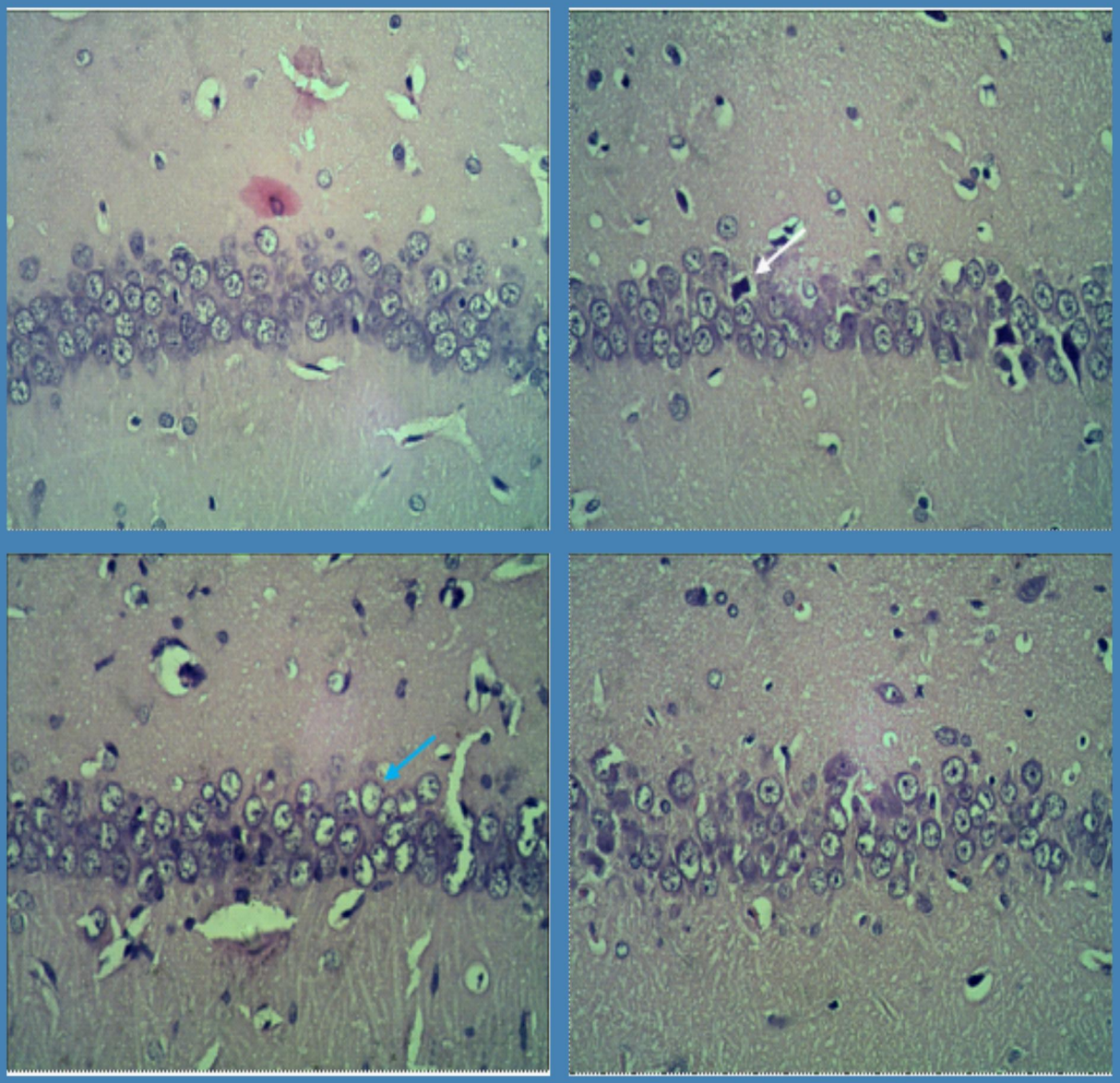
ISSN: 2356-3931 (Online)

Journal of the Medical Sciences

(Berkala Ilmu Kedokteran)

Gadjah Mada Journal of the Medical Sciences

Volume 54 No 2, 2022



Published by
Faculty of Medicine, Public Health, and Nursing,
Universitas Gadjah Mada, Yogyakarta, Indonesia

Credits

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JMedSci

Journal of the Medical Sciences
(Berkala Ilmu Kedokteran)

Volume 54, Number 2, 2022; 103-113
http://dx.doi.org/10.19106/JMedSci005402202201

Increased blood-brain barrier permeability correlate with microglial activation at hippocampal CA1 region in acute and chronic bilateral common carotid artery ligation in rats

Dian Prasetyo Wibisono[1], Nur Arfian[2], Handoyo Pramusinto[1], Fauziyatul Munawaroh[3], Yeshua Putra Krisnugraha[3], Daniel Agriva Tamba[1], Dwi Cahyani Ratna Sari[2]

[1]Division of Neurosurgery, Department of Surgery, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada/Dr. Sardjito General Hospital, Yogyakarta, [2]Department of Anatomy, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, [3]Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia

Submitted: 2022-01-31
Accepted : 2022-03-09

ABSTRACT

Inflammatory processes might play a key role in the pathogenesis of post-stroke epilepsy. The activation of microglia and release of vascular cell adhesion molecule-1 (*VCAM1*) might induce blood-brain barrier (BBB) disintegration. However, the influence of such pathomechanisms in the generation of post-stroke epilepsy is still not clear. We investigated whether cerebral ischemia exerts effects on inflammation in the hippocampus by measuring the hippocampal injury score, expression of a microglial marker, and expression of *VCAM1* in rats. A total of 24 Sprague Dawley rats were randomized into four groups with 6 rats in eachgroup i.e. sham operation (SO) as control, carotid ligation 1 (GCL1) as an acute model, carotid ligation 3 (GCL3) as a subacute model, and carotid ligation 7 (GCL7) as a chronic model. Immunostaining for microglia marker (CD68) was measured in rat brain tissue sections. The *VCAM1* expression was evaluated by reverse transcription-polymerase chain reaction (RT-PCR). Cerebral ischemia increased the amount of microglial immunostaining and expression of *VCAM1*. The hippocampal injury score and microglial immunopositivity were significantly correlated with the duration of brain ischemia. We conclude that cerebral ischemia is correlated with neuroinflammatory reaction and disturbance of BBB permeability, and the correlation of those molecular impairments with the generation of post-stroke epilepsy remains to be elucidated.

Keywords:
blood-brain barrier;
hippocampus;
microglia;
post-stroke epilepsy;
VCAM1

ABSTRACT

Proses inflamasi kemungkinan berperan penting dalam patogenesis epilepsi pasca stroke. Aktivasi mikroglia dan pelepasan *vascular cell adhesion molecule-1* (VCAM1) dapat menurunkan fungsi sawar darah otak. Namun, pengaruh setiap mekanisme patogensis tersebut dengan munculnya epilepsi pasca stroke masih belum diketahui dengan baik. Dalam penelitian ini, peneliti mengkaji apakah iskemia cerebri mencetuskan inflamasi di hippocampus dengan menetapkan skor luka hippocampal, ekspresi marker mikroglia, dan ekspresi dari VCAM1 pada tikus. Total 24 ekor tikus Sprague Dawley dibagi secara acak dalam empat kelompok yaitu *sham operation* (SO) sebagai kontrol, *carotid ligation 1* (GCL1) sebagai model iskemia akut, *carotid ligation 3* (GCL3) seebagai model iskemia subakut, dan *carotid ligation 7* (GCL7) sebagai model iskemia kronik. Pemeriksaan imunohistokimia marker mikroglia (CD68) dilakukan pada potongan otak tikus. Ekspresi *VCAM1* diperiksa dengan *reverse transcription-polymerase chain reaction* (RT-PCR). Iskemia serebral meningkatkan imunopositivitas mikroglia dan ekspresi *VCAM1*. Skor luka hippocampal dan imunopositivitas mikroglia berkorelasi nyata terhadap durasi iskemia serebral. Peneliti berkesimpulan bahwa iskemia serebral berkaitan dengan rekasi neuroinflamasi dan gangguan permeabilitas sawar darh otak, dan hubungan antara proses molekular tersebut dengan munculnya epilepsi pasca stroke masih harus diteliti lebih lanjut.

*corresponding author: dr.dianprasetyo@gmail.com

INTRODUCTION

Epilepsy is one of the neurological conditions that cause disability in patients, with an incidence rate of 61.4 per 100,000 person-years.[1] Epilepsy might be associated with genetic abnormality (i.e., idiopathic epilepsy) or may be caused by a wide array of intracranial disorders, including cerebrovascular attack or stroke.[2] The cerebrovascular attack or stroke are considered as the most common causes of seizures and epilepsy in the elderly.[3] Research conducted by the Oxfordshire Community Stroke Project (OCSP), reported that 11.5% of patients with stroke had a risk of experiencing a post-stroke seizure within 5 y after a stroke.[4]

Following the stroke event, an inflammatory cascade occurs, which leads to post-stroke glial cell proliferation.[5] Microglia act as resident macrophages and are key modulators of the brain immune response. This cell is one type of glial cell that increased in number after stroke.[6] The role of microglia in epileptogenesis is still uncertain, but several studies have shown that reactive microglia are found in the brains of temporal lobe epilepsy animal model.[7] Furthermore, increased activity of microglia also have been shown in the brain tissue section of epileptic patients.[8,9]

Several molecular dysregulations were postulated as the impact of the increasing number of microglial expressions, including the impairment of BBB integrity.[10-13] Human brain endothelial cells forming the BBB can release *VCAM1* and the level of microglial was positively correlated with *VCAM1* expression level.[14] High level of *VCAM1* was associated with the breakdown of the BBB, but to date, it is unknown whether *VCAM1* itself modulates BBB permeability.[15]

In an ischemic condition after stroke, several regions in the hippocampus, including CA1 region, are known to be the region with high vulnerability.[16] This variability was primarily associated with the difference in N-methyl-D-aspartate (NMDA) receptor activation, a type of glutamate receptor.[17] An increased activity of NMDA receptor might increase the excitotoxicity following ischemia.[17] In such conditions, the presence of extensive and multiple injuries, cortical damage, and hippocampal involvement are predictors of post-stroke epilepsy.[4] However, the mechanism that drives this condition is still unclear.

Currently, only a few studies focused on the correlation between the incidence of inflammation and the blood-brain barrier damage as the basis for the mechanism of post-stroke epilepsy, especially related to the hippocampus as the most vulnerable structure in ischemic injury. This mechanism may provide a basic important mechanism for prevention, pharmacological, and surgical therapy in cases of post-stroke epilepsy.

We hypothesized that the ischemic condition following stroke could increase the number of microglia in the brain, which leads to an increase in BBB permeability and ultimately increased the incidence of post-stroke epilepsy. To address this hypothesis, a model of cerebral ischemia (i.e., bilateral common carotid artery ligation model) in rats was utilized. The changes in the number of microglia as the marker of brain inflammation were then assessed using immunohistochemistry. In addition to this, we also hypothesized that the increased expression of microglial activity after experimentally-induced stroke increases the expression of *VCAM1*, which might be associated with BBB breakdown.

MATERIALS AND METHODS

This was a quasi-experimental study with a post-test only controlled

group design using 24 Sprague Dawley male rats, 4 wk, weighing 100 g. Rats were obtained from Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia. The animals were randomized and maintained with standard laboratory conditions and given access to an ad libitum diet and tap water. The protocol of study was approved (FIGURE 1) the Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada (ref. KE/FK/0222/EC/2021).

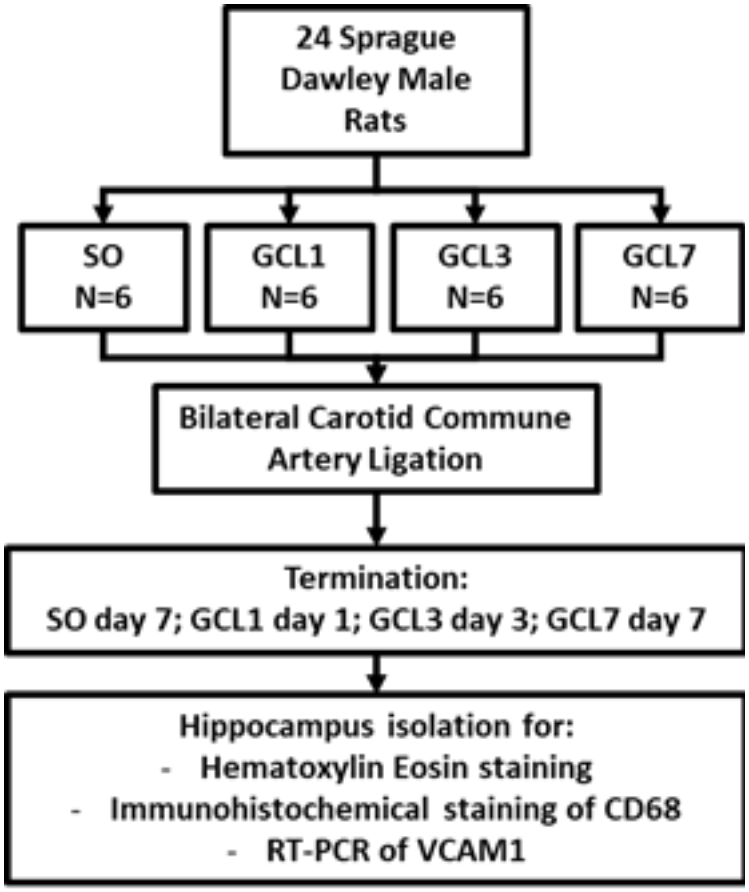

FIGURE 1. Experimental steps

Bilateral common carotid artery ligation for stroke model rats

Rats were divided into 4 groups containing 6 rats in each group i.e. sham operation (SO) as control, carotid ligation 1 (GCL1) as an acute model, carotid ligation 3 (GCL3) as a subacute model, and carotid ligation 7 (GCL7) as a chronic model. All experiments were performed under general anesthesia. Intraperitoneal injection of pentobarbital solution 1:10 (0.1 mg/10 g BW) was used as the anesthesia agent. In this model, we performed clamping of the right and left common carotid artery using a non-traumatic vascular clamp p (Karl Hammacher GmbH, Solingen, Germany) for 30 min. After 30 min, the clamp was released. The incised skin was then closed using surgical thread silk 3/0 (OneMed-Healthcare, Surabaya, Indonesia). In the SO group, no artery clamping was performed and the rats only underwent a cervical incision followed by the closing of the

incised skin. All rats in this group were euthanized on day 7. In the remainder groups, bilateral common carotid artery clamping was performed on all rats. All subjects were then euthanized on day 1 in CGL 1 group, day 3 in the CGL3 group, and day 7 in CGL7group.

Brain sample preparation for immunohistochemistry and hematoxylin-eosin staining

All animals were transcardially perfused with 4% paraformaldehyde (PAM) in 50 mM phosphate buffer and decapitated. The brains were kept in 4% PAM. Rats' brains were paraffinized and cut at 4 μm with a microtome (Leica Biosystems, UK). Rats' brain fixation and the cutting process were performed by a laboratory assistant and this author.

Microscopic analysis

Hippocampal injury score

Brain sections were stained with hematoxylin-eosin (HE) and examined with an Olympus CX22 light microscope (Olympus Corporation, Tokyo, Japan). The images were then portrayed using the Optilab software at 400x magnification at the CA1 area. As mentioned earlier, this hippocampal region has been recognized as an injury-prone area in an ischemic condition.[17] Hippocampal injury scores were determined using a semi-quantitative scoring system, as suggested by Møller *et al.*[18] Fifteen fields per hippocampus were chosen as region of interests (ROI). In each ROI, the injuries were graded from 0–4 (0: normal; 1: injury affecting <25%; 2: injury affecting 25-50%; 3: injury affecting 50-75%; 4 injury affecting >75%). According to these variables, the thickness of the pyramidal layer, pyramidal cell distribution, pyramidal cell clumping, gaps, and cytoplasmic color were evaluated.[18]

Immunohistochemical staining of CD68

Microglia were evaluated by immunohistochemistry on 4 μm brain slices using antibodies against CD68. The sections were deparaffinized and rehydrated using 100, 90, 80, and 70% alcohol, followed by the heating process in citrate buffer (pH 6) for antigen retrieval and blocking endogenous peroxidase using H_2O_2 3% in PBS solution. The slides were then incubated using Background Sniper, rabbit 1st monoclonal antibody CD68 with 1:200 dilution (Abcam, ab32570, Cambridge, United Kingdom), TrekAvidin-HRP, 2nd antibody anti-rabbit Trekkie Universal Link (Biocare Medical, STUHRP700, California, United States), and diaminobenzidine tetrahydrochloride (Biocare Medical, STUHRP700H L10). The results were analyzed using the ImageJ software, examined with a light microscope (Olympus CX22), and portrayed with the Optilab software at 400x magnification.

Reverse transcriptase PCR analysis

We performed RT-PCR analysis and electrophoresis to assess the changes in the expression of *VCAM1* after the experimentally-induced stroke.

RNA extraction and cDNA synthesis

Total RNA was extracted using Genezol (Geneaid GZR100, Geneaid Biotech Ltd, New Taipei City, Taiwan), followed by quantification of RNA concentration using spectrophotometry. We used 3,000 ng RNA for making cDNA. The cDNA was made using Rever Tra Ace® (Toy-obo Cat. No. TRT-101, Osaka,

Japan) and random primer (Toyobo Cat. No. 3801), with PCR conditions: 30°C for 10 min (denaturation), 42°C for 60 min (annealing) and 99°C for 5 min (extension).

