terjangkau dipusat pelayanan kesehatan. Penelitian ini bertujuan untuk membandingkan skor tingkat keparahan klinik Vesikari pada anak gastroenteritis dengan rotavirus-positif dan rotavirus negatif yang di rawat di rumah sakit. Selanjutnya perbedaan tingkat pengobatan antara

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rotavirus-positif dan rotavirus-negatif juga akan dikaji. Penelitian ini merupakan penelitian potong lintang menggunakan data sekunder dari rekam medis di lima rumah sakit pendidikan di Indonesia. Subjek penelitian adalah anak usia < 5 tahun dengan diare akut yang masuk rumah sakit. Analisis statistik yang digunakan adalah uji chi square, U-Mann Whitney, dan Kruskal Wallis. Hasil penelitian menunjukkan bahwa pasien dengan rotavirus positif sebagian besar mengalami tingkat dehidrasi lebih tinggi (80.2%) daripada pasien dengan rotavirus negatif (70%). Tingkat keparahan klinis diare lebih tinggi pada rotavirus positif dari pada non rotavirus (11,47 ± 2,89 vs 10,41 ± 2,70; p<0,000). Pengobatan penderita yang terinfeksi rotavirus positif sebagian besar menggunakan perlakuan plan C (47,7%) lebih tinggi dari pada plan B dan A (45,6% dan 30,9%). Hal ini berbeda penderita yang terinfeksi rotavirus negatif yang mayoritas menggunakan plan A (69,1%) lebih tinggi dari plan B dan C (54,4% dan 52,3%; p <0,001). Dapat disimpulkan, tingkat keparahan gastroentrities pada anak usia <5 tahun menggunakan skor vesikari lebih tinggi pada diare akibat rotavirus positif daripada non rotavirus. Tingkat perlakuan dengan plan C lebih tinggi dari plan B dan A pada diare karena rotavirus. Hal ini berlawanan dengan mayoritas non rotavirus yang menggunakan plan A lebih tinggi dari plan B dan C.

Keywords: rotavirus - acute gastroentrities - treatment level - vesikari score - children

INTRODUCTION

Acute gastroenteritis is an inflammation of the stomach and intestines *caused by* viral or non viral infections leading to diarrhoea, vomiting and abdominal discomfort. Non viral acute gastroenteritis can be caused by bacteria, protozoa and helminths, whereas viral acute gastroenteritis can be caused by rotavirus, enteric adenovirus, calciviruses, astroviruses and enteroviruses.[1,2] Acute gastroenteritis remains a major cause of morbidity and mortality in children worldwide, accounting for 124 million clinic visits, 9 million hospitalizations, and 1.34 million deaths annually in children under 5 years old with more than 98% of these deaths occurring in the developing countries.[3-5]

Among causes of viral acute gastroenteritis in children, rotavirus is the most common cause with the most severe clinical manifestations and rapidly progressive lethal dehydration especially in infants and young children.[5-7] It causes approximately 111 million cases requiring only home care, 25 million clinic visits, 2 million hospitalizations, and 352,000–592,000 deaths in children under 5 years old.[8] Rotavirus gastroenteritis is transmitted primarily via fecal-oral contamination through person-to-person contact or contact with rotavirus contaminated items such as respiratory secretions. In developing countries 75% of children are infected prior to 12 months of age and attack rates peak at 6 months of age, but in developed countries, the first episode usually does not occur between 2 and 5 years of age. Once infection has occurred there is an approximate 24 to 72 hour incubation period followed by between 3 and 8 days of vomiting and diarrhea that may be accompanied by fever and abdominal pain and may last for as long as 3 weeks.[9]

The severity of clinical features of acute gastroenteritis is associated with its etiology. It was reported that patients with rotavirus-positive gastroenteritis have a higher incidence of vomiting compared to patients with rotavirus-negative gastroenteritis lead to the higher need for intravenous rehydration therapy and the duration of hospitalization.[10,11] However, the confirmation of viral etiology has not been applied in the clinical practice due to the limitations of laboratory facilities,

time-consuming and economical raisons. To overcome the limitations, the clinical severity scoring systems in viral gastroenteritis has been proposed as clinical predictors.[12,13] The Vesikari clinical severity scoring system is currently considered the best predictor tool for identifying the severity of acute gastroenteritis. In this study, we reported the Vesikari clinical severity score rotavirus-positive gastroenteritis compared to rotavirus-negative gastroenteritis in hospitalized children in Indonesia.

The severity of clinical features of acute gastroenteritis will determine the treatment level. World Health Organization (WHO) recommended the level of treatment for acute gastroenteritis based on its severity of dehydration i.e. treatment plan A, B and C. In this study, we also reported the level of treatment for diarrheal rotavirus-positive gastroenteritis compared to that rotavirus-negative gastroenteritis.

MATERIALS AND METHODS

Subjects

This was observational study with a cross sectional design using secondary data from Rotavirus Surveillance Study conducted by Soenarto *et al.*[14] from the Pediatric Research Office, Department of Pediatric, Dr. Sardjito General Hospital/Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta from year 13 in five academic hospital in Indonesia i.e. Dr. Hasan Sadikin General Hospital, Bandung, West Java, Mataram General Hospital, West Nusa Tenggara, Dr. Sardjito General, Hospital, Yogyakarta, Sanglah General Hospital, Denpasar, Bali and Kulon Progo District Hospital, Kulon Progo, Yogyakarta. All children under 5 years old who experienced acute diarrhea and fulfil the inclusion and exclusion criteria were involved in this study. The inclusion criteria were all the children aged < 5 years with acute watary diarrhea who visited the 5 hospitals. The exclusion criteria were the stool sample was not enough to do the experiment in laboratory testing, incomplete variable on data or the parents and children did not agree to participate in the study.

Protocol of study

Subjects who fulfil the inclusion and exclusion were grouped into diarrheal rotavirus-positive gastroenteritis and rotavirus-negative gastroenteritis based on the laboratory viral examination results. A standardized clinical data of all subjects included the date of admission, age and sex of the patient, nutritional status, duration and frequency of diarrhea, duration and number of vomiting, previous treatment, status of dehydration, symptoms of illness were then collected. Nutritional status was determined based on ratio between weight and height according to WHO criteria i.e. malnutrition if weight and height ratio ≤ -2 SD; under nutrition if weight and height ratio -2 SD; well nutrition if weight and height ratio >2 SD. Acute diarrhea was defined as ≥ 3 loose stools within 24 h and for a duration of < 2 weeks. The clinical data were then used to calculate Vesikari clinical severity score as presented in TABLE 1.