Reverse transcriptase PCR and electrophoresis

The RT-PCR was carried out to amplify the following specific cDNAs: *VCAM1* (F: GTCTACACCTCCCCAAGAAT and R: GGAGATGTCAACAGTAAATGGTTTC); and *GAPDH* (F: GGCACAGTCAAGGCTGAGAATG and R: TCTCGCTCCTGGAAGATGGTGA). The RT-PCR was performed by mixing 2 μL cDNA, 12.5 μL of Taq Master Mix (Bioron, Germany, Cat. No. S101705), 0.6 μL of forward and reverse primer, and 9.3 μL of PCR water.

The cDNA was amplified to the following conditions: 94°C for 2 s (initial denaturation), 94°C for 10 s (denaturation), 60°C for 20 seconds (annealing), 72°C for 1 min (extension), and 72°C for 10 min (last extension) for 35 cycles. The PCR products were analyzed in 2% agarose gel along with a 100 bp DNA ladder (Bioron Cat. No. 306009, Germany). Expressions of the gene were quantified with densitometry analysis using the ImageJ software. The housekeeping gene used was *GAPDH*.

Statistical analysis

The data obtained were analyzed using the Shapiro Wilk test for distribution analysis. The Pearson correlation and Spearman correlation tests were used if the data were normally and abnormally distributed, respectively. Multiple comparisons among the groups were made using one-way Anova and followed by post hoc LSD tests if the data were normally distributed. If the data were abnormally distributed, Kruskal Wallis and post-hoc Mann Whitney tests were used. A p value < 0.05 was considered to be significant.

RESULTS

Microscopy analysis

Bilateral common carotid artery ligation induced hippocampal injury. Such occurrence was characterized by thickness decreasing of the pyramidal layer, increased gap between pyramidal cell, pyramidal cell clumping, and increased pyramidal cell with pale cytoplasm (FIGURE 2a). Hematoxylin-eosin staining revealed there was a progressive injury from acute, sub-acute, to the chronic phase. Quantification of the hippocampal injury score showed a significantly higher hippocampal injury score in the GCL1, GCL3, and GCL7 groups compared with the SO group (p=0.000). The GCL7 group demonstrated the highest hippocampal injury score followed by GCL3 and GCL1 groups (FIGURE 2a and 2b). This condition suggested that injury progressed and still occurred in the chronic phase.

Bilateral common carotid artery ligation induced a significant increase in the number of positive CD68 (microglia) fraction areas in GCL1, GCL3, and GCL7 groups compared with the SO group (p=0.002) (FIGURE 3a and 3b). This condition was associated with the increased number of activated microglia in the hippocampus following ischemia. In the chronic phase, the microglia presents in its active form, as the amoeboid form increased compared to the processed form (FIGURE 3a).

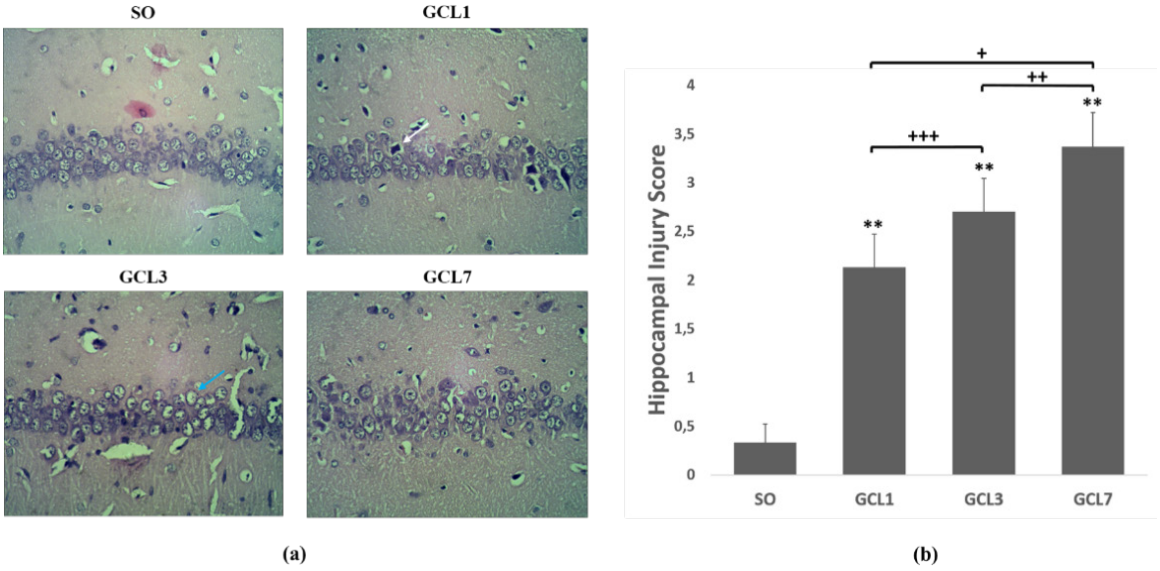

FIGURE 2. Histological quantification of hippocampal injury. (a). Representative FIGURE of Hematoxylin Eosin staining. The presence of pyramidal cell clumping (white arrow) and the increasing number of pyramidal cells with pale cytoplasm (blue arrow) are more frequent in the group with longer duration of cerebral ischemia. In such group, the pyramidal layer is thinner and gap between pyramidal gap is increased. This will lead to a higher hippocampal injury score in the chronic ischemic group. (b). Results of hippocampal injury score. The difference of hippocampal injury score was analyzed using one-way Anova (p=0.000). Asterisks show significance between SO and GCL groups (**, p<0.001). Elbow connectors show significance between GCL groups (+, p<0.001; ++, p<0.01; +, p<0.05).

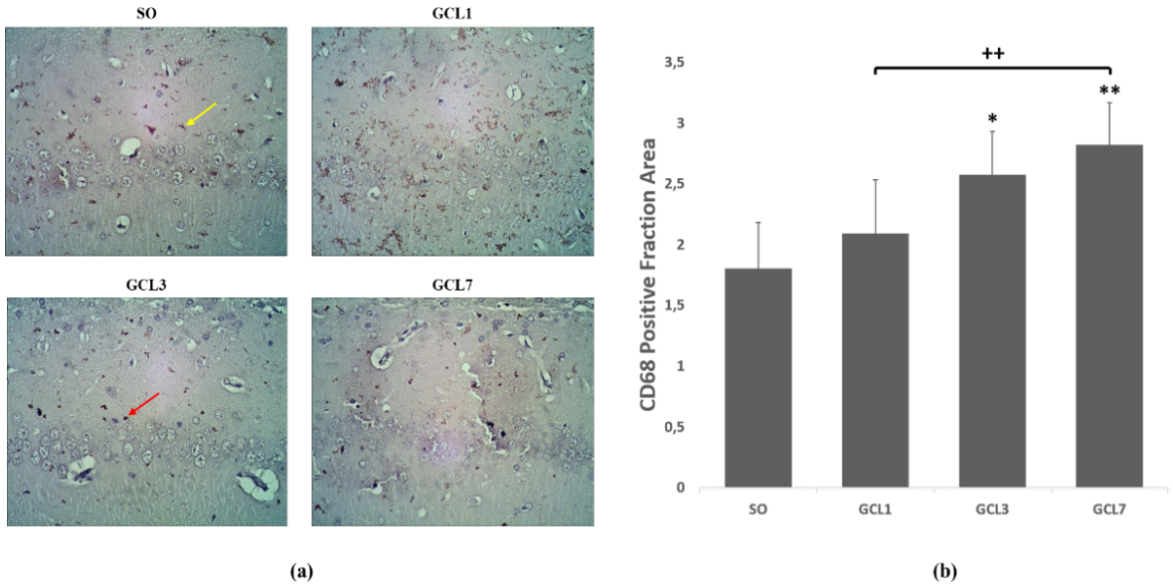

FIGURE 3. Immunohistochemical quantification of CD68. (a). Microscopic FIGUREs of CD68 positive fraction area. CD68 was observed in control and all ischemic groups. Microglial activity is shown in the form of ameboid cells (red arrow) and the appearance of cell processes (yellow arrow). (b). CD68 positive fraction area were analysed using one-way ANOVA (p=0.000). Asterisks show significance between SO and GCL groups (**, p<0.001; *, p<0.01). Elbow connectors show significance between GCL groups (++, p<0.01).

RT-PCR analysis of *VCAM1* expression

Bilateral common carotid artery ligation induced a significantly higher mRNA expression of *VCAM1* in the GCL1, GCL3, and GCL7 groups, compared to the SO group. This suggests that ischemia, either in acute or chronic conditions, leads to increasing *VCAM1* production (p=0.000) (FIGURE 4a and 4b).

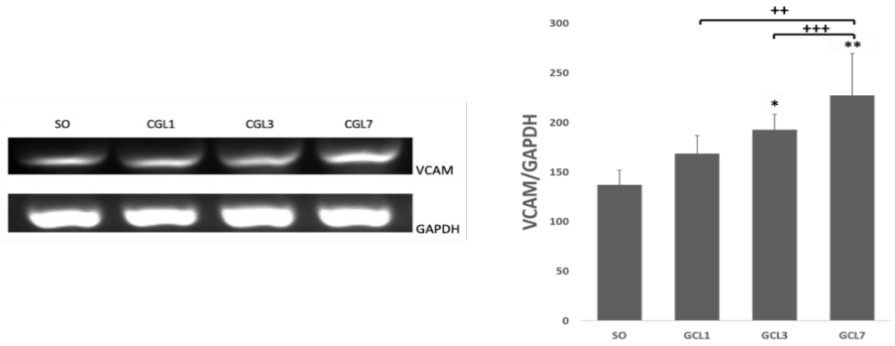

FIGURE 4. (a) Gel electrophoresis FIGUREs of RT-PCR analyses of *VCAM1* and *GAPDH* gene after 48 hours incubation. (b). Bar charts of relative quantification for mean *VCAM1/GAPDH* mRNA expressions. Data were analysed using one-way ANOVA (p=0.000). Asterisks show significance between SO and GCL groups (**, p<0.001; *, p<0.01). Elbow connectors show significance between GCL groups (++, p<0.01; +++, p<0.05).

Correlation test

The Pearson correlation test showed a strong positive correlation between CD68-positive fraction area with the *VCAM1* expression (p=0.000; r=0.661), *VCAM1* expression with the hippocampal injury score (p=0.000; r=0.761), and CD68-positive fraction area with the hippocampal injury score (p=0.000; r=0.681).

DISCUSSION

Until now, the impact of the inflammatory response on the pathogenesis of post-stroke epilepsy is unclear. In this study, arterial ligation to trigger ischemic conditions in the rat's brain was performed. The temporary blood flow cessation led to a caused significant neuronal death. Such damage was assessed microscopically at CA1 region of the hippocampus using hippocampal injury score quantification. The hippocampal injury scores were significantly higher in all groups with arterial ligation procedures than in SO group. Also, this project focused on the role of microglia on the inflammatory response following brain stroke. It was found that the neurological deterioration observed in the ischemic group might be associated with inflammation-mediated by microglia. CD68 immunopositivity, which marks the presence of microglia, was significantly increased in rats undergoing arterial ligation. Moreover,

this study assessed the integrity of BBB after the ischemic insult by evaluating the level of *VCAM1* expression in the brain ischemia models. *VCAM1* expression was raised in two ischemic groups (i.e., CGL 3 and CGL 7), indicating a disruption in BBB integrity.

Microglial activation occurred at acute, sub-acute, and chronic phases of ischemic injury.[19] In the present study, CD68 immunopositivity was highest in GCL7 group (i.e., a model of chronic ischemic phase), followed by GCL3, GCL1, and SO group. This condition suggested that the amount of microglia after ischemic condition might be graded temporally. As shown in a previously published article, the amount of microglial activation was positively correlated with the number of the day spent following the ischemic insult.[20] Moreover, inflammatory response post-ischemia is characterized by morphological alterations and increased mobility of microglia.[21] In this study, the evolution of microglial form into an amoeboid type might be observed in GCL3 and GCL7 groups.

On the other hand, *VCAM1* expression also showed a progressive increase from the acute phase toward the chronic phase. The highest expression showed in the GCL7 group, followed by GCL3, GCL1, and SO group. *VCAM1* is a glycoprotein that is inducible and expressed in endothelial cells. Its expression is dramatically increased by hypoxia.[22] It is activated by proinflammatory cytokines and Reactive Oxygen Species.[23] In this study, microglial activation could lead to the expression of some proinflammatory cytokines such as IL1β, IL6, and TNFα, depending on the polarization of the microglia. Microglia can undergo phenotypic changes in a process known as polarization.[24] Microglia, which are glial cells like macrophages, are rapidly activated and differentiate into M1 or M2.[25] M1 phenotype are the proinflammatory state and will secrete proinflammatory cytokines that induce tissue damage.[26] Several proinflammatory cytokines produced include IL1β, IL6, TNFα, and iNOS.[27,28] Related to the expression of proinflammatory cytokines, it is known that *VCAM1* was dramatically increased by IL1 β and TNFα.[29] In contrast, the M2 phenotype has anti-inflammatory properties that mainly work in debris clearance, extracellular matrix deposition, and angiogenesis.[30] In this study, we could not differentiate between these two types of microglial phenotypes.

A previous study revealed that epileptic conditions can be caused by inflammation cascade and BBB dysfunction, but the correlation between those two, is still unclear.[10] In this study, the Pearson correlation test showed a strong positive correlation between CD68 and *VCAM1* expression. It can be assumed that increased proinflammatory cytokines such as IL1β, produced by activated microglia, may induce the increase of *VCAM1* expression. *VCAM1* acts as a mediator for peripheral immune cells such as leukocytes to infiltrate through the BBB. It will add inflammatory response and also endothelium dysfunction and BBB leakage. The contribution of brain intrinsic inflammatory reactions compared with those mediated by peripheral immune cells was still unclear, but BBB failure could be the link between these two mechanisms. [31]

Due to its unique structural architecture, BBB and its permeability have a significant role in brain impairment.[32] Failure of BBB function could induce seizure activity through brain inflammation and BBB permeability.[10] Epilepsy could be the result of the inflammatory response and the endothelium impairments.[33] The inflammatory response includes the secretion of several inflammatory factors from neurons, astrocytes, and microglia. On the other hand,

infiltration of leukocytes through the BBB via adhesion molecules might be the reason for endothelium dysfunction and BBB leakage in epileptic patients.[31,33,34] The expression of cytokines and inflammatory chemokines and matrix metalloproteinases in activated microglial cells contribute directly or indirectly to BBB damage.[35] The M1-type microglial cells produce TNF-α and IL-1β, and affect the localization of VE-cadherin, occludin, and claudin-5, and therefore contribute to BBB disruption.[36] Therefore, both inflammatory reaction and BBB disruption contributes to epileptic condition originating from hippocampus. Moreover, such contribution occurs in acute to chronic phase of ischemic insult.

This study has some limitations. We did not assess the direct relationship between neuroinflammation (i.e., microglial activity and *VCAM1* expression) and post-stroke epilepsy. We suggest that future studies should include an analysis of the influence of post-stroke neuroinflammation in an animal model of epilepsy and its relation with the duration of cerebral ischemia.

CONCLUSION

Our data demonstrate that acute and chronic phases of ischemic injury in the hippocampal CA1 region might induce an inflammatory response. Accumulation of activated microglia and increased level of *VCAM1* expression might be correlated with post-ischemic neuroinflammation and BBB disruption, respectively. The association between those events and the pathogenesis of post-stroke epilepsy remains to be elucidated.

ACKNOWLEDGEMENTS

We would like to thank Mulyadi for his great assistance during the completion of this study.

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JMedSci

Journal of the Medical Sciences
(Berkala Ilmu Kedokteran)

Volume 54, Number 2, 2022; 114-124
http://dx.doi.org/10.19106/JMedSci005402202202

Potential secondary metabolite analysis of soil *Streptomyces* sp. GMR22 and antibacterial assay on *Porphyromonas gingivalis* ATCC 33277

Hera Nirwati[1]*, Ema Damayanti[2], Eti Nurwening Sholikhah[3], Mustofa[3], Jaka Widada[4]

[1]Department of Microbiology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, [2]Research Center for Food Technology and Processing, National Research and Innovation Agency, Gunungkidul, Indonesia [3]Department of Pharmacology and Therapy, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, [4]Department of Microbiology, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, Indonesia

Submitted: 2021-12-26
Accepted : 2022-03-03

ABSTRACT

Infectious diseases caused by oral pathogenic bacteria are currently a serious problem due to the increasing incidence of antimicrobial resistance. *Streptomyces* sp. GMR22, a soil actinobacterium which has large-genome size. In previous studies, it was known to have antifungal, and antibiofilm activity on *Candida albicans*. However, its antibacterial activity on oral pathogenic bacterium, *Porphyromonas gingivalis* is not clear. This study aimed to identify potential active compound based on genome mining analysis and to evaluate the antibacterial activity of GMR22 extract on *P. gingivalis* ATCC 33277. Potential active compounds and biosynthesis gene clusters were analysis using antiSMASH version 5. Antibacterial activity assay was carried out by the microdilution method on *P. gingivalis* ATCC 33277. Based on genome mining analysis polyketide synthase (PKS), the *Streptomyces sp.* GMR22 is the abundant BGCs (35%) and has large-predicted compounds which have antibiotic-antibacterial activity (22.9%). On antibacterial assay, chloroform extract of GMR22 at 7.8 – 62.5 µg/mL has high antibacterial activity on *P. gingivalis* compared to other extracts. Soil *Streptomyces* sp. GMR22 bacterium has biotechnological potential to produce active compounds for antibacterial.