TABLE 1. Vesikari clinical severity scoring system[13]

Parameter	1	2	3
Diarrhea			
Duration of diarrhea (day)	1-4	5	≥6
Maximum frequency per day	1-3	4-5	≥6
Vomiting			
Duration of vomiting (day)	1	2	≥3
Maximum number per day	1	2-4	≥5
Maximum body temperature (°C)	37.1-38.4	38.5-38.9	≥39
Degree of dehydration (%)	No	1-5	≥6
Treatment	Rehydration	Hospitalization	No
Severity rating scales	<7 (mild)	7-10 (moderate)	≥11 (severe)

Level of treatment was measured based on the WHO 2013 criteria that divided into 3 plans i.e. plan A for non dehydration, plan B for some dehydration and plan C for severe dehydration. The study has been approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta.

Statistical analysis

Data were analysed using statistical SPSS version 19.0. Chi-square, multivariate and U-Mann Whitney and Kruskal Wallis analysis were performed to determine the significance of difference observed between two different groups of patients. Statistical significance assigned to p value of <0.05.

RESULTS

In period of 12 months from January to December 2013, 592 data of acute diarrhea patients from the computerized data base of the 5 academic hospital were gathered and selected. As much as 586 (99%) data were available for analysis and only 6 data were excluded due to not available of rotavirus examination data of the stools. Among 586 data analysed, 242 data were rotavirus-positive and 344 data were rotavirus-negative acute gastroenteritis. Characteristics and clinical symptoms of the patients are presented in TABLE 2. This study showed an association between rotavirus-positive gastroenteritis and nutritional status, number of vomiting and degree of dehydration ($p<0.05$). In contrast, no an association between rotavirus-positive gastroenteritis and age, sex, duration of diarrhea, frequency of diarrhea, duration of vomiting, treatment and temperature was observed ($p>0.05$).

TABLE 2. Characteristic and clinical symptoms of acute gastroenteritis in children under 5 years old in 5 hospitals in Indonesia

Characteristic	Total n=586	Rotavirus (+) n=242 (41.3%)	Rotavirus (-) n=344 (58.7%)	p
Age (median in month)	378.5	370.0	383.0	0.975
Sex of patient (n/%)				
Male	362(61.8)	147(25.1)	215(36.7)	0.667
Female	224 (38.2)	95(16.2)	129(22.0)	
Nutritional status (n/%)				
Malnutrition	34 (5.8)	7(1.2)	27(4.6)	
Undernourished	120 (20.5)	43(7.3)	77(13.1)	0.010
Well nourished	432 (73.7)	195(32.8)	240(41.0)	
Duration of diarrhea (n/%)				
1-4	486 (82.9)	205(35.0)	281(48.0)	
5	42 (7.2)	18(3.1)	24(4.1)	0.379
≥ 6	58 (9.9)	19(3.2)	39 (6.7)	
Maximum frequency of diarrhea (n/%)				
1-3	38 (6.5)	9(1.6)	29(4.9)	
4-5	200 (34.1)	84(14.3)	116(19.8)	0.073
≥6	348(59.4)	149(25.4)	199(34.0)	
Duration of vomiting (n/%)				
1	175 (29.9)	77(31.8)	98(28.5)	
2	105 (17.9)	49 (20.2)	56(16.3)	0.368
≥ 3	92(15.7)	50(20.7)	42(12.1)	
Maximum number of vomiting (n/%)				
1	17 (2.9)	6(2.5)	11(3.2)	
2-4	187 (31.9)	73(30.1)	114(33.1)	0.001
≥5	168 (28.7)	97(40.1)	71(20.6)	
Treatment (n/%)				
Rehydration	41 (7.0)	17(2.9)	24(4.1)	0.982
Hospitalized	545 (93.0)	225(38.4)	320(54.6)	
Temperature (n/%)				
<37.1	269 (45.9)	99 (16.9)	170 (29.0)	
37.1-38.4	245 (41.8)	114(19.5)	131(22.3)	0.143
38.5-38.9	44 (7.5)	19(3.2)	25(4.3)	
≥39	28 (4.8)	10(1.7)	18(3.1)	
Degree of dehydration (n/%)				
Not dehydration	151 (25.8)	48(8.2)	103(17.6)	
1-5%	390 (66.6)	171(29.2)	219 (37.4)	0.015
≥6%	45 (7.6)	23(3.9)	22(3.7)	

A multivariate analysis showed that nutritional status, number of vomiting and degree of dehydration could be considered as strong predictor factors for rotavirus-positive gastroenteritis ($p<0.05$) as presented in TABLE 3. Furthermore, the Vesikari score and clinical severity level of rotavirus-positive acute gastroenteritis was significantly higher than that of rotavirus-negative acute gastroenteritis ($p<0.05$) as presented in TABLE 4.

TABLE 3. Multivariate analysis of characteristic and clinical symptoms of acute gastroentritis in children under 5 years old in 5 hospitals in Indonesia

Variables	Coefficient regression	OR	p
Nutritional status	0.46	1.58	0.041
Number of vomiting	0.59	1.82	0.001
Degree of dehydration	0.51	1.67	0.011

TABLE 4. Vesikari score and severity level of acute gastroentritis between rotavirus-positive and rotavirus-negative in children under 5 years in 5 hospitals in Indonesia

Vesikari score	Rotavirus (+) n = 242	Rotavirus (-) n = 586	p	Multivariate (OR; p)
Score (mean ± SD)	11.47± 2.89	10.41±2.70	0.000	(1.14; 0.000)
Severity level (n/%)				
Mild<7	13 (5.4)	28 (8.1)		
Moderate 7-10	67 (27.7)	133 (38.7)	0.004	
Severe ≥11	162 (66.9)	183 (53.2)		

TABLE 5 shows the differance of the treatment level between rotavirus-positive and rotavirus-negative acute gastroenteritis. The children with rotavirus-positive acute gastroenteritis had higher treatment level compared with those rotavirus-negative ($p<0.05$). The children with rotavirus-positive majority had treatment plan C higher than plan B and A, whereas the children with rotavirus-negative majority had treatment plan A higher than plan B and C ($p<0.05$).