Keywords:
actinobacteria;
antibacterial;
Streptomyces;
Porphyromonas gingivalis

ABSTRACT

Penyakit infeksi yang disebabkan oleh bakteri patogen rongga mulut hingga saat ini masih menjadi masalah serius yang disebabkan oleh peningkatan kejadian resistensi antimikroba. *Streptomyces* sp. GMR22 merupakan aktinobakteri tanah yang memiliki ukuran genom besar. Penelitian sebelumnya, bakteri mempunyai aktivitas antijamur dan antibiofilm pada *Candida albicans*. Namun, belum diketahui aktivitasnya terhadap bakteri patogen oral seperti *Porphyromonas gingivalis*. Penelitian ini bertujuan untuk mengidentifikasi senyawa aktif potensial berdasarkan analisis penambangan genom dan mengevaluasi aktivitas antibakteri dari ekstrak GMR22 terhadap *P. gingivalis* ATCC 33277. Senyawa aktif potensial dan klaster gen – gen biosintesis dianalisis dengan menggunakan antiSMASH version 5. Uji aktivitas antibakteri dilakukan dengan metode mikrodilusi terhadap bakteri *P. gingivalis* ATCC 33277. Berdasarkan analisis penambangan genom *polyketide synthase* (PKS), *Streptomyces sp.* mempunyai klaster gen – gen biosintesis dominan (33%) dan senyawa yang diprediksi memiliki kemampuan sebagai antibiotik – antibakteri (22,9%). Pada uji antibakteri, ekstrak kloroform GMR22 pada konsentrasi 7,8 – 62,5 µg/mL memiliki aktivitas antibakteri tertinggi pada *P. gingivalis* dibandingkan ekstrak lainnya. Bakteri tanah *Streptomyces* sp. GMRR22 memiliki potensi bioteknologi untuk menghasilkan senyawa aktif antibakteri.

*corresponding author: hera.nirwati@mail.ugm.ac.id

INTRODUCTION

Porphyromonas gingivalis is an opportunistic pathogenic bacterium, commonly found in the human body and especially in the oral cavity, where it is associated with periodontal diseases.[1] Several clinical strains of *P. gingivalis* show moderate susceptibility or resistance to amoxicillin and metronidazole.[1] The other study showed that the *P. gingivalis* samples isolated from periodontitis patients showed relatively similar rates of resistance to amoxicillin (24.6%), azithromycin (21.3%) and metronidazole (24.6%).[2] Some antibiotics maybe does not reach the bacteria in the biofilm, so it requires a higher dose to kill.[3] Efforts are needed to find new sources of antibiotics that are effective against *P. gingivalis*.

Actinobacteria especially genus *Streptomyces* have produced bioactive compounds for more than 10,000 of the 18,000 known bacterial bioactive compounds.[4] These bacteria have a huge genome size, between 6.2 and 12.7 Mb and 5% of their genome is devoted to the synthesis of secondary metabolites.[5] The discovery of new active compounds from Actinobacteria especially genus *Streptomyces* has become an important research because of the resulting novelty in chemical diversity and promising natural products for new drugs.[6]

In previous research, *Streptomyces* sp. GMR22, a soil bacterium[7] demonstrated promising antifungal activity.[8] Based on bioassay studies, this bacterium has the biotechnological potential for drug discovery. In recent years, the drug discovery from *Streptomyces* bacteria is not only based on bioassay procedure but also based on genome analysis and metabolite profiling. The genome mining techniques are currently genome analysis as the solution for accelerating the discovery of new drug candidates.[9] With this technique, biosynthetic gene clusters (BGCs) could be identified from genome analysis and used for the chemical core structures prediction.[10] Over the last 10 years, several new active compounds from *Streptomyces* have been revealed using genome mining approaches.[11]

The huge biotechnology potential of the soil bacteria *Streptomyces* sp. GMR22 drives exploratory research on this bacterium as a source of new antibiotics. This study aimed to identify the potential active compounds of Streptomyces sp. GMR22 using a genome mining approach and to evaluate its activity against *P. gingivalis*. This research is expected to provide information on the potential of *Streptomyces* sp. GMR22 as a source of antibiotics to treat *P. gingivalis* infection.

MATERIALS AND METHODS

Biological material

Streptomyces sp. GMR22, a soil bacterium isolated from the rhizosphere of the Cajuput medicinal plant, *Melaleuca leucadendra* (L), Myrtaceae, in Wanagama Forest, Gunung Kidul, Yogyakarta, Indonesia.[7] The GMR22 isolate has been deposited at InaCC A148, Indonesian Institute of Sciences, and NBRC Japan (NBRC 110112). GMR22 16S RNA sequence has been submitted in the National Center for Biotechnology Information (NCBI), accession code MN922646. The whole-genome shotgun projects have been deposited in Data Bank of Japan/ European Nucleotide Archive/GenBank with accession number JACGSQ000000000 for *Streptomyces* sp. GMR22. *Porphyromonas gingivalis* ATCC® 33277 (Culti-loops™) purchased from Thermo Scientific, Lenexa, USA. *P. gingivalis* was maintained in Brain Hearth Infusion (BHI) Broth at the Microbiology Laboratory, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Morphological analysis

The bacterial cell is grown at 28°C for 14 d on International *Streptomyces* Project-2 (ISP-2) medium (Difco, Spark, USA) were observed by scanning electron microscopy (SEM) (Hitachi SU3500, Japan). The SEM analysis in this study was performed without any chemical fixative.[12] One loop of bacterial biomass was added evenly to the surface of the carbon tape-covered stub. The sample was air-dried and coated with Au (Hitachi MC1000 Au ion sputter, Japan) with a setting of 10 mA for 60 sec and observed using SEM. The SEM settings operated in high vacuum mode, an accelerating voltage of 5 kV, a spot intensity of 30%, and magnifications of 5,000 and 10,000 x. The SEM analysis was conducted at Research Center for Food Technology and Processing, National Research, and Innovation Agency, Gunung Kidul, Indonesia.

Genome mining analysis

The identification, annotation, and analysis of gene clusters involved in the biosynthesis of secondary metabolites and predictions of the core structures produced were conducted by using antiSMASH 5.0,[13]genome mining has seen broad applications in identifying and characterizing new compounds as well as in metabolic engineering. Since 2011, the 'antibiotics and secondary metabolite analysis shell-antiSMASH' (https://antismash.secondarymetabolites.org available at http://antismash.secondarymetabolites.org.

Fermentation and extraction

Streptomyces sp. GMR22 was maintained on ISP-2 Agar medium (Difco, Sparks, USA). *Streptomyces* spp. was cultured at 28 °C with 180 rpm agitation for 2 - 3 d in a 250 mL Erlenmeyer flask containing 100 mL of tryptic soy broth (TSB) (Difco, Sparks, USA) as the seed medium. Then, the cells were transferred into four 1000 mL flasks containing 500 mL of starch nitrate broth (SNB) as the production medium and incubated for 8 d at 28 – 29 °C with 180 rpm agitation in a shaker incubator (Stuart S1500). The SNB medium contained 0.5 g of NaCl, 1 g of KNO_3, 0.5 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$ and 20 g of soluble starch in 1000 mL of distilled water.[14] All media was sterilized using autoclave machine (Tommy) at 121 °C, 2 atm, for 15 min.

Secondary metabolites were obtained by separating the cell biomass from the liquid using refrigerated centrifugation at 4137 × g at 4 °C for 15 min. The supernatant was extracted two times with an equal volume of ethyl acetate, and evaporated using a rotary vacuum evaporator (Buchi, Switzerland) to obtain the crude extract. The crude ethyl acetate extract was fractionated using chloroform, *n*-hexane, ethyl acetate and methanol to separate the polar and nonpolar fractions. All extracts were then evaporated using a vacuum evaporator (Buchi, Germany). The crude extracts were weighed for yield extract analysis and stored in the refrigerator (Gea). All chemical reagents and solvents were purchased from Merck KGaA, Darmstadt, Germany.

Antibacterial assay

The antibacterial assay against *P. gingivalis* ATCC® 33277 was conducted using the microdilution protocol described in a previous study with minor modification.[15] The extract was prepared in six levels of concentration (7.8 – 62 µg/mL in 0.1% of dimethyl sulfoxide (DMSO)) as solvent and re-diluted using Brain Hearth Infusion (BHI) medium (Merck, Germany). The initial bacterial suspension used for inoculation for assay was adjusted to 5 x 10^5 CFU/mL in BHI medium using

McFarland standard. An untreated growth control (without extract) was included. The plates were incubated at 37°C for 24 h, anerobic condition (AnaeroGen™). Growth inhibition was determined by a spectrophotometer at 0 and 24 h. Optical densities at 600 nm (OD_{600} nm) were measured using a multi-scan reader (Thermo Scientific). All experiments were performed in triplicate. Growth inhibition (%) was determined by change in OD (ΔOD) from the start of incubation to the final time point (24 hours). Growth inhibition was calculated with the following formula: [(ΔOD control –ΔOD test)/ΔOD control] x 100. The IC_{50} was defined as the lowest concentration at which 50% of growth was inhibited. The IC_{50} were determined by nonlinear regression analysis of log_{10} concentrations of the extract against percent *P. gingivalis* inhibition. Statistical analysis was performed using One-way Anova followed by Dunnett's multiple comparison test for analysis of treatment. Nonlinear regression and statistical analysis were done by using GraphPad Prism 9.0.1 software.

Targeted liquid chromatography high-resolution mass spectrometry (LC-HRMS)

Metabolite analysis of the active extract was carried out using ultra-high-performance liquid chromatography (TS Vanquish UHPLC) coupled to targeted high-performance mass spectrometry (Thermo Scientific Dionex Ultimate 3000 RSLC Nano UHPLC paired with Thermo Scientific Q Exactive) (Thermo Fisher Scientific, Bremen, Germany). Targeted LC-HRMS was based on a predicted compound formula which was obtained from the genome mining analysis of *Streptomyces* sp. GMR22 whole-genome sequence using antiSMASH version 5[13] available online at https://antismash.secondarymetabolites.org/. HRMS was carried out with mobile phases A (water + 0.1% formic acid) and B (acetonitrile + 0.1% formic acid). The column used was an Accucore™ Phenyl Hexyl analytical column (2.1 mm × 2.6 µm) (Thermo Fisher Scientific) with a flow rate of 40 µL/min, an injection volume of 5 µL, and a gradient with an analysis time of 25 min. The gradient was programmed as follows: 2 min, 5% B; 15 min, 60% B; 22 min, 95% B; 25 min, 95% B; 25.1 min, 5% B; and 30 min, 5% B. Experiments were carried out in full MS data dependent MSMS at 70,000 the full width at half maximum (FWHF) resolution, heated electrospray ionization, positive ionization, and data processing with Thermo Scientific XCalibur and Compound Discover 3.2. The data was analysis using procedures for targeted processing workflows for expected compounds. The mol file of expected compounds was selected and was input in analysis system based on manual procedures of Compound Discoverer version 3.2, user guide for LC studies (ThermoFisher Scientific, 2020).

Ethical clearance

This study has been approved by the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health and Nursing, the Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia (ref. No. KE/0927/09/2020).

RESULT

The GMR22 bacterial strain was observed to be Gram-positive and aerobic, grew well on ISP-2 Agar medium, ISP-4 Agar medium, and TSA after 5 – 7 d at 28 - 29 °C. Morphological observations of the 7-day-old culture grown on ISP2 medium showed a smooth spore surface with aerial and vegetative hyphae. Soil bacterium GMR22 has white aerial spores at young culture and changed into gray spores at old culture. *Streptomyces* sp.

GMR22 spores have a round shape and form a spirals chain (FIGURE 1).

Based on genome mining analysis (FIGURE 2), *Streptomyces* sp. GMR22 had the highest number of BGCs among the large genome-sized *Streptomyces* group. GMR22 has the highest BGCs (65 of BGCs) with PKS as dominant BGCs. The other BGCs, hybrid NRPS – PKS, NRPS, and terpene also as major BGCs.

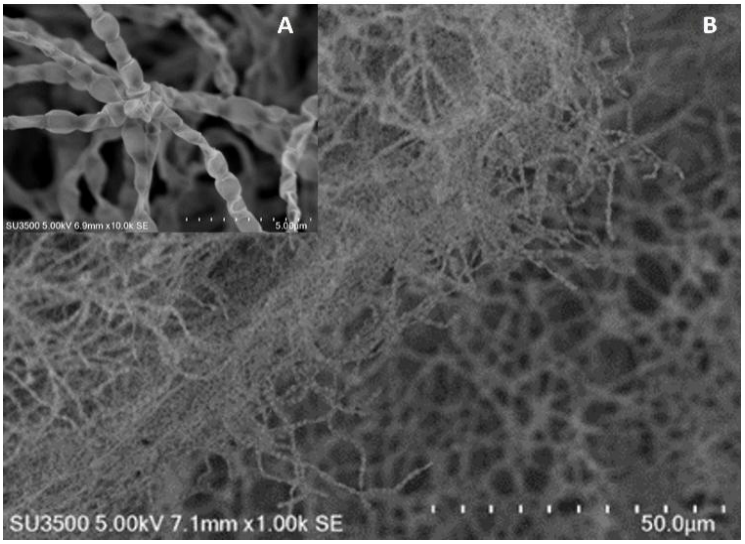

FIGURE 1. Morphology of *Streptomyces* sp. GMR22 using scanning electron microscope with 10,000x of magnification (A) and 1,000x of magnification (B)

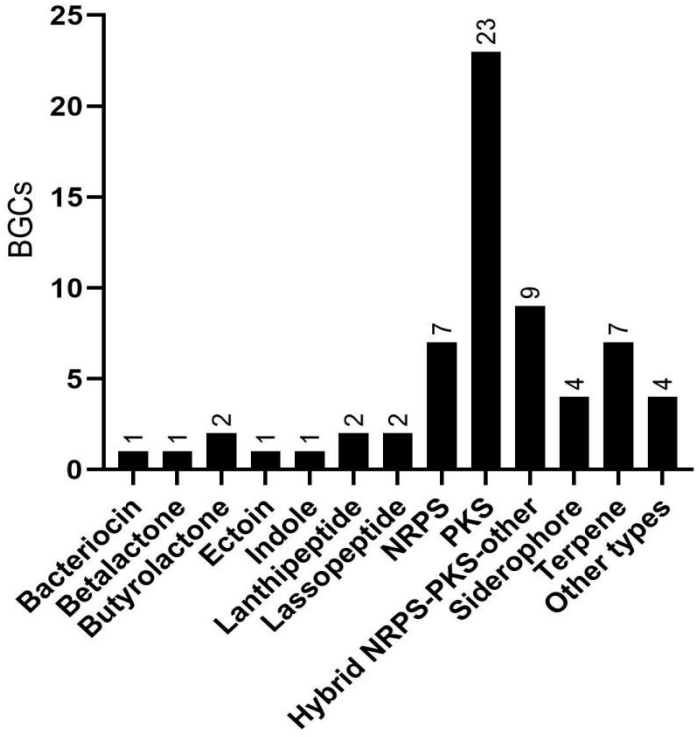

FIGURE 2. Biosynthesis gene clusters of secondary metabolites distribution of *Streptomyces* sp. GMR22 based on genome mining using AntiSMASH 5[13]

Based on their biological activities, most of the compounds had known biological activities, while some compounds had no known specific activity. The biological activities possessed by the compounds predicted to be produced by this bacterium was shown in FIGURE 3. Based on this FIGURE 3, it is dominated by antibiotic compounds that have antibacterial activities.

FIGURE 4 showed that the chloroform extract (CE) at 7.8 – 62.5 µg/mL has the significant antibacterial activity against *P. gingivalis*. This extract has the higher antibacterial activity than *n*-hexane, ethyl acetate and methanol extracts. The fungal growth was 56.16 – 72.65% and it was lower than bacterial growth in the control (100 %). The statistical analysis showed that there was significant difference at 7.8 – 15.62 µg/mL ($p = 0.001$) and at 31.25 – 62.5 µg/mL ($p<0.0001$) with control without CE ($p<0.05$).