TABLE 5. Treatment level of acute gastroentritis in children under 5 years in 5 hospitals in Indonesia

Treatment level	Rotavirus (+)	Rotavirus (-)	p	Multivariate (OR; p)
Mean rank	376.61	277.24	0.001	(1.59; 0.002)
Plan A (n/%)	55 (30.9)	123 (69.1)	0.003	
Plan B (n/%)	166 (45.6)	198 (54.4)		
Plan C (n/%)	21 (47.7)	23 (52.3)		

DISCUSSION

Acute gastroenteritis in children remains a major health problem in both developing and developed countries.[8,9,15] Although the diseases is usually self-limited, it can cause severe clinical manifestations that need hospitalization especially in infants and young children. This study showed that rotavirus-positive gastroenteritis was more prevalent in male children than in female children in this study indicating that male children was more susceptible to rotavirus infection than female children. This result is in agreement with previous studies that reported boys are twice more suffered from rotavirus infection than girls and are more likely to be hospitalized.[16] Junaid *et al.*[17] reported that male children excrete rotavirus at a significant higher rate than female children in Nigeria with the ratio 1.8:1. Shim *et al*.[18] also reported that the number of rotavirus infected male was higher the number of rotavirus infected female in Korea.

Significantly association between rota-virus-positive gastroenteritis and nutritional status, number of vomiting and degree of dehydration was observed in this study ($p<0.05$). The children with rotavirus-positive gastroenteritis had low nutritional status compared to those with rotavirus-negative gastroenteritis. The association between nutritional status and susceptibility to rotavirus infection remains not well understood. Some studies provide evidences for the different association between nutritional status and rotavirus infection. Nitiema *et al.*[19] also reported that acute malnutrition is significantly associated with more severe symptoms in rotavirus-induced diarrhea and undernourished children also exhibits a prolonged duration of diarrheal episodes. In contrast, Mpabalwanit *et al.*[20] reported that rotavirus infection is more common in the hospitalized children with normal nutritional status than in those with malnutrition in Zambia. Furthermore, Das *et al.*[21] reported that rotavirus infection among overweight and obese children is higher compared to those well-nourished and malnourished children attending at Dhaka Hospital, Bangladesh. A recent longitudinal study in Bangladesh reported that healthy growth and development over the first 3 years of life are positively associated with a risk of symptomatic rotavirus infection.[22]

The identification of the etiology acute gastroenteritis is very useful to help determine appropriate therapy. Unfortunately, clinicians often have difficulties to distinguish between viral or non viral causes of acute gastroenteritis. Stool culture examination has been considered as a standard diagnostic to identify the etiology. However, it is time-consuming, expensive and not applicable. The clinical severity scoring systems has been applied as clinical predictors to determine clinical conditions of patients with acute gastroenteritis. The Vesikari clinical severity scoring system is the severity scale that was originally developed to evaluate the effectiveness and efficacy of rotavirus vaccines.[16] Recently, the system is used for predicting the viral or non viral pathogens in acute gastroenteritis.

This study showed that the Vesikari clinical severity score of rotavirus-positive acute gastroenteritis (11.47 ± 2.89) was significantly higher than that of rotavirus-negative (10.41 ± 2.70) ($p<0.05$) indicating severe symptoms were observed in children with rotavirus-positive. The Vesikari clinical severity score was supported with the clinical symptoms of patients where the children with rotavirus-positive gastroenteritis suffered more often vomiting (71.9% vs. 56.9%) and dehydration (80.2% vs. 70%) compared to

those with rotavirus-negative. This results showed that there is association between the Vesikari clinical severity score and clinical severity symptoms of the rotavirus infections indicating it could be used as diagnostic tool for predicting the rotavirus infection in acute gastroenteritis in children. However, the cut-off point values to achieve an acceptable overall diagnostic to distinguish between retrovirus and non retrovirus in acute gastroenteritis should be further optimized.

Vomoting and dehydration appeared to be more common in children with rotavirus-positive gastroenteritis in this study. This result is in agreement with previous studies reported by some authors.[16,17] These symptoms could determine the different of treatment level. Results of this study showed that the children with rotavirus-positive had treatment plan C higher than plan B and A, whereas the children with rotavirus-negative had treatment plan A higher than plan B and C. It was indicated that children with rotavirus-positive was more effective to be treated with treatment plan C, whereas children with negative-rotavirus was still effective to be treated with treatment plan A (at home) and plan B (treat some dehydration with oral rehydration salts/ORS).

Some diagnostic tools for the confirmation of rotavirus infection in children with gastroenteritis have been used routinely in diagnostic laboratories include enzyme linked immunosorbent assay (ELISA), latex agglutination assay (LA), polyacrylamide gel electrophoresis (PAGE), electron microscopy (EM) and real-time reverse transcription-polymerase chain reaction (RT-PCR).[23-26] However, these diagnostic tools are not always applicable in hospitals with limited laboratory facilities. Moreover, some of these diagnostic tools are expensive and time consuming. In regard of these conditions, the Vesikari clinical severity score system could be alternative diagnostic tool. The Vesikari clinical severity score system as a noninvasive test is recommended for children to avoid painful procedures such as venipuncture or invasive endoscopy. The Vesikari clinical severity score system could be useful to standardize assessment and to guide decision making among clinicians with differing levels of training by scoring the symptoms patients, because it can be calculated using clinical findings by trainees and experienced staff alike.

CONCLUSION

In conclusion, the Vesikari clinical severity score of rotavirus-positive acute gastroenteritis is significantly higher than that of rotavirus-negative. The children with rotavirus-positive majority receive treatment level plan C higher than plan B and A, whereas the children with rotavirus-negative majority recieve treatment plan A higher than plan B and C. It is demonstrated that the Vesikari clinical severity score can be used as a diagnostic tool for rotavirus acute gastroenteritis.

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Staphylococcus epidermidis: how to turn from commensal to be a pathogen lifestyle

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ABSTRACT

Staphylococcus epidermidis normally is a commensal inhabitant of healthy human skin and mucosa, but also a common nosocomial pathogen in immunocompromised patients, neonates, and patients with indwelling medical devices. To distinguish the pathogen and commensal strain is a big challenge when identifying this agent with its related infection. This mini-review aims to summarize recent research in this area with a special emphasis on the virulence factor of generating genotypic and phenotypic diversity in *S. epidermidis*. By living between a commensal and pathogen, *S. epidermidis* needed to establish many strategies to face different clinical environments, including the new ecological niche of biomaterials. In addition, the growing number of immunocompromised patients increased the risk for a very sensitive host. However, further exploration of the relationship between virulence factor and *in vivo* pathogenesis is still needed. According to the virulence factor of these bacteria, which are considered as a real pathogen, strict control measures should be taken for *S. epidermidis* infection.