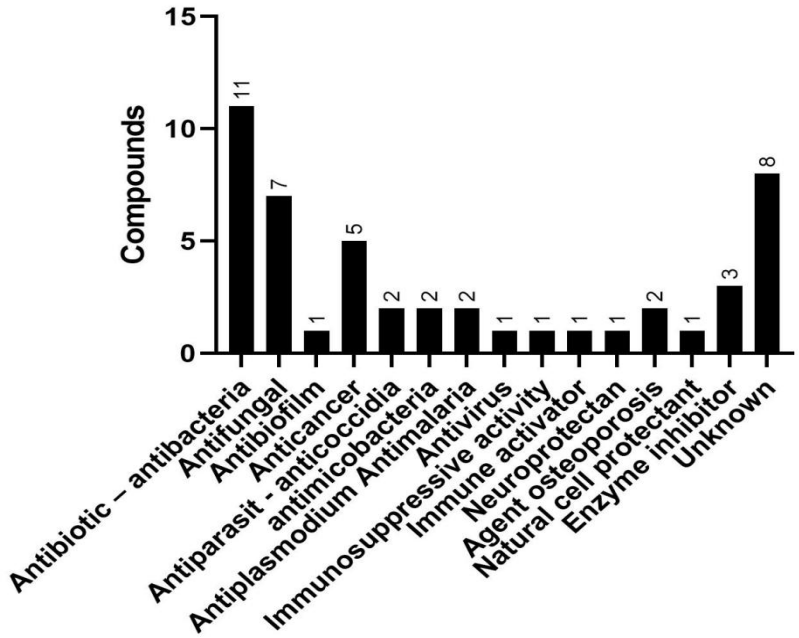

FIGURE 3. The biological activities of known compounds of *Streptomyces* sp. GMR22 based on genome mining analysis

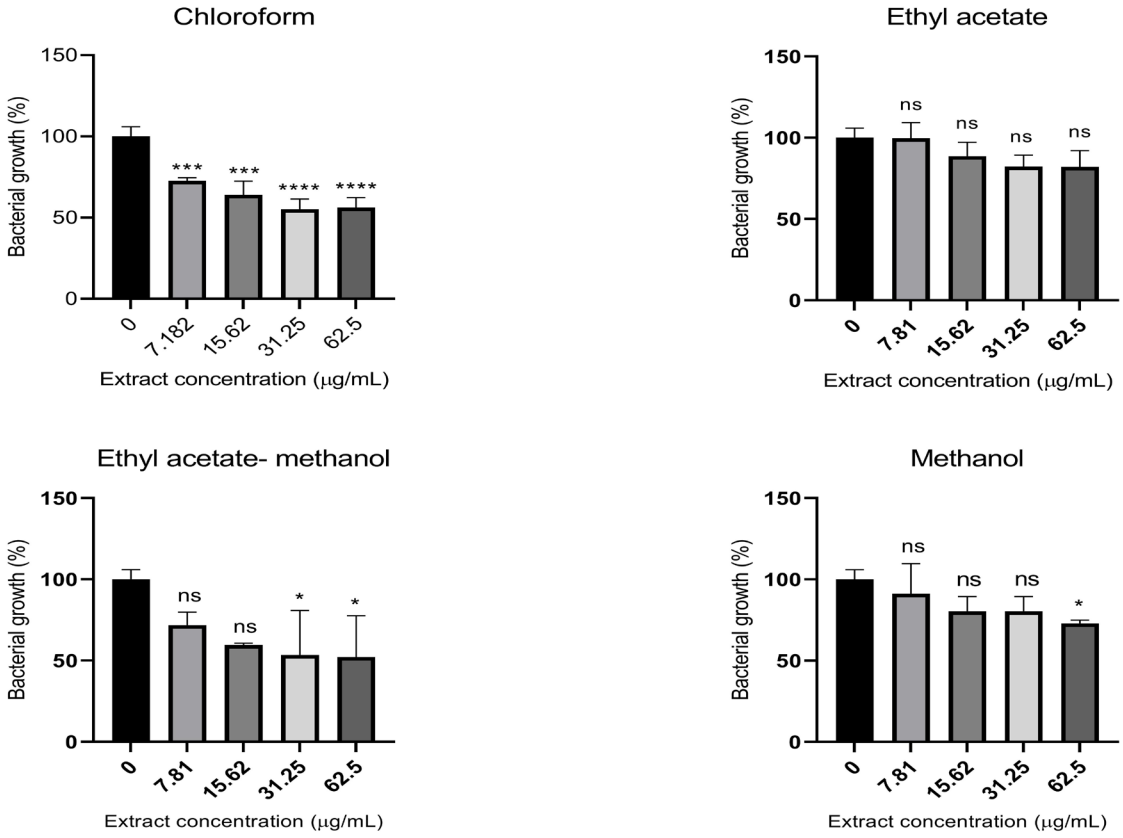

FIGURE 4. Antibacterial activity of soil *Streptomyces* sp. GMR22 extract on *Porphyromonas gingivalis*. Value are expressed as mean ± SD ($^{ns}p>0.5$, $^{*}p<0.1$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$) compared to control without any extract.

Prediction of the content of active compounds from chloroform extract was carried out using targeted LC-HRMS. TABLE 1 shows that the five selected targeted compounds had undergone a transformation and had changes in both formula and molecular weight.

TABLE 1. Targeted LC-HRMS of *Streptomyces* sp. GMRR chloroform extract

Parent compound	Formula	Molecular weight	Transformations	Composition change	RT (min)	Area (Max)
Hygrocin A	$C_{28}H_{31}NO_6$	477.21514	Dehydration, nitro reduction	-(O2)	17.426	23582396.96531
Amipurimycin	$C_{20}H_{35}N_7O_5$	453.26997	Hydration, nitro reduction, nitro reduction	-(O3) +(H6)	23.35	259957945.55138
Geldamycin	$C_{29}H_{42}N_2O_{11}$	594.27886	Hydration, oxidation	+(H2 O2)	23.82	42389047.14245
Bicyclomycin	$C_{12}H_{20}N_2O_2$	224.15248	Dehydration, nitro reduction, nitro reduction	-(O5) +(H2)	25.24	23911337.10273
Nigericin	$C_{40}H_{74}O_{10}$	714.5282	Hydration, nitro reduction, reduction	-(O) +(H6)	25.34	48124828.60041

DISCUSSION

The *Streptomyces* sp. GMR22 isolated from rhizopshere soil has the closest relationship to *S. lactacystinicus* strains OM-6519^T. The *S. lactacystinicus* strains OM-6519^T was isolated from soil samples near Lake Inba, Chiba, Japan.[16]respectively.Here, the taxonomic positions of these two strains were determined. The morphological and chemical features of strains OM-6519T and K04-0144T indicated that they belonged to the genus Streptomyces. Strain OM-6519T showed the highest 16S rRNA gene sequence similarities with Streptomyces xanthocidicus NBRC 13469T (99.7% GMR22 has high BGCs with PKS as dominant BGCs (FIGURE 2). This result was similar to the other previous studies. Genome mining analysis using antiSMASH version 3 revealed that the genome of *Streptomyces* sp. GMR22 harbored at least 63 BGCs with polyketide synthetase (PKS) as the major group of the identified gene cluster products.[17] GMR22 also exhibited the presence of PKS-I and NRPS genes were amplified by PCR[7] These genes were predicted to have a correlation with the antifungal activity of GMR22.[7]

Streptomyces rapamycinicus NRRL 5491 (12.7 Mbp) (accession number QYCY00000000)[18] has 53 BGCs, and *S. bingchenggensis* BCW-1 (11.9 Mbp) (accession number CP002047)[19] has 49 BGCs. The considerable genome potential of GMR22 suggest that this Indonesian *Streptomyces* species may be a promising source of new drugs.

The most antibiotic compounds produced by GMR22 bacteria such as meilingmycin,[20] daptomycin,[21] feglymycin as anti-HIV,[22] antinomycin,[23] glycinocin,[24] bafilomycin B1,[25] chlorothricinc,[26] medermycin,[27] paromomycin,[28] cadaside,[29] and azalomycin F3a,[30] GMR22 soil bacteria are also known to produce the most antifungal compounds such as, ECO-02301,[31] selvamicin,[32] mediomycin A,[33] natamycin,[34] This is in line with the previous studies demonstrating that GMR22 produces antifungal compounds against molds and yeasts.[7,17,35] GMR22 is also known to be the only bacterium that is predicted to produce antiplasmodial compounds, namely geldamycin[36,37] and salinomycin.[38]

The antibacterial activity in this study was similar with previous studies that showed chloroform extract has the highest inhibition against *C. albicans* (>20 mm of clear zone diameter) within n-hexane, benzene, and ethyl acetate extracts.[35] The IC_{50} value of antifungal against *C. albicans* was 62.5 µg/mL.[17]

Therefore, the active compound belonging Streptomyces sp. GMR22 could be predicted to be a new compound and has nothing in common with the predicted compound based on genome mining analysis. Further research related to the isolation of active compounds from the active fraction of chloroform extract is very important. In addition, the mechanism of antibacterial action of the active compounds found is also important to know.

CONCLUSION

Streptomyces sp. GMR22 has abundant biosynthetic cluster genes and has the potential to produce new types of antibiotics. Chloroform extract of GMR22 fermentation product has the potential to produce new compounds as antibacterial against *P. gingivalis*.

ACKNOWLEDGEMENTS

This work was supported by Indonesian Ministry of Research and Technology and Higher Education through Excellent Basic Research for Higher Education (Penelitian Dasar Unggulan Perguruan Tinggi) (Project No. 2757/UN1.DITLIT/DIT-LIT/PT/2020).

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JMedSci

Journal of the Medical Sciences
(Berkala Ilmu Kedokteran)

Volume 54, Number 2, 2022; 125-131
http://dx.doi.org/10.19106/JMedSci005402202203

Analysis of systemic risk factors of occipital stroke-related vision loss

Indra Tri Mahayana*, Nyssa Alexandra Tedjonegoro, Tatang Talka Gani

Department of Ophthalmology, Faculty of Medicine, Public Health, and Nursing Universitas Gadjah Mada/Dr. Sardjito General Hospital, Yogyakarta, Indonesia

Submitted: 2022-01-09
Accepted : 2022-02-12

ABSTRACT

Occipital stroke is associated with visual field defects and other visual perceptual deficits that might lead to detrimental effects on health-related quality of life. This study aimed to evaluate the possible association between systemic risk factors and the features of occipital stroke. It was a retrospective observational study involving patients diagnosed with occipital stroke in Dr. Yap Eye Hospital, Yogyakarta, Indonesia, between 2013 and 2014. A total of 72 patients with occipital stroke proven with CT/MRI who underwent detailed evaluation of ocular and systemic risk factors were included in this study. The patients were divided into two groups with or without risk factors. The risk factors were hypertension, diabetes mellitus, and cardiac diseases. The effect of risk factors on sex, age groups (≤40, >40 and ≤60, >60 years old), stroke lesions (left occipital, right occipital, bilateral), and visual field defects (homonymous hemianopia, homonymous quadrantanopia, and others) were analyzed. Out of 72 patients, there were 56 males (77.8 %), and 16 females (22.2 %), with a mean age of 58.46 ± 11.83 years old. The study found a significant difference in age with risk factors compared with those without risk factors (p = 0.025), as well as visual acuity with age groups (p = 0.005) and stroke lesions (p = 0.024). Analysis of risk factors showed that hypertension was significantly correlated with stroke lesions, whereas cardiac disease was significantly associated with age groups (p<0.05). Predictive analysis was performed using a linear regression model, and it showed that risk factors could predict the occurrence of stroke lesions. In conclusion, patients' systemic risk factors are significantly associated with the older onset of occipital stroke and lower visual acuity, although not significantly associated with stroke location and visual field defect characteristics.

ABSTRAK

Stroke oksipital berhubungan dengan defek pada bidang visual dan defisit persepsi visual lainnya yang dapat merugikan kualitas hidup yang berkaitan dengan kesehatan. Penelitian ini bertujuan untuk mengkaji hubungan antara faktor risiko sistemik dan karakteristik stroke oksipital. Penelitian ini merupakan penelitian retrospektif observasi pada pasien dengan stroke oksipital yang berobat di RSM Dr. Yap, Yogyakarta, Indonesia, pada tahun 2013 sampai 2014. Total sebanyak 72 pasien stroke oksipital yang dibuktikan dengan CT/MRI dan menjalani evaluasi faktor risiko okuler dan sistemik secara detail dilibatkan dalam penelitian. Pasien dibagi menjadi dua kelompok, yaitu kelompok dengan faktor risiko (hipertensi, diabetes mellitus, dan penyakit jantung) dan tanpa faktor risiko. Pengaruh faktor risiko pada jenis kelamin, kelompok usia (≤40, >40 - ≤60, >60 tahun), lesi stroke (oksipital kiri, oksipital kanan, bilateral), dan defek lapang pandang (hemianopia homonim, quadrantanopia homonim, dan lain-lain) dianalisa. Dari 72 pasien, 56 laki-laki (77,8%) dan 16 perempuan (22,2%) dengan usia rata-rata 58,46 ± 11,83 tahun. Terdapat perbedaan nyata pada usia dengan faktor risiko dibandingkan tanpa faktor risiko (p = 0,025), dan juga pada ketajaman penglihatan pada kelompok usia (p = 0,005) dan lesi stroke (p = 0,024). Analisa faktor risiko menunjukkan bahwa hipertensi berhubungan bermakna dengan lesi stroke, sedangkan penyakit jantung secara bermakna berhubungan dengan kelompok usia (p< 0,05). Analisa prediktif dilakukan menggunakan model regresi linear. Analisa tersebut menunjukkan bahwa faktor risiko dapat menentukan keberadaan lesi stroke. Kesimpulan, faktor risiko sistemik yang dimiliki pasien berhubungan dengan onset stroke oksipital yang lebih lama dan ketajaman visual yang lebih rendah, namun tidak berhubungan dengan lokasi stroke dan karakteristik defek bidang visual.

Keywords:
aging;
neuro-ophthalmology;
occipital stroke;
visual acuity;
visual cortex

*corresponding author: tri.mahayana@gmail.com

INTRODUCTION

Stroke is a common problem in the elderly that leads to significant disability. Although most patients have no other neurological deficits aside from visual-field defects, occipital stroke has significant impacts on their quality of life, which include changes to independent living, ability to drive, loss of confidence, and some links to depression.[1-3]

The most common cause of occipital lobe infarct is posterior cerebral artery (PCA) ischemia, also known as posterior circulation ischemic stroke, which is caused by a cardiac embolism and blocked local artery to artery sources.[4] Cardiac disease is the most common source of embolism (41%), and patients with a cardiac source of an embolism usually have pure PCA infarcts (81%).[5] Vascular disease due to hypertension and diabetes mellitus may be the main underlying cause of stroke.[6-8] A study by Subramanian[9] in 2009 reported that diabetes mellitus is associated with the increased odds of posterior circulation ischemic stroke. This study is supported by Kim *et al*. in 2012, who found that hypertension and diabetes mellitus were more related to posterior than anterior circulation ischemic stroke.[10]

Occipital stroke is associated with visual field damage with detrimental effects on health-related quality of life.[3] The PCA infarction may lead to homonymous hemianopia (HH) and other visual perceptual deficits, which is expected since it creates damage to the areas responsible for the central visual pathway.[11,12]

This study aimed to evaluate the association of systemic risk factors i.e. hypertension, cardiac disease, and diabetes mellitus and the characteristics of occipital stroke such as patients' age, visual loss, visual field defects, and stroke laterality.

SUBJECTS AND METHODS

Design and study population

This retrospective observational study used the medical records of occipital stroke patients who came to the outpatient clinic at Dr. Yap Eye Hospital between January 2013 and December 2014.

Protocol of study

The diagnosis of occipital stroke was performed through clinical examinations (complete ophthalmic evaluation: visual acuity, anterior segment examination, funduscopy, and visual field examination) by an ophthalmologist and then continued by reviewing the results of imaging modalities such as computerized tomography (CT)-scan, magnetic resonance imagery (MRI), or both, performed by the radiologists.

Basic characteristics of patients and systemic risk factors (hypertension, diabetes mellitus, and cardiac diseases) were collected from the medical records. The laterality and location of the stroke in the occipital cortex were also recorded for analysis. Visual acuity was assessed at a fixed distance with the Snellen chart, and the visual field examination used Goldmann kinetic perimetry. Patients with pre-existing ophthalmological problems were excluded, such as cataracts, glaucoma, retinopathy, or other ocular pathologies that may affect the visual field. The study was conducted in accordance with the Declaration of Helsinki. The institutional ethics board of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia, approved the study and waived individual consent for this retrospective analysis by issuing the ethical clearance number KE/FK/0749/EC/2017.

Statistical analysis

Statistical analysis was performed using the IBM SPSS Statistic for Windows Version 20.0 (IBM Corp. Armonk, NY, USA). The patients were divided into two groups with (hypertension, diabetes mellitus, and cardiac diseases) or without risk factors. The effect of risk factors on sex, age groups (≤40, >40 and ≤60, >60 years old), stroke lesions (left occipital, right occipital, bilateral), and visual field defects (HH, homonymous quadrantanopia, and others) were analyzed using Chi-square tests, whereas age and visual acuity were analyzed using independent t-tests if the data distribution was normal. The association between stroke lesions and visual acuity were analyzed using one-way Anova followed by post-hoc analysis. Predictive analysis using linear regression models was performed to predict the possibility of systemic risk factors affecting the stroke lesions and the visual field defects. A value of $p < 0.05$ was considered statistically significant.

RESULTS

A total of 72 patients (mean age 58.46 ± 11.83 years) were included in this study with 56 males (77.8 %) and 16 females (22.2 %). TABLE 1 describes the subjects' characteristics, which indicate almost half of the subjects had a right occipital lesion (45.05%). The most frequent visual field defect in patients with occipital stroke was hemianopia (69.44%). Data showed that most patients had hypertension (61%) as their main risk factor for occipital stroke.

TABLE 1. Characteristic of subjects

Variables	Number
Sex [n (%)]	
▪ Male	56 (77.8)
▪ Female	16 (22.2)
Age (mean ± SD years)	58.46 ± 11.83
Visual acuity (mean ± SD dec)	0.37 ± 0.35
Stroke lesions [n (%)]	
▪ Left occipital	24 (33)
▪ Right occipital	31 (45.05)
▪ Bilateral	10 (13.88)
▪ Others	7 (9.72)
Visual field defects [n (%)]	
▪ Hemianopia	50 (69.44)
▪ Quadrantanopia	10 (13.88)
▪ Others	12 (16.67)
Risk factors [n (%)]	
▪ Hypertension	44 (61.10)
▪ Diabetes mellitus	22 (30.60)
▪ Cardiac disease	9 (12.50)

TABLE 2 shows the association of sex, age, and visual acuity with the presence of systemic risk factors. The visual acuity of the right eye (VOD) was significantly correlated with age groups (F=5.630, p=0.005). In comparison, the visual acuity of the left eye (VOS) was not (F=2.036, p=0.138) (TABLE 3). Post hoc pairwise comparisons (Bonferroni adjusted) showed a significant difference between the two age groups: ≤40 years old versus >60 years old (p=0.012). Significant results were also observed between the locations of stroke lesions and the visual acuity for both eyes (VOD (F=3.338, p=0.024) and VOS (F=3.682, p=0.016)). Post hoc pairwise (Bonferroni adjusted) comparison was significant on VOD and VOS for left occipital stroke versus bilateral occipital stroke (p=0.039 and p=0.023, respectively).

TABLE 2. The correlation of subject characteristics with the presence of systemic risk factors

Variables	Without risk factors (n=15)	With risk factors (n=57)	p
Sex [n (%)]			
▪ Male	10 (66.67)	46 (80.7)	0.245
▪ Female	5 (33.33)	11 (19.3)	(OR:0.478, CI:0.136-1.684)
Age (mean ± SD years)	52.4 ± 16.22	60.05 ± 9.95	0.025
▪ ≤40	2 (13.33)	1 (1.75)	
▪ >40 and ≤60	9 (60)	31 (54.38)	0.093*
▪ >60	4 (26.67)	25 (43.85)	
VOD (mean ± SD)	0.54 ± 0.37	0.32 ± 0.33	0.028*
VOS (mean ± SD)	0.51 ± 0.36	0.34 ± 0.35	0.090

Note: Sex was analyzed by Chi-square test; Age, VOD, and VOS (shown in decimal) were analyzed by independent samples t-tests; visual acuity: means ± standard deviation; *significant (p<0.05); CI= confidence interval.

TABLE 3. Risk factors analyses based on laterality of the stroke and visual field defects

Variables	Without risk factors (n=15)	With risk factors (n=57)	p
Stroke lesions [n (%)]			
▪ Left occipital	5 (33.33)	19 (33.33)	
▪ Right occipital	8 (53.33)	23 (40.35)	0.715
▪ Bilateral	1 (6.67)	9 (15.78)	
▪ Others	1 (6.67)	6 (10.53)	
Visual field defects [n (%)]			
▪ Hemianopia	11 (73.3)	39 (68.42)	
▪ Quadrantanopia	3 (20)	7 (12.28)	0.430
▪ Others	1 (6.67)	11 (19.33)	

Note : p value : Chi-square tests

TABLE 4 shows the analysis of the risk factors based on stroke characteristics. The results showed that there were no significant differences observed. Spearman's correlation of risk factors and stroke lesions, visual field defects, and age groups showed that hypertension had a significant correlation with stroke lesions (r= 0.317, p=0.007), the cardiac disease had a significant correlation

with age groups (r= 0.288, p=0.014), and diabetes mellitus had marginally significant correlation with visual field defects (r=-0.199, p=0.095). Furthermore, predictive analysis using a linear regression model of stroke lesions and risk factors showed that risk factors could predict the stroke lesions (R^2 = 0.102, p=0.049), where hypertension was the most common systemic risk factor (t=2.610, p=0.011). However, the visual field defects did not have a significant result (diabetes mellitus and visual field defects: t= -1.749, p=0.085).

TABLE 4. The correlation of systemic risk factors, visual field defects, and age groups

Systemic risk factors	r	p
Hypertension	0.317	0.07
Cardiac disease	0.288	0.014*
Diabetes mellitus	-0.199	0.095

*significant (p<.05)

DISCUSSION

This study found that patients with risk factors were associated with older age and lower visual acuity, but it was not associated with stroke laterality and visual field defect characteristics. Previous studies revealed that patients with hypertension and diabetes mellitus were more significantly associated with posterior circulations stroke.[9,10] Although the contribution of systemic risk factors might be combined with the occurrence of a specific condition such as vertebral artery hypoplasia.[13]

The significant difference in visual acuity between right and left occipital stroke was presumably due to coincidence since occipital stroke might not affect visual acuity. However, this finding was in line with a study conducted by Rowe *et al.*,[14] which also mentioned that this asymmetry might affect patients' ability to read in the future. This study also found that sex was not associated with systemic risk factors. On the contrary, age groups and visual acuity were associated with risk factors in which older age had more underlying systemic diseases and lower visual acuity. A study conducted by Naess *et al.*,[15] revealed that occipital infarction was associated with younger patients. Although occipital stroke may not create any changes to visual acuity, further detailed visual acuity analyses showed associations of visual acuity with the location of occipital stroke lesions (TABLE 3). The visual acuity of patients with bilateral lesions and involvement of parietal or temporal lesions were lower compared to patients who only have unilateral occipital stroke lesions. This finding showed that there might be other underlying risk factors. Poor visual acuity is a risk factor for falls and a common impediment to rehabilitation.[16,17] After a stroke, visual impairment may exacerbate the impact of other impairments on overall disability.[16,17]

TABLE 4 shows there was no association between stroke lesions and visual field defects with the underlying risk factors. Hypertension and diabetes mellitus might create an effect on the development of stroke-associated visual field defects, although the frequency distribution analysis of risk factors based on visual field defects was not statistically significant. However, predictive analysis using linear regression showed that systemic risk factors could predict the occurrence of stroke lesions, with hypertension being the most frequently

found factor. This finding shows that vascular factors, such as hypertension, might be one of the significant risk factors in the occurrence of occipital stroke.

Rowe *et al*.[14] found that cortical strokes which are associated with visual field loss, particularly in the occipital, temporal, and parietal lobes, were mainly caused by PCA infarcts. Infarcts on PCA's territory are common, and their clinical signs and symptoms are well-known.[18,19] Occipital strokes may produce HH visual field defects although, other visual field defects such as quadrantanopia (HQ) and other defects might occur due to lesions affecting the occipital white matter or defects with the adjacent temporal or parietal area, such as occipitotemporal and occipitoparietal lesions.[1,14] Lack of visual field improvement is the most accurate prediction for the high risk of cortical blindness. Ischemic stroke due to occipital lobe lesions causes most HH, which generally does not produce any other neurologic manifestations. The HH configuration does not predict the location of the lesion within the retrochiasmal visual pathway.[18]

There are several limitations in this study. First, this is not a population-based prospective study. Therefore, it is not possible to analyze the improvement and treatment outcome of patients with occipital stroke over time. Second, the results and conclusions should be interpreted cautiously due to the small sample size. With a larger sample population, some marginally trending results could become more significant. Further, a larger number of patients and a population-based study are needed to disclose the association of occipital stroke more accurately with certain risk factors.

CONCLUSION

Risk factors are associated with older patients and lower visual acuity, although they are not associated with stroke location and visual field defect characteristics. Younger patients might suffer from an occipital stroke that is not underlined by systemic risk factors, and therefore, further study is important to investigate the exact mechanism of occipital stroke.

ACKNOWLEDGEMENTS

The authors declare no conflict of interest regarding this paper's publication. The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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JMedSci

Journal of the Medical Sciences
(Berkala Ilmu Kedokteran)

Volume 54, Number 2, 2022; 132-140
http://dx.doi.org/10.19106/JMedSci005402202204

The role of clinical reminder system to drug prescribing on patients of the National Health Insurance with ischemic stroke

Faramita Hiola[1]*, Iwan Dwiprahasto[2], Rizaldy Pinzon[3]

[1]Faculty of Sports and Health, Universitas Negeri Gorontalo, Gorontalo, Indonesia [2]Department of Pharmacology and Therapy, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Yogyakarta, [3]Department of Neurology, Bethesda Hospital, Yogyakarta, Indonesia

Submitted: 2021-01-28
Accepted : 2021-10-12

ABSTRACT

Since 2014 Indonesia has entered the era of universal health coverage (UHC) and public health financing system managed by the Social Security Organizing Agency Law/SSOAL (*Badan Penyelenggara Jaminan Sosial*/BPJS) . In this system, a national formulary was used as the basis for prescribing drugs by clinicians. One effort for quality and cost control in UHC was to develop a clinical reminder system (CRS) to help prescriber set treatment options in accordance with the national formulary. The aim of this study was to measure the role of CRS to the compatibility of drug prescribing in patients with ischemic stroke in Bethesda Hospital Yogyakarta, Indonesia. This study was carried out using quasi-experimental with pre-test and post-test design. The subjects of this study were outpatient ischemic stroke and the National Health Insurance (NHI) participant, age >18 years and had complete medication data. Prescribing data were compared between stroke patients treated at the hospital before and after implementation of CRS. The study was performed in 200 National Health Insurance (NHI) scheme outpatients with ischemic stroke. The groups consisted of 100 patients without CRS and 100 patients with CRS. The basic characteristics of both groups were similar. The results showed that after implementation of CRS, a significant improvement in the compliance of the neurologist prescribing medicine used to be available only in national formulary (RR: 1.02; 95% CI=1.00-1.04; *p*=0.015). Among others the most significant improvement was the prescription of antidyslipidemic using HMG-CoA medicine available in formulary. In conclusion, CRS can improve the compliance of prescribing with national formulary in stroke ischemic patients.

Keywords:
clinical reminder system;
stroke;
prescription;
universal health coverage;
national formulary

ABSTRAK

Sejak tahun 2014, Indonesia telah memasuki era jaminan kesehatan universal (JKU) dan sistem pembiayaan kesehatan masyarakat dikelola oleh Badan Penyelenggara Jaminan Sosial (BPJS). Dalam sistem ini, digunakan formularium nasional sebagai dasar peresepan obat oleh para klinisi. Salah satu upaya untuk kendali mutu dan kendali biaya dalam JKU adalah dengan mengembangkan sistem pengingat klinis (SPK) untuk membantu *prescriber* menetapkan pilihan terapi sesuai dengan yang terdapat dalam formularium nasional. Tujuan dari penelitian ini adalah untuk mengukur peran SPK terhadap kesesuaian peresepan obat pada pasien stroke iskemik di rumah sakit Bethesda, Yogyakarta. Penelitian ini adalah penelitian quasi eksperimental dengan desain *pre-test and post-test*. Subjek penelitian adalah pasien stroke iskemik rawat jalan peserta jaminan keseahtan nasional (JKN), usia >18 tahun dan memiliki data pengobatan yang lengkap. Data peresepan dibandingkan antara pasien stroke yang berobat pada saat sebelum dan setelah penerapan SPK. Penelitian dilakukan pada 200 pasien stroke iskemik peserta JKN rawat jalan di rumah sakit, 100 pasien pada saat sebelum penerapan SPK dan 100 pasien setelah penerapan SPK. Karakteristik kedua kelompok penelitian sebanding. Hasil penelitian menunjukkan bahwa setelah penerapan SPK, terdapat peningkatan kepatuhan dokter dalam meresepkan obat sesuai formularium nasional (RR: 1.02; 95% CI=1.00-1.04; *p*=0.015). Perbaikan yang signifikan terdapat pada peresepan antidislipidemia jenis HMG-CoA. Simpulan, SPK dapat meningkatkan kesesuaian peresepan obat dengan formularium nasional pada pasien stroke iskemik di rumah sakit.

*corresponding author: Faramita0702@gmail.com

INTRODUCTION

Indonesia has entered the era of universal health coverage (UHC) since 2014. The public health financing system is managed by the Social Security Organizing Agency Law/SSOAL (Badan Penyelenggara Jaminan Sosial/BPJS) with a publicly subsidized or premium system.1 Recently, there are more than 178 million inhabitants of about 256 million have become UHC members managed by BPJS.[1] In this system, the Minister of Health of Republic of Indonesia establishes a national formulary as a basis for prescribing medicines by clinicians. The national formulary is a list of selected drugs are needed and must be available at health service facilities in the context of implementing the National Health Insurance (NHI).[2]

Since the introduction of UHC, one of the diseases classified as high cost is stroke. Stroke is the 5th leading cause of death and the first leading cause of disability. There are two main types of strokes. The commoner type is an ischemic stroke, caused by interruption of blood flow to a certain area of the brain, and hemorrhagic stroke caused by leak or interruption of a blood flow in brain.[3,4] The incidence of stroke in Indonesia is high. Therefore, various efforts to optimize stroke management in Indonesia are required.[1] In an effort to quality and cost control in the execution of NHI schema, the Ministry of Health of Republic of Indonesia set up a drug price list and as outlined in a national formulary.[5] According to the applicable provisions, every health service should use the National Formulary to establish the type of drug for NHI patients.[5]

Stroke is one of the diseases that the number of events is increasing from time to time (8.3%), then in 2013 the incidence of stroke increased by 12.1%.[6] In NHI, stroke is included in high-cost disease from the diagnosis, therapy, up to rehabilitation of physiotherapy to improve patient's quality of life. An attempt that can be done to control the quality and the cost of disease implementation is using clinical reminder system (CRS). This system is a computer-based reminder system that is used to inform the doctor about the drug, indication of use, restrictions and maximum prescription of each type of drug.

Clinical reminder system is an information system for improving health services to patients in hospitals and helps in clinical evaluation, decision making, assisting doctors in diagnosing and reducing prescribing errors. It is adapted from treatment guidelines both locally and nationally by providing complete access to information on patient treatment data to obtain quality treatment.[7] This reminder system is integrated in a computerized provider order entry (CPOE), therefore every doctor determines the types and items of the drug accompanied by a reminder that explains what drugs can be given to NHI patients. This clinical reminder is expected to help control hospital costs and select therapy according to the National Formulary.[8]

This study aimed to evaluate the usefulness of the CRS in improving physician compliance to prescribe drugs contained in the National Formulary.

MATERIALS AND METHODS

Protocol of the study

It was a quasi-experimental study with pre-test and post-test design. The inclusion criteria of this study were all ischemic stroke patients with diagnostic criteria based on CT scans and in accordance with ischemic stroke characteristics of the American Heart Association.[9] Subjects were outpatients NHI participants who underwent treatment during the period January

2014 to December 2015, subjects aged > 18 years and had complete treatment data. They were excluded from the study if not SSOAL patients, died, and had incomplete medical record data. Prescribing data were compared between ischemic stroke patients treated before and after CRS application. The protocol of the study has been approved by the Medical and Health Research Ethics Committee, Universitas Gadjah Mada, Yogyakarta.

The CRS was developed by the team at Bethesda Hospital, based on the list of drugs in the National Formulary. One of the outcomes of this study was an increase in physician compliance to use the National Formulary, and reduce the use of drugs outside the National Formulary which is the burden of hospitals.

Statistical analysis

The sample size used in this study was calculated using the power and sample size software. Data of ischemic stroke patients that have been collected were analyzed using data processing software. For comparisons of dichotomous outcomes, the relative risk (RR) with their 95% confidence interval (95% CI) was calculated. The exact p value was calculated using Yates-corrected chi-square test. The outcome with continuous data was first checked for normality of distribution. For normally distributed data, comparison of means was performed using an independent t-test; otherwise, the non parametric Mann-Whitney U test was used. Patients factors such as age, sex, onset, and comorbidity was assessed to determine the amount of influence on the study outcome.

RESULT

Based on data from the electronic stroke register, there are 6 neurologists who treat outpatient ischemic stroke patients. The subjects were divided into two groups i.e. before implementation of the CRS in 2014 and after implementation of the CRS in 2015. The sample size used can be seen in FIGURE 1 that each group consists of 100 subjects.

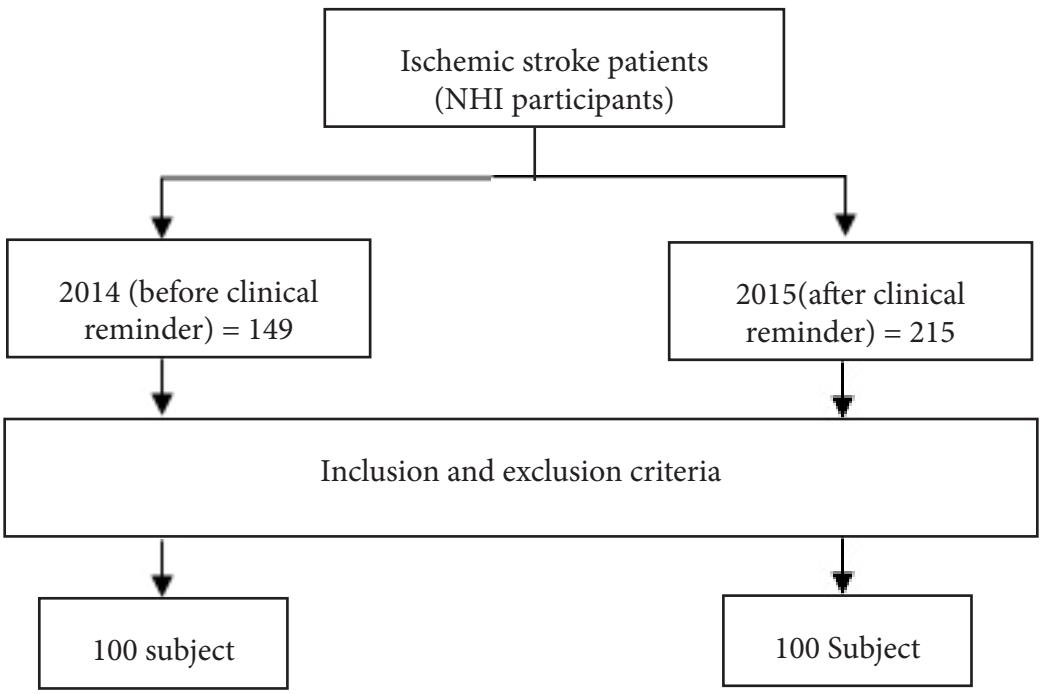

FIGURE 1. The flowchart of the study data selection process

The subjects of this study were 100 in each observation group showing that between the two groups was comparable in terms of age, gender, onset of attack, number of comorbidities and visits. The characteristics of the subject are presented in TABLE 1.