ABSTRAK

Staphylococcus epidermidis merupakan bakteri komensal kulit dan mukosa pada manusia, tetapi akhir-akhir ini banyak ditemukan sebagai agen patogen infeksi nosokomial terutama pada pasien *immunocompromised*, neonatus, dan pasien dengan peralatan medis invasif. Saat ini, bagaimana membedakan *S. epidermidis* strain patogen dan komensal masih merupakan tantangan besar, baik di laboratorium maupun bagi klinisi. Tinjauan ini bertujuan untuk mendiskusikan peran faktor virulensi *S. epidermidis* dalam menyebabkan keragaman genotipik dan fenotipik serta keterkaitannya dengan perubahan karakterisitik *S. epidermidis*, sebagai bakteri komensal maupun patogen. Dengan hidup di antara pola komensal dan patogen, *S. epidermidis* perlu menyusun banyak strategi untuk menghadapi lingkungan klinis yang beragam, termasuk beradaptasi dengan permukaan biomaterial yang merupakan bahan dari peralatan medis invasif. Selain itu, meningkatnya jumlah penderita immunocompromised, menyebabkan peningkatan kepekaan host terhadap infeksi *S. epidermidis.* Namun, penelitian lebih lanjut tentang hubungan antara faktor virulensi dan patogenesis infeksi *in vivo* masih diperlukan. Dengan pertimbangan bisa berperan sebagai bakteri patogen, tindakan pengendalian yang ketat harus dilakukan untuk infeksi *S. epidermidis*.

Keywords: *Staphylococcus epidermidis* – commensal – pathogen - virulence factor - biofilm

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INTRODUCTION

Staphylococcus epidermidis is a coagulase-negative Staphylococcus, considered as a part of the normal mucosa and skin microflora of humans and other mammals.[1,2] It is considered as a member of the Staphylococci genus, which are gram-positive bacteria belonging to the family Staphylococcaceae. They are clustering, non-motile and non-spore forming cocci, facultative anaerobes and produce catalase. Currently, there are 35 known species of the genus Staphylococcus, from which 15 species are indigenous to humans, while the others are non-human pathogens.[2,3] Coagulase-negative staphylococci (CNS) are grouped together as *Staphylococcus saprophyticus (S. saprophyticus), Staphylococcus lugdunensis (S. lugdunensis), Staphylococcus schleiferi (S. schleiferi), Staphylococcus haemolyticus (S. haemolyticus), Staphylococcus caprae (S. caprae)* or *S. epidermidis* based on their inability to clot blood plasma. Coagulase-negative staphylococci are widely distributed over the surface of the human body, where they constitute the majority of the commensal bacterial skin microflora.[1]

Culture analysis has revealed that *Staphylococcus* spp. are the most abundant organisms colonizing moist areas. These moist sites include the umbilicus, the axillary vault, the inguinal crease (side of the groin), the gluteal crease, the sole of the foot, the popliteal fossa (behind the knee), nares anterior, and the antecubital fossa (inner elbow).[1] Staphylococci occupy an aerobic niche on the skin and probably use the urea present in sweat as a nitrogen source.[3] In spite of being a saprophyte and opportunistic bacterium, this bacteria is involved in balancing the epithelial microflora and serves as a reservoir of resistance genes, which might be transferred to the closely related but more virulent organisms, such as *Staphylococcus aureus (S. aureus)*.[4] Accordingly, *S. epidermidis* maintains a commonly mutualism relationship with its host and serves as a shield, preventing colonization of potentially more harmful bacteria by producing lantibiotics, which are lanthionine-containing antibacterial peptides, also known as bacteriocins that may provide an added level of protection against certain common pathogens. Additionally, acting as skin microbiome, this bacteria promote the integrity of cutaneous defence through elicitation of host immune responses. [4,5] As an innocuous commensal microorganism, *S. epidermidis* was for a long time seen as an virulent species. However, today this bacterium is considered the most frequent cause of healthcare associated infections (HAIs), namely those related with indwelling medical devices. Overall, *S. epidermidis* is the most common species in HAIs, followed by *S. haemolyticus, S. hominis, and S. capitis*.[6–8] it has not been established that adherence and biofilm formation are closely linked phenotypes for clinical isolates. In this study, the initial adhesion to different materials (acrylic and glass For example, *S. epidermidis* may be involved in prosthetic joint, vascular graft, surgical site, central nervous system shunt and cardiac device infections.[5,9–11]

In contrast to *S. aureus, S. epidermidis* does not produce many aggressive virulence factors, and consequently the infections caused are, at least in immunocompetent patients, often low-grade and chronic. For severely immunocompromised patients, *S. epidermidis* may develop into a life-threatening pathogen triggering septicaemia, meningitis, and other serious conditions[1,12] *Staphylococcus epidermidis* infections mostly are considered as being extremely recalcitrant to therapy. This is due to high antibiotic resistance rates among nosocomial *S. epidermidis* isolates,

but treatment failure is also associated with the ability of *S. epidermidis* to form biofilms on inert surfaces of medical devices from where these sticky, multilayered aggregates of bacteria are hard, if at all possible, to completely remove.[13,14]

Additionally, the increasing use of biomaterials in modern medicine has improved the quality of life of many patients. However, as a drawback, the occurrence of biomaterial-associated infections (BAI) is increasing and now becoming a serious health threat to patients, as well as a financial burden to the society. *S. epidermidis*, generally regarded as an opportunist pathogen, is now recognised as a real "new" pathogen, since it is the major etiologic agent of BAI.[10,11,15] Biomaterial-associated infections is generally related to microbial biofilm formation, defined as a microbial community encased in a matrix of self-produced extracellular polymeric substances (slime). Slime affects antimicrobial resistance as well as the effectiveness of the host immune system[14,16]. Currently, no effective non-invasive technique exists to prevent or destroy biofilms associated with BAI. Systemic antibiotics predominantly attack a biofilm infection through the outermost layers of the biofilm, which are usually ineffective as bacteria continue to grow from the inner layers combined with an increased production of extracellular polymeric substances. This virulence constitutes the main reason why biomaterial implants related to an infection nearly always have to be removed.[17] In addition, the use of antibiotics and disinfectants in hospitals puts a high selective pressure on bacteria to select for resistant and well-adapted variants.[18,19] However, this unique pattern does not yet explain why just *S. epidermidis* and not any other bacteria, was able to conquer and occupy this novel ecological niche.