TABLE 1 shows that men (60.5%) and age (63.0%) have a high risk of having ischemic stroke. Based on the basic characteristics of patients including age, gender, onset, number of comorbidities, and frequency of visits between groups before and after the application of the CRS was the same. It can be seen that the widely prescribed antiplatelet type is acetylsalicylic acid, clopidogrel and cilostazol (TABLE 2).

TABLE 1. Study subject characteristics

Characteristics	Total n = 200	Before CRS [n =100 (%)]	After CRS [n = 100 (%)]	p
Gender				
▪ Male	121 (60.5)	58 (58)	63 (63)	0.470
▪ Female	79 (39.5)	42 (42)	37 (37)	
Age (y.o.)				
▪ ≤ 60 years	74 (37)	33 (33)	41 (41)	0.241
▪ > 60 years	126 (63)	67 (67)	59 (59)	
Onset (h)				
▪ ≥ 3	164 (82)	84 (84)	80 (80)	0.462
▪ < 3	36 (18)	16 (16)	20 (20)	
Comorbidity				
▪ 1	99 (49.5)	49 (49)	50 (50)	0.888
▪ > 1	101 (50.5)	51 (51)	50 (50)	
Visitation frequency (times)				
▪ ≥ 3	77 (38.5)	39 (39)	38 (38)	0.884
▪ < 3	123 (61.5)	61 (61)	62 (62)	

TABLE 2. Type of single-antiplatelet prescribed in patients with ischemic stroke

Variable	Before CRS	After CRS
	[n = 257 (%)]	[n = 273 (%)]
Acetylsalicylic acid	193 (75.1)	200 (73.3)
Clopidogrel	53 (20.6)	65 (23.8)
Cilostazol	11 (4.3)	8 (2.9)

There were various types of antihypertensive prescribed for stroke patients, most of which are the calcium channel blocker groups which is amlodipine (TABLE 3). From the two study groups, it can be seen that in 2014 the prescribed amlodipine was 93 drugs (52.5%) compared to others, while in 2015 it was 85 (56.3%). In addition, there were also groups of angiotensin II antagonists' namely irbesartan, valsartan and candesartan were widely prescribed for patients with ischemic stroke.

TABLE 3. Types of single-antihypertensive prescribed in patients with ischemic stroke

Variable	Before CRS	After CRS
	[n = 177 (%)]	[n = 151 (%)]
Amlodipine	93 (52.5)	85 (56.3)
Irbesartan	13 (7.3)	18 (11.9)
Valsartan	12 (6.8)	12 (7.9)
Candesartan	10 (5.6)	10 (6.6)
Spironolacton	11 (6.2)	6 (4.0)
Telmisartan	14 (7.9)	2 (1.3)
Ramipril	2 (1.1)	8 (5.3)
Bisoprolol	6 (3.4)	2 (1.3)
Losartan	7 (4.0)	0 (0.0)
Furosemide	2 (1.1)	5 (3.3)
Lisinopril	4 (2.3)	0 (0.0)
Captopril	1 (0.6)	1 (0.7)
Nifedipine	1 (0.6)	1 (0.7)
Telmisartan + amlodipine	1 (0.6)	0 (0.0)
Clonidine	0 (0.0)	1 (0.7)

TABLE 4 shows the most prescribed statin group which was 90 simvastatin (67.2%) of the prescribed drugs in 2015. Atorvastatin was the most prescribed in 2014 of 57 (38%), followed by fenofibrate and gemfibrozil.

TABLE 4. Type of single-antidyslipidemic prescribed in patients with ischemic stroke

Variable	Before CRS	After CRS
	[n = 150 (%)]	[n = 134 (%)]
Simvastatin	82 (54.7)	90 (67.2)
Atorvastatin	57 (38)	37 (27.6)
Fenofibrate	9 (6)	7 (5.2)
Gemfibrozil	2 (1.3)	0 (0)

This study found that the amount of national formulary drugs prescribed in 2015 was higher than in 2014 before the implementation of the CRS (76.3% vs. 61.3%). In 2014 (before CRS) the amount of generic drugs prescribed was 31.6%, while in 2015 (after CRS) there was an increase of 43.9% (TABLE 5).

The results in TABLE 6 illustrate the compliance of neurologist prescription at Bethesda Hospital. It appears that the application of CRS has been well utilized, and proved significant in CRS used by doctors with DS-A, DS-B and DS-D codes.

TABLE 5. Prescription of drugs in ischemic stroke patients

Variable	Before CRS [n = 1254 (%)]	After CRS [n = 1083 (%)]
National formulary		
▪ Yes	769 (61.3)	826 (76.3)
▪ No	485 (38.7)	257 (23.7)
Generic		
▪ Yes	396 (31.6)	475 (43.9)
▪ No	858 (68.4)	608 (56.1)

TABLE 6. The compatibility of physicians prescription with a list of drugs in national formulary

Variable	Before CRS [n (%)]	After CRS [n (%)]	p
DS-A			
▪ National formulary	297 (654)	185 (80.8)	<0.001
▪ No	157 (34.6)	44 (19.2)	
DS-B			
▪ National formulary	90 (54.5)	114 (73.1)	0.001
▪ No	75 (45.5)	42 (26.9)	
DS-C			
▪ National formulary	84 (54.5)	117 (65.4)	0.056
▪ No	70 (45.5)	62 (34.6)	
DS-D			
▪ National formulary	231 (58.9)	264 (80.2)	<0.001
▪ No	161 (41.1)	65 (19.8)	
DS-E			
▪ National formulary	44 (67.7)	111 (75.5)	0.244
▪ No	21 (32.3)	36 (24.5)	
DS-F			
▪ National formulary	23 (95.8)	35 (81.4)	0.142
▪ No	1 (4.2)	8 (18.6)	

Assessment of prescribing compatibility was only carried on drugs included in the national formulary, based on drug strength, restriction, and maximum prescribing. Drugs prescribed outside from national formulary were categorized as inappropriate. The results in TABLE 7 show that the application of CRS significantly improves the prescribing compatibility with the national formulary of 1.02 than without CRS. The prescribing of antiplatelet and antihypertensive was also increased but these results were not significant. The prescribing antidyslipidemic is proven to increase the compatibility (p<0.001).

TABLE 7. Compatibility of drugs prescription with the National Formulary

Variable	National formulary restriction		Total	RR (95% CI)	p
	Compatible [n (%)]	Not compatible [n (%)]			
CRS					
▪ After	809 (97.9)	17 (2.1)	826	1.02 (1.00-1.04)	0.015
▪ Before	737 (95.8)	32 (4.2)	769		
Antiplatelets					
▪ After	267 (97.8)	6 (2.2)	273	1.01 (0.98-1.03)	0.512
▪ Before	249 (96.9)	8 (3.1)	257		
Antihypertensive					
▪ After	144 (95.4)	7 (4.6)	151	1.02 (0.97-1.08)	0.307
▪ Before	164 (92.7)	13 (7.3)	177		
Antidyslipidemic					
▪ After	119 (88.8)	15 (11.2)	134	1.43 (1.24-1.64)	<0.001
▪ Before	93 (62)	57 (38)	150		

DISCUSSION

The CRS started as a prototype system and evolved into an integrated and daily used software in outpatient care services. It can be used simultaneously and also available to multiple clinics and physician practices through a web-based system.[10] This study aimed to evaluate the effectiveness of CRS application to physician prescribing in outpatient ischemic stroke patients. The results suggest that CRS can improve the compliance of drugs prescribing with national formulary for NHI patients compared without CRS. This reminder would appear in the form of a reminder spot that is automatically displayed on the computer of each physician when prescribing drugs. Therefore it can help in reminding the list of drugs and restrictions according to the formulary.

The results in this study were similar to the result obtained by Youssef *et al.*[11] The use of clinical reminders has been proven to reduce 30% patient treatment costs and medication errors in hospitals.[11] Other study by Foster *et al*[12] in their 6-month study showed that adherence to prescribing was significantly higher in the group receiving CRS than without CRS. Prescribing compatibility to formulary rules can improve medication safety and assist hospitals in managing drugs as quality and cost control. Clinical reminder systems can increase the percentage of generic drugs prescription, generic drugs have the same benefits and efficacy compared to branded drugs.[13] The application of clinical reminder systems can assist in controlling the medication cost in hospitals.

Analysis result of the use antidyslipidemic drugs showed that the CRS could improve prescribing compatibility with the national formulary than without the CRS, because the prescribing of antidyslipidemic should be based on the results of patient's laboratory, and attached when taking drugs at the pharmacy.

There was no significant difference in antiplatelet and antihypertensive prescribing. These results were different from those conducted by Sequist *et al.*[14] on the use of aspirin in diabetic patients and coronary arteries, suggesting that CRS can improve the appropriateness of aspirin than without CRS. Other research conducted by Filippi *et al.*[15] suggests that the compatibility of antiplatelet prescribing was increased

in the intervention group than in the control group but these results were not significant. Incompatibility in this study occurred in the prescription of acetylsalicylic acid 100 mg. In formulary restriction, the maximum prescription is 30 tablets for 30 days, but in this case 40 tablets were given.

Incompatibility of antihypertensive prescribing occurs in telmisartan 80 mg and valsartan 80 mg. This drug is only used in hypertension patients who are intolerant to ACE inhibitors with prescribing maximum 30 tablets for 30 days, but in this study 60 tablets were given. In addition, another class of angiotensin II antagonists used was losartan 50 mg. However this drug is not attached to the list in the national formulary, so it is categorized as inappropriate. In prescribing amlodipine 5 mg there is also incompatibility because it exceeds the maximum prescriptions.

CONCLUSION

Application of CRS can improve the compatibility of prescribing drugs based on the restriction of the national formulary.

ACKNOWLEDGEMENT

We are grateful to Bethesda Hospital, Yogyakarta and the staffs of Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Yogyakarta and all who involved in the data collection and analysis in this study.

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JMedSci

Journal of the Medical Sciences
(Berkala Ilmu Kedokteran)

Volume 54, Number 2, 2022; 141-147
http://dx.doi.org/10.19106/JMedSci005402202205

Hearing loss in pediatric patients with congenital rubella syndrome

Muyassaroh[1*], Nisa Alifia Rahmi[2]

[1]Department of Otolaryngology-Head and Neck Surgery, Dr. Kariadi Central Hospital Semarang, [2]Department of Pediatric, Faculty of Medicine, Universitas Diponegoro/Dr. Kariadi Central Hospital Semarang, Indonesia

Submitted: 2021-11-01
Accepted : 2022-03-09

ABSTRACT

Congenital rubella syndrome (CRS) is caused by rubella infection in pregnant women. It was estimated 100.000 children with CRS, with 46% found in developing countries. The CRS consists of symptoms like sensorineural hearing loss (SNHL), congenital heart disease, cataract or congenital glaucoma, and other symptoms. Sensorineural hearing loss is the commonest symptoms compared with others. This study aimed to determine the hearing loss in CRS in Dr. Kariadi Central Hospital, Semarang, Central Java. This was a descriptive study using data from medical records from CRS pediatric patients who had BERA examination from January 2019 until December 2020. The CRS was characterized with one or more symptoms: SNHL, congenital cataract, congenital heart disease, and laboratory IgG and/or IgM Rubella (+). The SNHL was described from refer OAE result, tympanometry A, and BERA with hearing threshold ≥40 dB. Follow-up evaluation was performed with Google form questionnaire. There were 55 CRS cases with 70.9% had bilateral SNHL, mostly with very severe hearing loss. Normal hearing was found in 16 children (28.1%). From 30 children who were followed up, there were 20 children who had bilateral SNHL with 30% were moderate-severe degree and 70% profound. With further multidisciplinary management in children with CRS and parental support, 65% children used hearing aid and 40% had auditory-verbal therapy (AVT). Evaluation of the AVT showed progress in 30% children. In conclusion, SNHL is found in 70.9% children with CRS. Further management using hearing aid and AVT shows progress on few children.

ABSTRAK

Sindrom rubella kongenital (SRK) terjadi akibat adanya infeksi rubella pada ibu hamil. Diperkirakan lebih dari 100.000 anak menderita SRK setiap tahunnya. Sindrom rubella kongenital ditandai adanya *sensorineural hearing loss* (SNHL), cacat jantung bawaan, katarak atau glaukoma kongenital, dan symptom lain. *Sensorineural hearing loss* yang paling umum didapatkan dibanding yang lainnya. Penelitian ini melaporkan kejadian kurang pendengaran pada SRK di RSUP Dr. Kariadi, Semarang. Penelitian deskriptif ini mengambil data rekam medis anak SRK yang diperiksa BERA pada Januari 2019 sampai Desember 2020. Sindrom rubella kongenital ditandai dengan salah satu atau lebih adanya SNHL, katarak kongenital, penyakit jantung bawaan, hasil laborat IgG dan atau IgM rubella (+). SNHL dinilai dari hasil OAE refer, timpanometri A, BERA ambang dengar ≥40dB. Evaluasi tindak lanjut penanganan dengan kuesioner *Google form*. Terdapat 55 (70,9%) anak dengan SRK. Sebagian besar SNHL bilateral berderajat sangat berat. Pendengaran normal dijumpai pada 16 anak (28,1%). Dari 30 anak yang dievaluasi, terdapat 20 anak dengan SNHL bilateral derajat sedang-berat sebanyak 30% dan sangat berat sebanyak 70%. Tindak lanjut dan tatalaksana multidisiplin pada anak SRK disertai dukungan orang tua didapatkan 65% menggunakan alat bantu dengar (ABD) dan menjalani *auditory-verbal therapy* (AVT) rutin (40%). Evaluasi AVT terdapat kemajuan pada 30% anak. Dapat disimpulkan, dijumpai SNHL pada 70,9% anak dengan SRK. Tindak lanjut dan tatalaksana penggunaan ABD dan AVT menunjukkan perbaikan pada sebagian kecil anak.

Keywords:
hearing loss;
congenital rubella syndrome;
habilitation;
auditory-verbal therapy;
hearing aid

*corresponding author: muyastht@gmail.com

INTRODUCTION

Rubella infection in pregnancy can cause miscarriage, fetal death or congenital abnormalities after birth. Risk level and types of disability depend on gestational age when infected. When the infection of rubella happens in first trimester (12 weeks), about 85% babies had risks to be born with congenital abnormalities; if infection happens in week 13-16 of pregnancy, risks were dropped into 10-20%, while malformation rarely happens after 20 weeks of pregnancy.[1,2] In Indonesia, not many studies on the incidence of rubella are conducted. However, it is estimated that the incidence of rubella infection is quite high. Data from the National Basic Health Research (*Riset Kesehatan Dasar*/Riskesdas) 2011, about 400 cases of congenital rubella syndrome (CRS) were reported, while according to World Health Organization (WHO) in 2012 around 5,000-10,000 babies were born with deafness annually.[3]

Congenital rubella syndrome is characterized with sensorineural hearing loss (SNHL), congenital cataract or glaucoma, congenital heart disease, and developmental delay. Other symptoms are craniofacial anomalies, purpura, and meningoencephalitis. Hearing loss is the most common symptom of CRS. Children with CRS who survive, some will have developmental delay, diabetes mellitus type 1 or thyroiditis.[2,4,5] Some of these impairments can appear or worsen later in the lives of these children. Early introduction and continuation of speech, occupational, physical, and behavior therapies and training with appropriate medical interventions by a multidisciplinary team approach are required to maximize quality of life.[6]

World Health Organization reported more than 100.000 babies were born with CRS worldwide every year and around 46% were reported from southeast Asia including Indonesia. The CRS incidence increased 10 times when epidemic happened.[7] Hearing loss was estimated happened around 80 – 96% in CRS patients and it can be unilateral or bilateral with various degrees in severity.[4] Rubella virus can directly affect cochlea by inducing apoptosis in stria vascularis, cochlear duct and the organ of corti. Stria tissue can be infected and change endolymph structure. Vasculitis occurs and directly damages cells in cochlea, which disrupts myelinization in auditory nerves.[2,8]

Speech and language development are severely affected when hearing impairment is present.[9,10] Auditory–verbal therapy (AVT) is a listening and spoken language (LSL) instructional approach. The AG Bell Academy for Listening and Spoken Language provides certification to specialists who deal with hearing loss children. A previous study revealed that children who participated in AVT can achieve linguistic skills at the same level as their hearing peers.[11] This study aimed to report hearing loss in children with CRS at Dr. Kariadi Central Hospital, Semarang, Indonesia.

MATERIALS AND METHODS

Subjects

This was a descriptive study conducted in Dr. Kariadi Central Hospital, Semarang, Central Java. Data were gathered from medical records from CRS pediatric patients with age 6.2 – 57 months who had BERA examination from January 2019 until December 2020 and met the inclusion and exclusion criteria.