Notably, it has been shown that the genomic structure of *S. epidermidis* represents an amazingly versatile microorganism living in a grey area between commensalism and pathogenicity. *S. epidermidis* employs sophisticated regulatory networks to quickly adapt its metabolism to changing external conditions, to communicate with its neighbours in the same ecological niche, or to escape the host's immune response.[8,20] Genomic analyses demonstrated the presence of numerous mobile genetic elements in *S. epidermidis* genomes, including methicillin resistance-mediating *SCCmec* elements and insertion sequences (IS). IS elements seem to be important driving forces that keep the *S. epidermidis* genome extremely flexible and trigger heterogeneous gene expression.[21]It is suggested that well-adaptability properties both on the regulatory and genetic level might have contributed to the evolutionary success of *S. epidermidis* as a nosocomial pathogen.[8] Meanwhile, due to the ubiquitous prevalence of *S. epidermidis* as a commensal bacterium, clinicians often face the challenge to decide whether an isolate represents the causative agent of an infection or an unspecific culture contamination. Nowadays, our understanding of how *S. epidermidis* becomes a commensal or pathogen is far from complete and many questions still remain. This review addresses the questions concerning how the recent mechanism of commensal and infectious lifestyles of *S. epidermidis* takes place, which more focusing on literature about virulence properties of *S. epidermidis* i.e biofilm formation, *icaADBC* presence *and* the mechanism of regulating gene expression, the role of small colony variant and methicillin resistance gene, as well as its genomic flexibility.

DISCUSSION

Biofilm formation, major pathomechanism of HAIs infection

One of the main virulence characteristics of *S. epidermidis* is related with their adhesion to substratum surfaces and subsequent biofilm formation.[5,10] A biofilm is a population of cells growing on a surface and enclosed in a self-produced matrix of extracellular polymeric substance (EPS). Biofilms are notoriously difficult to eradicate and are a source of many recalcitrant infections.[14] Bacterial biofilm formation comprises a number of physical, biological, and chemical processes. The relative contribution of each process changes throughout biofilm development and depends on prevailing environmental and hydrodynamic conditions.[22] In general, biofilm formation can be described in five phases [5,23,24] as shown in FIGURE 1.

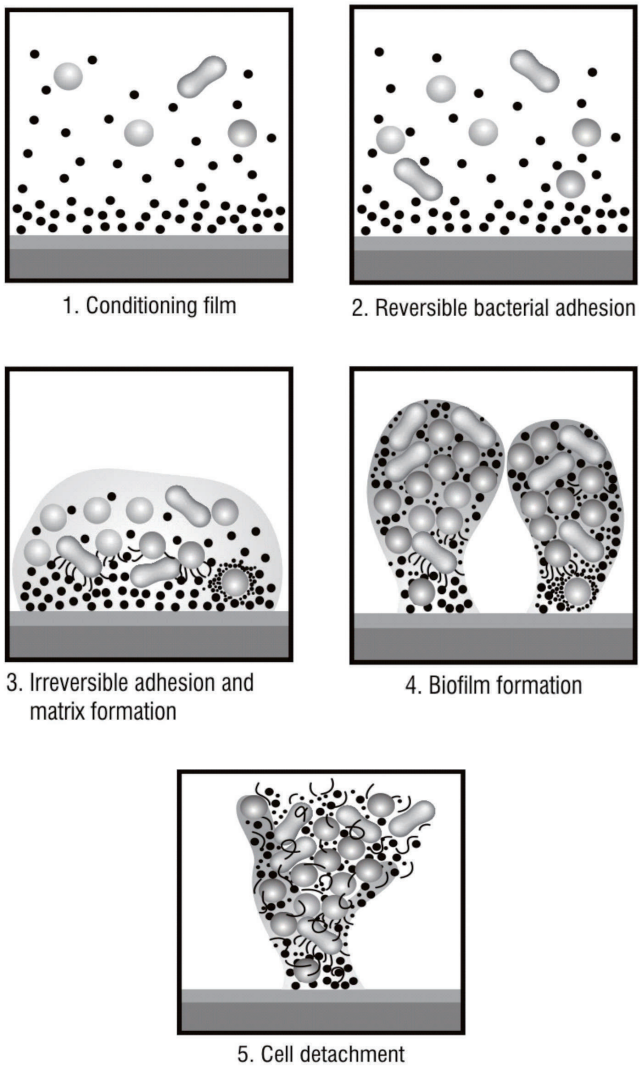

FIGURE 1. The phases of biofilm formation in *S. epidermidis*. Graphs were modified from Vuong et al., Nuryastuti, and Bos et al.5,23,24

Substratum surfaces will first become covered with a conditioning film consisting of proteins and glycoproteins, such as fibronectin, vitronectin, fibrinogen, albumin, and immuno-globulins, many of which serve as binding ligands to receptors on colonizing bacteria, although adhesion can also occur to bare substratum surfaces. Biofilm formation continues with the transport of bacteria to the substratum-liquid interface, which is governed by a combination of transport mechanisms, including Brownian motion, gravity, diffusion, convection, or the intrinsic motility of a microorganism.[23,24] Subsequently, in the second phase, microbial adhesion may occur which is initially of a reversible nature. Factors involved in the initial adhesion to a substratum surface include non-specific interactions originating from both the bacterial cell and substratum surfaces. These non-specific interactions are governed by physicochemical properties such as surface charge, hydrophobicity, and chemical structure of both the bacteria and substratum surface. In the third phase, reversible adhesion of bacteria changes to irreversible, amongst others due to protein-protein interactions and the production of EPS. The fourth phase in biofilm formation is surface colonization. Adhering bacteria grow and divide, forming microcolonies that are considered to be the basic organizational units of a biofilm. Entrapment of other planktonic bacteria in the EPS also occurs, resulting in a multi-layered and mature biofilm.[5,23] The last step is detachment of individual bacteria or aggregates, which allows bacteria to disseminate into other areas for further surface colonization. In the clinical setting, this last step generally leads to severe systemic infections.[5] As a pivotal structural component of microbial biofilms, EPS has received much attention. In general, EPS consists of polysaccharides, eDNA and proteins in a hydrated environment.[26,27] Recently eDNA was found to be a major structural component of bacterial EPS where it plays a role in bacterium-surface and bacterium-bacterium interactions. The EPS produced by *S. epidermidis* consists mostly of polysaccharide intercellular adhesin (PIA).[26]

icaADBC and the mechanism of regulation of expression

Production of PIA, a key virulence factor of *S. epidermidis*, is subject to on-off switching, resulting in phenotypic variability (phase variants).[11,15,28] Polysaccharide intercellular adhesion production is stimulated through the action of membrane bound sensory proteins within the bacterial cell wall. Polysaccharide intercellular adhesion synthesis is catalyzed by proteins encoded within the *ica* operon, a gene cluster consisting of *icaADBC*. The *icaA* gene product is a transmembrane protein with homology to N-acetyl-glucosaminyltransferases. The functions of *icaB* and *icaC* are less well defined. However, *icaB* is likely to be secreted while *icaC* is predicted to be an integral membrane protein. *icaD* might act as a link between *icaA* and *icaC* and represent a novel enzyme combination. When *icaA* is co-expressed with *icaD*, the transferase activity increases 20 fold.[11,29]