Protocol of study

Initial diagnosis of CRS was made

from medical records if characterized with one or more symptoms i.e. SNHL, congenital cataract, congenital heart disease, and laboratory IgG and/ or IgM Rubella (+). The CRS diagnostic was conducted by pediatricion and examined hearing function was conducted by otolaringologist. The SNHL was scored with refer OAE result, tympanometry A, and BERA with hearing threshold ≥40 dB. Follow-up evaluation was performed by using Google form questionnaire.

Statistical analysis

Data were presented as frequency or percentages and continued by descriptively analysis.

RESULT

Initial data from medical records in period January 2019 until December 2020 showed 55 children with CRS. Boys were found more than girls, mostly aged more than 1 year old. Hearing loss was found in 39 patients (70.9%), all affected bilaterally and 70.9% with very severe hearing loss. The characteristics of subjects are presented in TABLE 1.

Evaluation was performed in September 2021 using Google form, among 30 children with CRS had responses, consisted of 20 CRS children with hearing loss and 10 CRS children with normal hearing. Questionnaire results of 20 CRS children are shown in TABLE 2.

TABLE 1 Basic characteristics of the subjects

Variable	Total [n (%)]
Gender	
▪ Male	32 (59.2)
▪ Female	23 (41.8)
Age of having BERA	
▪ 0 - 1 years old	25 (45.4)
▪ > 1 years old	30 (54.6)
BERA results	
▪ Hearing threshold < 40 dB	16 (29.1)
▪ Hearing threshold ≥ 40 dB	39 (70.9)
▪ SNHL moderate - severe	8 (20.5)
▪ Profound	31 (79.5)
▪ Unilateral	0 (0)
▪ Bilateral	39 (100)
▪ Organ abnormalities	
Hearing loss	14 (35.9)
▪ Hearing loss +1 organ abnormality	19 (48.7)
▪ Hearing loss + > 1 organ abnormalities	6 (15.4)

TABLE 2. Questionnaire results of CRS children (n=30)

Variable	Moderate – severe [n=6 (%)]	Profound [n=14 (%)]	Total [n (%)]
Age (years)			
▪ 0-1	2 (10)	6 (30)	8 (40)
▪ >1	4 (20)	8 (40)	12 (60)
Gender			
▪ Male	3 (15)	8 (40)	11 (55)
▪ Female	3 (15)	6 (30)	9 (45)
Hearing aid type			
▪ Implant	0 (0)	3 (15)	3 (15)
▪ Hearing aid	4 (20)	6 (30)	10 (50)
▪ Without hearing aid	2 (10)	5 (25)	7 (35)
Habilitation			
▪ Speech therapy/AVT	2 (10)	6 (30)	8 (40)
▪ Non-therapy	4 (20)	8 (40)	12 (60)
▪ Therapy result			
▪ No improvement	6(30%)	8(40%)	14(70%)
▪ Improvement	0(0%)	6(30%)	6(30%)

Moderate severe hearing loss was found in 6 children, 4 children used hearing aid, while 2 children only had speech therapy resulted with no improvement. Profound hearing loss were identified in 14 children, 9 children had hearing aid (3 implant, 6 conventional hearing aid device), 6 children had speech therapy resulted with improvement. Parent's occupation and education which supports further management of hearing loss in CRS children (TABLE 3).

TABLE 3. Parent's occupation and education related to hearing loss management

Variable	Implant [n=3 (%)]	Hearing aid [n=10 (%)]	Non Implant/ Hearing aid [n=7 (%)	Total [n=20 (%)
Parent's occupation				
▪ Both parents work	2 (10)	3 (15)	3 (15)	8 (40)
▪ Only father works	1 (5)	7 (35)	4 (20)	12 (60)
Parent's education				
▪ Both parents with scholar degree	3 (15)	4 (20)	1 (5)	8 (40)
▪ Father/mother with scholar degree	0 (0)	3 (15)	3 (15)	6 (30)
▪ Father/mother with non-degree	0 (0)	3 (15)	3 (15)	6 (30)

Parent's occupation and education may affect compliance in follow up control (TABLE 4). Among 20 children with hearing loss (13 children with hearing aid and 7 children with routine speech therapy), 6 children had improve of hearing, 2 children from non hearing aid group, that be conducted on routin speech therapy. All children did no improve of hearing.

TABLE 4. Parent's compliance

Variable	Therapy		Result	
	Routine [n=9 (%)]	Not routine [n=11 (%)]	No improvement [n=14 (%)]	Improvement [n=6 (%)]
Hearing aid type				
▪ Implant	3 (15)	0 (0)	0 (0)	3 (15)
▪ Hearing aid	4 (20)	6 (30)	7 (35)	3 (15)
▪ Without hearing aid	2 (10)	5 (25)	7 (35)	0 (0)
Parent's occupation				
▪ Both parents work	3 (15)	5 (25)	4 (20)	4 (20)
▪ Only father works	6 (30)	6 (30)	10 (50)	2 (10)
▪ Parent's education				
▪ Both parents with scholar degree	6 (30)	2 (10)	5 (25)	3 (15)
▪ Father/ mother with scholar degree	2 (10)	4 (20)	4 (20)	2 (10)
▪ Father/ mother with non-degree	1 (5)	5 (25)	5 (25)	1 (5)

DISCUSSION

Rubella infection in pregnancy can cause miscarriage, stillbirth, congenital abnormalities or asymptomatic infection. It can affect some organs and causes congenital abnormalities which called CRS. Prevalence of CRS in Indonesia is still high. Rubella vaccination can decrease CRS prevalence in developed countries, but can not be conducted completely in some of developing countries including Indonesia.[5] Impact of CRS can cause growth and developmental delay in children, hearing organ impairment can disturb speech and language development, which affect greatly on communication disorder and opportunity on decent education and job.

The result of this study showed that 70.9% children experience bilateral SNHL with mostly profound hearing loss. Age ranged from 6.2 – 57 months. Age 0 – 1 years old were found in 45.4% children and age more than 1 years were found in 56.4% children. A male and female ratio was 1.4:1. It was different with study conducted in Surabaya which found 1.06:1.[12] A study conducted in Bandung reported that 88% of CRS patients experienced hearing loss in which 75% were bilateral. The ratio male and female was 1:1 and mostly occurred in age group of 1-3 months old (22.11%), whereas very severe SNHL were found in children aged 2-14 months old (22%). Furthermore, a study conducted in Tokyo reported that SNHL occurred in CRS patients with ratio of 1:1.[14] Nazme *et al.*[15] reported that SNHL in CRS patients are dominated by male in Bangladesh. A study in Yogyakarta showed severe SNHL in CRS patients were found in 36% children with mostly aged 2-6 months old.[5]

This study also showed hearing loss with and without other organ abnormalities were 70.9%, only hearing loss were 35.9%, hearing loss with one organ abnormality were 48.7%, and hearing loss with more than one organ

abnormalities were 15.4%. This result is similar with the study conducted in Surabaya which reported that bilateral hearing loss was the most common hearing abnormality found with 71.43% cases, hearing loss and congenital heart disease were found in 17.89% cases, hearing loss, congenital heart disease, and ophthalmology abnormalities were found in 16.84% cases, while hearing loss and ophthalmology abnormalities were found in 13.68% cases.[12]

Most CRS study in Indonesia conducted in patients less than 6 months old.[5] The different result of this study compared with other studies may due to still a lot of children who were admitted in Dr. Kariadi Center Hospital, were referred for auditory examination when aged more than 1 years old. Some cases were referred to ENT Departement with complaint of speech delay. The parents realized after their children were enable to talk like the others.

Further follow up and management for CRS patients need multidisciplinary approach involving pediatrician, ophthalmologist, physical medicine and rehabilitation, physicians, and parents support. Parents occupation and education were one of the factors which determines further management of CRS. Parents with good education and socio-economics will get well information about the problem of hearing loss in children and its management. Therefore, they can provided psychosocial and academic development supports of the child and to his or her ultimate quality of life. Children with working fathers and mothers will reduce the focus on children's needs. Among 30 children who gave responses in follow up this study, there were 20 children with hearing loss.

Habilitation with implant or conventional hearing aid device was conducted in 13 children (65%). Eight children had speech therapy/AVT, 6 showed improvement. Success rate of speech therapy for SNHL in children with CRS who used hearing aid only 6 form 13 (46%), 7 children doing to routine speech therapy, 6 children showed hearing improvement, 2 children from non hearing aid group, that be conducted on routin speech therapy. All children no improving of hearing. This may cause by non-routine therapy, parents' commitment and occupation limitation, limitation was cost burden, abnormalities in organs involved and limited time and distance from therapy clinic to home. This result similar with previous study which showed that AVT is an effective intervention option for the AVT group.[16]

CONCLUSION

Hearing loss in CRS patients are commonly found (70.9%). Habilitation with speech therapy/AVT conducted in some children shows improvement in listening and speech. These findings support the positive effect of creating an appropriate educational environment by considering individualized needs. Also, exploring parental needs is very important for planning and making decisions in the rehabilitation process.

ACKNOWLEDGMENT

We would like to express our gratitude to all our colleagues of the Department of Otolaryngology-Head and Neck Surgery, Dr. Kariadi Central Hospital, Semarang, Indonesia, for their full supports in this study.

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Journal of the Medical Sciences
(Berkala Ilmu Kedokteran)

Volume 54, Number 2, 2022; 148-160
http://dx.doi.org/10.19106/JMedSci005402202206

Relationship between high-risk human papilloma virus (HPV) and subclinical condyloma acuminata (CA) in the cervix of high-risk women

Benjamin Ricardo Rubirosa Lumban Tobing*, Richard Hutapea, Kristina Nadeak

Department of Dermatology and Venerology, Faculty of Medicine, Universitas Sumatra Utara, Medan, Indonesia

Submitted: 2021-10-15
Accepted : 2021-12-02

ABSTRACT

Condyloma acuminata (CA) is a sexually transmitted infection (STI) caused by both high- and low risk human papilloma virus (HPV) infection. Subclinical CA looks like a white spot after an acetic acid test. High-risk women are women who have a high risk of STIs, including CA. The aim of this study was to evaluate the relationship between high-risk HPV and subclinical CA in the cervix of high-risk women. This was a cross-sectional analytic study involving 30 high-risk women. The age, the age at first sexual intercourse, the frequency of sexual intercourse, the number of sexual partners and the use of condoms were recorded. Subclinical CA was diagnosed by the 5% acetowhite test. HPV infection was detected by PCR. Kruskal Wallis test was conducted to evaluate the relationship between variables and the results were considered to be significant if $p<0.05$. The HPV infection was detected in 15 high-risk women with subclinical CA and most subjects showed the high-risk type. No significant relationship between age and using of condom with high-risk HPV or with subclinical CA ($p> 0.05$). No significant relationship between age at first sexual intercourse and frequency of sexual intercourse with high-risk HPV was also observed ($p> 0.05$), however, a significant relationship with subclinical CA was observed ($p < 0.05$). In conclusion, there is a relationship between high-risk HPV and subclinical CA in the cervix of high-risk women.

ABSTRAK

Kondiloma akuminata (KA) merupakan infeksi menular seksual (IMS) yang disebabkan oleh *Human Papilloma Virus* (HPV) baik tipe risiko tinggi atau rendah. Kondiloma subklinis terlihat seperti bercak putih setelah dilakukan tes asam asetat. Wanita berisiko tinggi adalah wanita yang memiliki risiko tinggi terhadap IMS, termasuk KA. Tujuan penelitian ini adalah mengkaji hubungan antara HPV tipe risiko tinggi dengan KA subklinis pada serviks wanita risiko tinggi. Penelitian ini merupakan penelitian analitik potong lintang yang melibatkan 30 pasien wanita berisiko tinggi. Data usia, usia pertama melakukan hubungan seksual, frekuensi hubungan seksual, jumlah pasangan seksual dan penggunaan kondom dicatat. Kondiloma akuminata subklinis didiagnosis dengan tes *acetowhite* 5%. Infeksi HPV dideteksi dengan PCR. Uji Kruskal Wallis dilakukan untuk menilai hubungan antar variabel dan hubungan dinyatakan signifikan bisa nilai $p<0,05$. Infeksi HPV terdeteksi pada 15 orang (50%) wanita berisiko tinggi yang menderita KA subklinis dengan tipe risiko terbanyak, yaitu 7 orang (23,3%). Tidak terdapat hubungan nyata antara usia dan penggunaan kondom dengan infeksi HPV tipe risiko tinggi maupun KA subklinis ($p> 0,05$). Tidak terdapat hubungan nyata antara usia pertama melakukan hubungan seksual dan frekuensi hubungan seksual dengan HPV tipe risiko tinggi ($p> 0,05$), namun terdapat hubungan nyata dengan KA subklinis ($p<0,05$). Dapat disimpulkan terdapat hubungan HPV tipe risiko tinggi dengan kejadian KA subklinis di serviks pada wanita risiko tinggi.

Keywords:
condyloma acuminate;
subclinical;
high risk HPV;
PCR;
acetowhite;

*corresponding author: osatobing@yahoo.co.id

INTRODUCTION

Condyloma acuminata (CA) is a sexually transmitted infection (STI) characterized by changes in mucosal and skin hyperplasia and caused by the human papilloma virus (HPV) infection.[1] Basal cells are the first site of HPV infection after inoculation through minor trauma, HPV virions will enter the basal cell layer of the epithelium.[2] Several clinical features of CA are reported. However, subclinical form of CA that only looks like white spots (positive acetowhite) after the 5% acetic acid test performed is also reported. Therefore, the patient often goes unnoticed even undiagnosed.[1-4] This infection is also demonstrated by identification of viral DNA on genital skin.[5] Many studies estimated that subclinical HPV infection rates have a higher prevalence than clinical infections.[6,7]

High-risk women are women who have a high risk of STIs, including CA.[8] One of the groups of women at high risk of CA is female commercial sex workers.[9-11] The prevalence of CA in each country is different. In developed countries, the prevalence of CA is around 11-46%.[12] It was estimated among 122 million people aged 15-49 years in United State, more than 1% of them suffers from CA and around 2% have subclinical infection.[13] In Indonesia, the prevalence of CA ranges from 5 to 19%.[14] In Medan, North Sumatera the prevalence of CA at Haji Adam Malik General Hospital in 2008-2011 was 76 people and in 2012-2017 was 39 people.[15,16]

Based on the possibility of epithelial dysplasia and malignancy, HPV infection is divided into low-risk HPV and high-risk HPV. Low-risk HPV types, such as type 6, 11, 42, 43, 44, 54, 61, 70, 72 and 81 tend to cause benign tumors such as verrucae and CA. Meanwhile, high-risk HV types tend to cause anogenital malignant tumors, such as cervical, vulvar, vaginal, anal and penile cancers. Whereas, HPV types 16 and 18 are often found in high-grade dysplasia and malignancies.[1,17,18] A study conducted on high-risk women in Lagos showed a high prevalence of high-risk HPV types with most having one type of HPV. Age at first sexual intercourse and an increase in the number of sexual partners are the most important factors for high-risk genital HPV infection in this study.[19] A study on Italian women who have previously been diagnosed with cervical HPV showed a high risk of developing subclinical oral HPV in their sexual partners.[20] Another study in Brazil also reported that patients with genital HPV infection are at risk for oral HPV infection.[21]

The correlation between HPV infection and CA is well known. However, studies concerning high risks HPV and subclinical CA is still limited. This study was conducted to evaluate the relationship between high-risk HPV and subclinical CA in the cervix of high-risk women.

MATERIALS AND METHODS

Design of study

This was an analytic observational study with a cross-sectional design involving high risk women in Public Health Center Glugur Darat, Medan, North Sumatra from Mei 2021 to July 2021.

Protocol of study

A total of 30 high risk women whom met the inclusion and exclusion criteria were involved in this study. The inclusion criteria included high risk women and signed informed consent. According to WHO, high-risk women are those who have risk factors, including husband or sexual partner suffers from STI; husband or sexual partner or patient himself has had another sexual partner in the last one month have a new partner in the

last three month have had one or more STI episodes in the past one mo; high-risk sexual partner/husband behavior.[8] The exclusion criteria included patients who had sex, used vaginal rinses, creams and tampons the day before, patients who have a mass in the birth canal, pregnancy and breastfeeding.

The characteristics of patients included the patient's age, the age at first sexual intercourse, the frequency of sexual intercourse, the number of sexual partners and the use of condoms were recorded. Subclinical CA was diagnosed by the 5% acetowhite test, if there was a white color (positive acetowhite) on the cervix after applying 5% acetic acid.

The research samples were collected at Prodia S. Parman Laboratory in Medan, North Sumatra and would be sent to Prodia Kramat Jaya Laboratory in Jakarta to be examined for HPV types using standard PCR. The protocol of the study was approved by the Health Research Ethics Committee, Faculty of Medicine, Universitas Sumatera Utara, Medan, North Sumatra.

Statistical analysis

The collected data was tabulated and presented as frequency and percentage. The relationship between high-risk HPV types and subclinical CA in the cervix of high-risk women was statistical analyzed using the Chi Square test. If the Chi Square test conditions did not meet criteria, the Fisher's Exact test would be applied. However, if the two conditions did not meet criteria, then the Kruskal Wallis test would be applied. The results of statistical analysis were considered significant if a p value of <0.05.

RESULTS

The HPV infection was detected in 15 high-risk women with subclinical CA (TABLE 1 and 2).