Extracellular polymeric substance production is vital, but metabolically expensive for *S. epidermidis* and therefore well-regulated (FIGURE 2). Regulation of *ica*-expression and biofilm formation is negatively controlled by the *ica* operon regulator, *IcaR* and teiocoplanin-associated locus regulator, *TcaR*. It is also influenced by environmental conditions that are potentially toxic for the bacterial cell. The exposure of *S. epidermidis* to a high osmolarity, high temperature, detergents, urea, ethanol, the presence of sub-MIC (Minimal Inhibitory Concentration)

concentrations of certain antibiotics, glucose, iron limitation and oxidative stress have all been shown to elevate *ica*-expression and biofilm formation.[30–32] Moreover, the global stress response factor σB, positively regulates *ica*-expression by negatively regulating *icaR* expression, while staphylococcal accessory regulator A (*sarA*) and regulators of sigmaB (*rsbU*) act similarly.[33] In addition, the LuxS system involved in quorum sensing in *S. epidermidis*, recently emerged as another negative regulator of biofilm formation.[11]

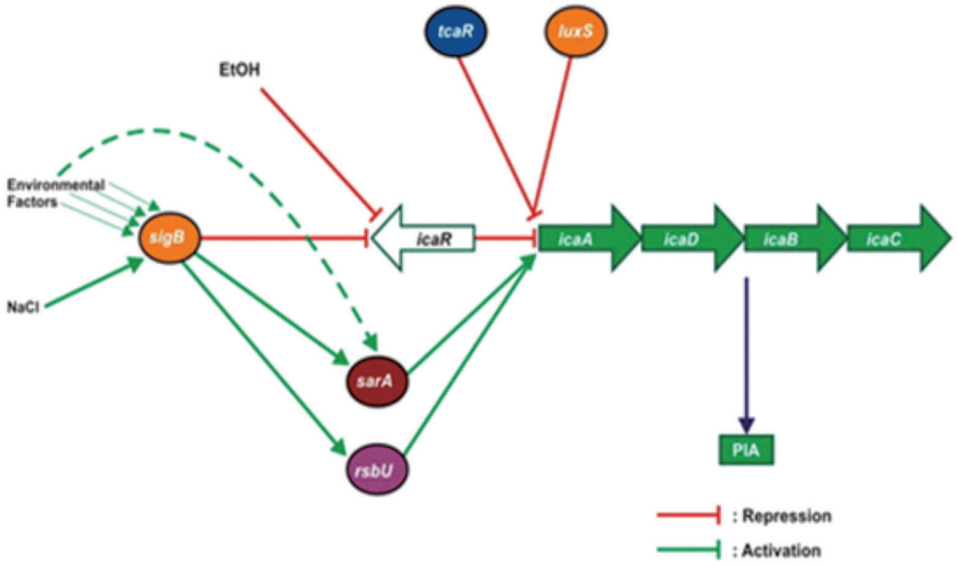

FIGURE 2. The schematic overview of regulatory network controlling expression of i*caADBC in S. epidermidis*. Graphs were modified from Nuryastuti.23

The genetic and molecular basis of biofilm formation in *S. epidermidis* is multi-faceted. It has been reviewed that there are two distinct mechanisms of biofilm development; through an *ica*-dependent and an *ica*-independent mechanism of biofilm development.[11] Biofilm production by *ica* operon-encoded enzymes is currently the best-understood biofilm mechanism in staphylococci,[11] which is regulated by several regulatory genes such as *icaR, σB, rsbU* and *sarA*, including the reversible integration of the IS256 into those genes.[28,34] Additionally, a surface protein homologous to biofilm-associated protein (Bap), accumulation-associated protein (Aap), and considerable amounts of extracellular teichoic acids (ECTA)[8] have been identified to be involved in *ica*-independent biofilm formation in some *S. epidermidis*, which is also under control of the *sarA* regulatory gene.[11]

Recent studies imply that the multicellular organization of bacteria in a biofilm is a crucial mechanism in resisting unfavorable conditions.[35] Heterogeneous gene expression is typically observed in clinical *S. epidermidis* strains, and it is assumed that this ability is an advantage for adaptation of staphylococci to changing environmental conditions.[8] Phase variation involves both regulatory pathways, e.g. in response to environmental signals, as well as genetic variations, by local genomic re-arrangements, altered activity of regulatory proteins or modulation of transcription or translation of the appropriate gene through strand slip mechanisms.[8,11,23]

Polysaccharide intercellular adhesin is the most important component of the staphylococcal slime and its production is catalyzed by proteins encoded within the *icaADBC* operon. Different *S. epidermidis* strains vary widely in the degree of PIA or slime, and biofilm they produce.[5,36] The importance of the *ica* operon has been confirmed in numerous epidemiological studies, which found a higher prevalence of the *ica* genes in clinical than in control skin isolates.[37,38] Clinical strains of *S. epidermidis* obtained from urinary tract infection,[39] as well as from paediatric cancer patients receiving chemotherapy are reported to be related with *ica*-presence.[25]

Epidemiological studies have shown that the icaADBC operon is a typical feature of nosocomial *S. epidermidis* strains obtained from device-associated infections.[10,33] It is shown that *icaADBC* operon is mostly prevalent in strains associated with intravascular catheter-associated bacteraemia and septicaemia.[10,39] A study of the occurrence of *ica* operon among *S. epidermidis* isolates obtained from various origins has indicated that the genetic information for biofilm formation is rarely found in isolates obtained outside of hospital settings.[18] Interestingly, many of these studies found that invasive *S. epidermidis* strains significantly more often carried *icaADBC* than colonizing commensal *S. epidermidis* strains. Therefore, *icaADBC*-negative *S. epidermidis* strains were regarded as non-virulent and it was proposed to use *icaADBC* as a genetic marker to distinguish invasive and contaminating *S. epidermidis* in blood cultures.[37,38]

Phenotypic and genotypic instability of biofilm-forming ability

Phenotypic variation in *ica*-presence is commonly observed in *S. epidermidis*.[8,28,40] Ziebuhr and coworkers identified an insertional element (IS256) that was capable of inserting itself into the *ica*-locus resulting in *ica*-negative phenotypes.[8] This disruption was shown to be reversible as precise excision from the *ica*-locus which observed at low incidence resulting in *ica*-positive phenotypes.

We and others have shown that a significant proportion (42-85%) of clinical isolates are *ica*-negative during culturing in the laboratory.[28,40] In contrast to studies showing a reversible switching (phenotypic switching) between *ica*-positive and *ica*-negative phenotypes, the *ica*-locus was permanently lost in these strains. The absence of IS256 and phenotypic variation in these clinical *S. epidermidis* isolates and the inability to switch back to *ica*-positive suggested a new mechanism of switching in terms of biofilm formation involving genetic instability.[28]

We showed that the presence of the *ica*-locus in clinical isolates represents a disadvantage for growth in laboratory conditions. In line with this, it was recently suggested that the presence of the *icaADBC* operon represents a disadvantage when *S. epidermidis* colonizes the skin.[3,28]

Strains that have a high level of PIA production have a significant growth disadvantage under commensal conditions and are therefore outcompeted by strains with more moderate or absent PIA production. Whereas PIA production enables staphylococci to survive and grow under hostile, infection related conditions (biofilms), during commensal colonization (as well as during planktonic growth), PIA production can be considered a burden that can easily be subsided. It is important to conclude that the ability to express different slime-producing phenotypes could provide staphylococci with a greater degree of flexibility for colonizing a range of different environments.[33] Too much or no PIA production is only favourable under specific conditions while the ability to regulate PIA production allows the organism to adapt to all conditions, both commensal and infectious.[23]

Other studies have found, in *S. epidermidis*, IS256 detection is attributed to the epidemic biofilm-forming clonal lineages, and the element has been shown to trigger heterogeneous biofilm expression by reversible transposition into biofilm-associated genes and regulators.[21,41]

Thus, IS256 was shown to cause phase variation of *icaADBC* operon expression by alternating insertion in and precise excision from the PIA synthesis-mediating gene locus.[20] While switch-off of PIA production through IS256 insertions occurs with a frequency of approximately 10^{-6} per cell and generation, restoration of PIA-dependent biofilm formation by precise IS256 excision was found to be an extremely rare event (10^{-11} per cell and generation).[42]

The role of small colony variant (SCV)

Small colony variants are naturally occurring subpopulations of bacteria demonstrating distinctive phenotypic characteristics and pathogenic traits. Phenotypically, SCVs have a slow growth rate, atypical colony morphology associated with the formation of pinpoint or 'fried egg' colonies and unusual biochemical features. It was most extensively studied for staphylococci, especially for *S. aureus* as well as *S. epidermidis*.[43] SCVs were recorded as being <1 mm in size (less than 1/10 of the normal cell size), with reduced pigmentation and haemolytic activity as described in literature.[43,44] The tiny size of SCVs on solid agar is often due to auxotrophy for haemin and/or menadione, two compounds involved in the biosynthesis of electron transport chain components, which is associated with defects in electron transport and, consequently, altered membrane potential. The abnormal membrane potential, in turn, may confer on these variants innate resistance to aminoglycosides, since the ability of these antibiotics to gain access to intracellular target sites depends on the proton motive force. More importantly, some reports have linked bacterial SCVs to several recurring infections that are intractable to conventional treatment antibiotic regimes.[44–46]

Small colony variants have been associated with long-lasting, chronic, and recurrent infections, and it was suggested that this property was linked to the ability of SCVs to survive intracellularly, thereby being protected from the host immune system and the action of antibiotics. Both biofilm formation and the SCV phenotype may contribute to the recurrence and persistence of staphylococcal infections; bacteria are either embedded in large, adherent biofilms on the surfaces of implanted foreign bodies or may persist intracellularly in phagocytes, such as epithelial or endothelial cells, and thus evade the host immune system.[44,45] Additionally, it was proved that in vitro experiment using menadione auxotrophs of *S. aureus* and haemin auxotrophs of *S. epidermidis* [46]

resulted in the upregulation of alternative *sigma factor B*, which plays a central role in the augmentation of *icaADBC* expression and PIA production.[43,47]

Methicillin resistance gene

In addition to biofilm formation, nosocomial *S. epidermidis* isolates are characterized by their pronounced resistance against commonly used antibiotics including methicillin. Methicillin resistance is, similar with *S. aureus*, mediated by the *mecA* gene encoding a penicillin binding protein with reduced affinity to β-lactam antibiotics.[48] However, in contrast to methicillin-resistant *S. aureus* (MRSA), attention paid to methicillin resistant *S. epidermidis* (MRSE) in hospital settings is not adequate enough, meaning they are not dealt with by using intense hygienic measures as those for MRSA. As a result, methicillin resistance rates among nosocomial *S. epidermidis* isolates and other CoNS are extremely high and regularly exceed those of MRSA.[48–50] It has been reported approximately 80% of *S. epidermidis* isolates from device-associated infections are considered as MRSE, and also found to be multiresistant; whereas commensal strains obtained from the community are mostly methicillin-sensitive *S. epidermidis*.[10]

The *mecA* gene and its regulators are located on large DNA elements that are termed staphylococcal cassette chromosome mec (*SCCmec*). In addition to the methicillin resistance determinant, *SCCmec* carry a set of recombinases and a wide variety of mobile DNA elements such as transposons, insertion sequences or integrated plasmids.[1,15] To date, five major *SCCmec* types have been identified, all of them can be distributed over the *S. epidermidis* genome. Interestingly, SCCmec have been shown to be transferable among staphylococcal species. These genes are now regarded as mobile elements in which extensive recombination and gene shuffling takes place.[15,51] Obviously, they do not only serve as shuttles for the transfer of methicillin resistance but can also carry other staphylococcal genes. MRSE is often associated with additional antibiotic resistance, such as erythromycin (encoded by *erm* genes), ciprofloxacin, clindamycin, aminoglycosides (encoded in *aacA/aphD* gene) or trimethoprim-sulfamethoxazole.[15] The recent findings of genomic research strongly suggest that *S. epidermidis* and other coagulase-negative staphylococci represent the gene pool for the ongoing generation of novel SCC types from which methicillin resistance in *S. aureus* might originate.[1,12] Accordingly, it would be meaningful and reasonable to control MRSE and MR-CoNS by appropriate hygiene measures in a similar manner for MRSA, in order to lower MRSA burden in medical facilities, due to their role as reservoirs for the spread of resistance genes within microbial communities.

Genomic flexibility

It was demonstrated that clonal diversification in *S. epidermidis* is mainly based on genetic recombination, which is in contrast to *S. aureus*, a species known to evolve preferentially by point mutations.[21,41]

Multilocus sequence typing (MLST) analysis of a representative collection of clinical *S. epidermidis* isolates revealed a high degree of genetic diversity within the species, but the most widespread clone was ST2 or ST27 (sequence types). Especially, clonal complex ST2 isolates were found to be highly flexible with respect to methicillin resistance and prone to take up these mobile genetic elements. [21] Possibly, the successful spread of ST2 may be due to the fact that all ST2 isolates contain IS256 insertion sequences and *ica* genes, two factors that may have determinants to enhance transmissibility, persistence, or

invasiveness in *S. epidermidis*. In addition, most ST2 isolates show in vitro capacity to form biofilms.[15,21]

Instability of genetic material is often an indication of mobility, and in this respect it is also conceivable that the *ica* operon represents mobile DNA that has been lost in the commensal strain. *S. epidermidis* isolates ST 2(ST27) represent an ideal genetic background for biofilm and resistance genes, resulting in well-adapted strains which are then selected in the hospital environment and causes device-related infection and bacteraemia. The presence of multiple copies of IS256 in the ST27 genome might support this adaptation process by an ongoing generation of novel phenotypic and genotypic variants. Therefore, the combination of biofilm formation, antibiotic resistance, and genetic flexibility may explain why ST2 has become the dominant clonal variant within medical facilities.[8,41]

Clinical manifestation of related infection

Staphylococcus epidermidis and other CoNS have for a long time been dismissed as culture contaminations which is mainly due to the fact that CoNS are primarily ubiquitous commensals of the human skin and mucosa. It is still a great challenge for the clinical microbiology laboratory to distinguish infecting strains from contaminants. In suspected *S. epidermidis* infections, where the pathogen is also a skin commensal that could contaminate skin swab or blood specimen if aseptic techniques are not followed, the same indistinguishable microorganism must be cultured from at least two separate specimens in order to differentiate a relevant infection from skin contamination.[52] In contrast, for virulent species such as *S. aureus* or gram negative bacteria, a single positive clinical specimen may be sufficient to determine the presence of a recent infection.[53,54] However, some groups of the population are prone to be infected with this microorganism. These higher risk groups include preterm neonates, immunocompromised individuals and patients with indwelling medical devices.[1,5,10]

The most important clinical manifestation associated with CoNS, particularly *S. epidermidis* is biomaterial-associated infections (BAI), which include a unique, complex constellation of many factors that have to be considered for their successful management.[33] The increasing use of foreign materials in almost all fields of modern medicine is associated with a risk of bacterial infection.[10] Morbidity and mortality of biomaterial-associated infections may vary according to the underlying patient condition, the microbial strain(s) that are implicated, and the type of device. Biomaterial-associated infections contribute significantly to the increasing problem of nosocomial infections. While a variety of microbial strains have been involved as causative organisms in biomaterial-associated infections, staphylococci, particularly *S. epidermidis*, account for the majority of infections related to both temporarily inserted and permanently implanted biomaterials.[5,10]

The presence of a biomaterial significantly compromises the host's ability to cope with infectious microorganisms. These microorganisms can reach a biomaterial implant in several ways and at different times post-implantation. Airborne microorganisms, inevitably present in the operating theater, can reach a biomaterial implant surface as early as before the implantation. Also during insertion of a biomaterial implant, microorganisms from the commensal microflora of the skin can contaminate a biomaterial implant. Peri-operative contamination is believed to be the most common cause of biomaterial associated infection.[10,18,33]

Bacteria that adhere to implanted medical

devices or damaged tissue can encase themselves in a hydrated matrix of extra cellular polymeric substances, a slimy layer, and start growing into a biofilm. Bacteria organized in biofilms are at least 10-1000 times more resistant to antibiotics [14,16] and can cope much better with unfavorable external conditions as the host immune system than their planktonic counterparts. The biofilm mode of growth represents a benefit for staphylococcal strains enabling them to colonize inert surfaces of medical devices.[8] Antibiotic resistance of bacteria in the biofilm mode of growth contributes to the chronic nature of these infections, which are notoriously difficult to resolve. The mechanisms of bacterial resistance in biofilms are different from the now familiar plasmids, transposons, and mutations that convey innate resistance to individual bacterial cells. In biofilms, resistance seems to depend on multicellular strategies resulting in an impaired penetration of antibiotics to the target organisms and a decreased immune response.[14,55]

Biomaterial-associated infections comprise local (e.g., exit site) and systemic infections. Originating from bacteremia or other systemic spread of causative organisms and depending on the nature and localization of the biomaterial inserted, sepsis, endocarditis, meningitis, joint sepsis, vertebral abscesses, and other local manifestations due to metastatic seeding may result.[1,19] These comprise infections commonly associated with prosthetic vascular grafts, prosthetic heart valves, cardiac devices, and coronary stents. Moreover, local inflammation signs include erythema, warmth, swelling, tenderness, and purulent drainage, which characterize exit-site infections.

It has been shown that *S. epidermidis* was the most frequent agents of central venous catheter (CVC) and umbilical catheter-associated BSIs (Blood Stream Infection) in neonatal ICUs.[5,25]the coagulase-negative staphylococci (CoNS Besides BSIs, the CoNS group may cause further invasive infections in preterm infants, such as infective endocarditis, meningitis, and necrotizing fasciitis.[5,12] Additionally, *S. epidermidis* is also considered as the main cause of septicemia in febrile patients who suffer from chemotherapy-induced neutropenia, which is accounting for approximately 20 to 40% of cases.[1,25]

CONCLUSION

So far it is still a great challenge for clinician to distinguish *S. epidermidis* strains that may cause infection from those that live on the skin. However, the virulence properties identified in this paper, such as the presence of biofilm formation phenotype including *icaADBC* operon, *IS256*, *mecA*, SCV properties, together with patient characteristics, might be used to consider the pathogenesis of infection caused by *S. epidermidis*. Nevertheless, up to date, the clues to distinguish between infectious and commensal strains of *S. epidermidis* are not clear yet. It is well understood the adhesion to host tissue is considered crucial during both these lifestyles.

By living on the verge of commensalism and pathogenicity, *S. epidermidis* has elaborated many strategies to overcome different clinical environments, including the new ecological niche of biomaterials. In addition, the growing number of immunocompromised patients increases the risk for a very sensitive host. The formation of biofilms, the acquisition of resistance characteristics and the enormous flexibility of the genome of staphylococci are characteristics that help their survival in specific environments and are the main reasons why staphylococci have become the most successful pathogens in clinical setting. With respect to their possible role as true pathogens, *S. epidermidis* infection should be

taken more seriously with adequate prevention applications for future infection control and hygiene measures.

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