TABLE 1. Distribution of subclinical CA in high-risk women after 5% acetowhite test

Subclinical CA	High-risk women [n (%)]
Positive	15 (50)
Negative	15 (50)

TABLE 2. Distribution of HPV infection in subclinical and non-subclinical CA

High-risk women	HPV infection	
	Positive [n (%)]	Negative [n (%)]
Subclinical CA	15 (50)	0 (0)
Non-subclinical CA	0 (0)	15 (50)

Most subjects showed the high-risk type (TABLE 3). Subclinical CA was most commonly found in the 26-35 y.o. age group (7 people or 46.7%). The 12-16 y.o. age group was the first age group to have sexual intercourse with the highest number of subclinical CA subjects (9 people or 60%). The frequency of sexual

intercourse in subclinical CA subjects was mostly found in the age group with the frequency of having sex 1-7 times a wk (8 people or 53.3%). All subjects suffering from subclinical CA had more than 1 sexual partner. Subclinical CA subjects were more commonly found in the age group who had never used condoms (12 people or 80%). High risk HPV types were found in 3 people (42.9%) in the 26-35 y.o. age group, 3 people (42.9%) in the 36-45 y.o. age group and 1 person (14.3%) in the 17-25 y.o. age group. Mix of high-risk and low-risk HPV types were found in the 46-55 y.o. age group (3 people or 75%). The 12-16 y.o. age group was the first age group to have sexual intercourse with the highest number of subjects infected with high-risk HPV types (4 people or 57.1%). Mix high risk and low risk HPV types were found in 2 people (50%) in the 12-16 y.o. age group, and 2 people (50%) in the 17-25 y.o age group. Subjects with high-risk HPV types who had sexual intercourse more than once a d were found in 3 people (42.9%), 1-7 times a wk 3 people (42.9%), and the least was found in the group with the highest frequency. Having sex 1-3 times a mo was found in 1 person (14.3%). Mix high-risk and low-risk HPV types were found in 2 people (50%) in the group who had sex more than once a d, and 2 people (50%) in the group who had sex 1-7 times a wk. All subjects infected with high-risk HPV types and mix high-risk and low-risk HPV types had more than 1 sexual partner. Subjects infected with high-risk HPV types were found in the group who had never used condoms (6 people or 85.7%) and all subjects infected with mix high risk and low risk HPV types never used condoms were 4 people.

TABLE 3. Characteristics of subclinical CA in high-risk women by HPV type

HPV type	n (%)
High risk	7 (23.3)
▪ Type 16	1
▪ Type 31	1
▪ Type 45	1
▪ Type 51, 52	2
▪ Type 52	1
▪ Type 66/68	1
Low risk	2 (6.7)
▪ Type 42 and type 70	1
▪ Type 43/44	1
Mix of high risk and low risk	4 (13.3)
▪ Type 33, type 44, and type 84/26	1
▪ Type 51 and type 6	1
▪ Type 53, type 6, type 43/44, type 54/55, type 70 and type 72	1
▪ Type 58, type 53, type 40/61, and type 42	1
Outside the type found	2 (6.7)

No significant relationship between the age, number of sexual partners and use of condoms was observed ($p > 0.05$). However, a significant relationship between the age at first sexual intercourse, and frequency of sexual intercourse with subclinical CA was observed ($p<0.05$) as presented in TABLE 4.

TABLE 4. Characteristics of high-risk women based on subclinical CA infection

Characteristics of high-risk women	Sub clinical CA		p
	Positive [n (%)]	Negative [n (%)]	
Age (y.o.)			
▪ 12-16	0 (0)	0 (0)	
▪ 17-25	1 (6.7)	1 (6.7)	
▪ 26-35	7 (46.7)	9 (60)	0.510[a]
▪ 36-45	4 (26.7)	3 (20)	
▪ 46-55	3 (20)	2 (13.3)	
Age at first sexual intercourse (y.o.)			
▪ 12-16	9 (60)	2 (13.3)	
▪ 17-25	5 (33.3)	10 (66.7)	
▪ 26-35	1 (6.7)	3 (20)	0.012[a]
▪ 36-45	0 (0)	0 (0)	
▪ 46-55	0 (0)	0 (0)	
Frequency of sexual intercourse			
▪ > 1 time a d	6 (40)	2 (13.3)	
▪ 1-7 times a wk	8 (53.3)	6 (40)	0.014[a]
▪ 1-3 times a mo	1 (6.7)	7 (46.7)	
▪ < 1 time a mo	0 (0)	0 (0)	
Number of sexual partners (person)			
▪ 1	0 (0)	0 (0)	-
▪ > 1	15 (15)	15 (50)	
Use of condoms			
▪ Always	0 (0)	0 (0)	
▪ Sometimes	3 (20)	6 (40)	0.213[b]
▪ Never	12 (80)	9 (60)	

d: day; wk: week; mo: month; y.o.: years old; [a]Kruskal-Wallis Test; [b]Fisher's Exact Test

No significant relationship between the characteristics of high risk women i.e. age, age at first sexual intercourse, frequency of sexual intercourse, number of sexual partners and use of condoms with high-risk HPV was observed ($p > 0.05$) as presented in TABLE 5. Furthermore, a significant relationship between high-risk HPV and subclinical CA in the cervix of high-risk women was observed ($p < 0.05$) as presented in TABLE 6.

TABLE 5. Characteristics of high-risk women based on high-risk HPV

Characteristics of high-risk women	High risk [n (%)]	Mix of high and low risk [n (%)]	Non high-risk [n (%)]	p
Age (y.o.)				
▪ 12-16	0 (0)	0 (0)	0 (0)	
▪ 17-25	1 (14.3)	0 (0)	1 (5.3)	0.094[a]
▪ 26-35	3 (42.9)	1 (25)	12 (63.2)	
▪ 36-45	3 (42.9)	0 (0)	4 (21.1)	
▪ 46-55	0 (0)	3 (75)	2 (10.5)	
Age at first sexual intercourse (y.o.)				
▪ 12-16	4 (57.1)	2 (50)	5 (26.3)	
▪ 17-25	2 (28.6)	2 (50)	11 (57.9)	
▪ 26-35	1 (14.3)	0 (0)	3 (15.8)	0.362[a]
▪ 36-45	0 (0)	0 (0)	0 (0)	
▪ 46-55	0 (0)	0 (0)	0 (0)	
Frequency of sexual intercourse				
▪ > 1 time a d	3 (42.9)	2 (50)	5 (15.8)	
▪ 1-7 times a wk	3 (42.9)	2 (50)	9 (57.4)	0.112[a]
▪ 1-3 times a mo	1 (14.3)	0 (0)	7 (36.8)	
▪ < 1 time a mo	0 (0)	0 (0)	0 (0)	
Number of sexual partners (person)				-
▪ 1	0 (0)	0 (0)	0 (0)	
▪ > 1	7 (100)	4 (100)	19 (100)	
Use of condoms				
▪ Always	0 (0)	0 (0)	0 (0)	
▪ Sometimes	1 (14.3)	0 (0)	8 (42.1)	0.155[a]
▪ Never	6 (85.7)	4 (100)	11 (57.9)	

d: day; wk: week; mo: month; y.o: years old; [a]Kruskal-Wallis test

TABLE 6. Relationship between HPV types and subclinical CA in the cervix of high-risk women.

HPV types	Subclinical CA		p
	Positive [n (%)]	Negative [n (%)]	
High risk - Mix of high and low risk			
High risk	7 (63.6)	0 (0)	-
Mix of high and low risk	4 (34.4)	0 (0)	
Total	11 (100)	0 (0)	
High risk - Non-high risk			
High risk	7 (63.6)	0 (0)	0.001[a]
Non-high risk	4 (36.4)	15 (100)	
Total	11 (100)	15 (100)	
Mix of high and low risk - Non-high risk			
Mix of high risk and low risk	4 (50)	0 (0)	0.008[a]
Non-high risk	4 (50)	15 (100)	
Total	15 (100)	15 (100)	

DISCUSSION

There were 15 high-risk women with positive subclinical CA (50%) and 15 women (50%) who with negative subclinical CA (TABLE 1). The results of this study are in accordance with previous study that reported an abnormal cytology showing an acetowhite area on the cervix of high-risk women.[22] Several studies also reported a fairly good correlation with histopathological findings indicating HPV and the presence of positive HPV DNA in the acetowhite area.[23,24]

The HPV infection was detected in 15 people (50%) with subclinical CA and not detected in 15 people (50%) with non-subclinical CA (TABLE 2). Yanofsky *et al.*[25] reported the subclinical HPV infection rate to be 40%, whereas DNA analysis of apparently uninfected genital skin was reported to be positive for HPV. Giraldo *et al.*[26] reported that 29 people (20.7%) with CA were positive HPV infection on PCR examination.

High risk HPV type was the most detected HPV type in women with subclinical CA (7 people or 23.3%), while low risk HPV types were found in 2 people (6.7%). A mixture of high risk and low risk HPV types were found in 4 people (13.3%) and 2 people (6.7%) outside the type of HPV found (TABLE 3). Condyloma acuminata is mostly caused by HPV types 6 or 11, but can also be caused by the high-risk HPV types coinfection.[27] This study is in line with the study conducted by Hawkins *et al.*[28] that reported the presence of DNA and mRNA from one HPV type to be the cause of the development of lesions with mixed infection of high risk. In addition, low risk types was found in 4 people (33.3%) and a single infection in 8 people (66.6%). The results of this study are also in accordance with the study conducted by Al-Awadhi *et al.*[29] It was reported that high-risk HPV types were found in 34.62% of patients and low-risk HPV in 14.4 % of patients. The highest prevalence (50.6%) was HPV 1a, 2, 4, 7, 27, 57b, 57c, 65. The prevalence of HVP infection with single types, two types and triple types were 88.4%, 9.0%, and 2.6%, respectively.[29]

Subclinical CA was most commonly found in the 26-35 y.o. age group (7 people or 46.7%). However, no significantly relationship between age and subclinical CA was observed in this study (p=0.510) (TABLE 4). These results are similar to previous studies. A study conducted by Puspawati *et al.*[30] at Sanglah Hospital Denpasar, Bali reported the age of patients with CA was 12-35 y.o. Likewise, Effendi *et al.*[31] reported patients with CA who come to Dr. H. Abdoel Moeloek Distric Hospital Lampung were dominated by the 20-40 y.o. age group. Peder *et al.*[32] reported that the increase of infection risk among the young age is associated with a lack of adaptive immune response and the area of cervical epithelium. In this young age, the cervical epithelium can undergo relatively larger metaplasia in which may increase the risk of HPV infection of the basal cell layer and then increase proliferation. However, Tamer *et al.*[33] reported that the older people can suffer from CA due to decreased immunity and reactivation of latent infection.

The 12-16 y.o. age group was the first age group to have sexual intercourse with the highest people with subclinical CA (9 people or 60%). A significant relationship between the age of first sexual intercourse and subclinical CA was observed (p=0.012) as presented in TABLE 4. This result is in line with the study conducted by Tamer *et al.*[33] that reported the age at first sexual intercourse is significantly associated with the people with CA. Abnormal cells in the cervix can lead to malignancy.[33-35]

The frequency of sexual intercourse in people with subclinical CA was mostly found in the group with the frequency of having sex 1-7 times a wk (8 people or 53.3%) in this study (TABLE 4). A

significant relationship between the frequency of sexual intercourse with subclinical CA was observed (p=0.014). A study conducted by Haseen *et al.*,[36] supported the results obtained in this study. It was found that the frequency of sexual intercourse could lead to a higher positive incidence of STIs included subclinical CA. Haseen *et al.*[36] reported that adolescents who visited female sex workers at least once a mo had a higher prevalence of symptoms than other adolescents.

All subjects suffering from subclinical CA had more than 1 sexual partner (TABLE 4). Tamer *et al.*[33] reported a significant number of sexual partners in patients with CA (3 people) compared to without CA (1 people) (p = 0.0001). Peder *et al.*[32] reported that women who had multiple partners are more likely to develop CA than those had single partners.

People with subclinical CA were more higher in the group never using condoms (12 people or 80%). However, it was no significant relationship between using condom and the subclinical CA prevalence (p=0.213) as presented in TABLE 4. The results is in line with study conducted by Nareswari *et al.*[35] It was reported that the majority of people with CA informed that they had never used a condom during sexual intercourse (51.1% in men vs. 72.3% in women).[35] Many studies report the function of condoms as a protector for the prevention of transmission of HPV infection. This function is especially important in women because they do not want to be seen as distrusting their partners so that they are more susceptible to infection.[32]

High risk HPV types were found in 3 people (42.9%) in the 26-35 y.o, age group 3 people (42.9%) in the 36-45 y.o. age group and at least 1 people (14.3%) in the 17-25 y.o. age group. High-risk and low-risk mixed HPV types were found in 3 people (75%) in the 46-55 y.o. age group. No significant relationship between age and high-risk HPV types (p=0.094) was resported (TABLE 5). Kang *et al.*[37] in China reported that the prevalence of high-risk HPV types is greater in older women. The prevalence of high-risk HPV types (p<0.001), HPV16/18/45 (p=0.002), and high-risk HPV types other than HPV16/18/45 (p=0.002) generally increased with age. High-risk HPV types more found in older women due to increased lifetime exposure, incidence of HPV, and/or viral viability in older women. In addition, the high incidence in older women was due to acquired HPV infection caused by changes in the sexual behavior of women themselves and their sexual partners and reactivation of previous latent HPV infections due to a decrease in the body's immune system at menopause.[37]

The 12-16 y.o. age group was the first age group to have sexual intercourse with the highest number of subjects infected with high-risk HPV types (4 people or 57.1%). High risk and low risk mixed HPV types were found in 2 people (50%) in the 12-16 y.o. age group, and 2 people (50%) in the 17-25 y.o. age group (TABLE 5). No significant relationship between age at first sexual intercourse with high-risk HPV types was observed (p=0.362). The results of this study were not supported by Kang *et al.*[37] who reported that younger age at initiation of sexual intercourse have a higher risk of developing high-risk HPV infection.

Subjects with high-risk HPV types having sexual intercourse more than once a day were found in 3 people (42.9%), 1-7 times a wk in 3 people (42.9%), and the least were found in the group with the frequency of having sex 1-3 times a month in 1 person (14.3%). High-risk and low-risk mixed HPV types were found in 2 people (50%) in the group having sex more than once a day, and 2 people (50%) in the group having sex 1-7 times a wk. No significant relationship between the frequency of sexual intercourse with high-risk HPV

types (p=0.112) as presented in TABLE 5. Haseen *et al.*[36] reported that the more frequent the frequency of sexual intercourse will increase the incidence of STI. Adolescents who frequently visited female sex workers had more STIs than other adolescents.[36]

All subjects infected with high-risk HPV types and mixed high-risk and low-risk HPV types had more than 1 sexual partner (TABLE 5). Kang *et al.*[37] reported the most high-risk HPV types in women had a number of sexual partners 5 or more. The women who have had more than 1 sexual partner for life have a 1.3-fold risk of experiencing high-risk HPV infection.[37]

Subjects infected with high-risk HPV types were found in the group never using condoms (6 people or 85.7%) and all subjects infected with mixed high risk and low risk HPV types had never used condoms (4 people or 100%). However, no significantly relationship between using condom and high-risk HPV types (p=0.155) as presented in TABLE 5. Tay *et al.*[38] reported that women not using condoms shows a high prevalence of HPV infection (28.6%) and infection with high-risk HPV types (23.8%). In addition, the use of condoms has been shown to be protective in preventing STIs and could reduce the prevalence of HPV infection.[39]

A significantly association between high-risk HPV types and subclinical cervical CA in high-risk women (p=0.001) was observed (TABLE 6). This results are in line with the study conducted by Al-Awadhi *et al.*[29] It was reported that there is a relationship between HPV types and the incidence of CA with 34.62% of patients infected with high-risk HPV types more than 14.4% of patients infected with low-risk HPV types. Another study in Austria reported that 42% of patients are infected with low-risk HPV and 21% of patients were positive for the high-risk HPV genotype. Multiple infections with low risk and high-risk genotypes were reported in 36% of patients.[40]

Based on the possibility of epithelial dysplasia and malignancy, HPV is divided into low-risk HPV and high-risk HPV. Low-risk HPV types, such as types 6, 11, 42, 43, 44, 54, 61, 70, 72 and 81 tend to cause benign tumors such as verrucae and CA. While high risk types tend to cause anogenital malignant tumors, such as cervical, vulvar, vaginal, anal and penile cancers, where HPV types 16 and 18 are often found in high-grade dysplasia and malignancy.[1,17,18]

Cervical cancer is one of the most common types of cancer in women which it is associated with HPV infection. Patients infected with HPV genotypes 6 or 11 had an increased risk of having CA (OR=2.34; 95%CI= 0.955-5.737; p=0.06). In addition, this association increased with the presence of high-risk HPV types and low-risk HPV types for having CA (OR= 2.814; 95% CI= 1.208-6.55; p=0.017) and cases with high-risk HPV types (OR= 2,329; 95% CI= 1.029-5.269; p=0.042). Patients with CA are usually found to have CIN2/3.[41] A cohort study involving 10,971 patients (1,685 men and 9,286 women) in the Swedish population reported that CA is strongly associated with an increased risk of cancers of the anogenital tract, such as the vulva, penis, and anus.[42]

CONCLUSION

There is a relationship between high-risk HPV and subclinical CA in the cervix of high-risk women.

ACKNOWLEDGMENT

We would like to express gratitude to the Head of the Department of Dermatology and Venereology of Faculty of Medicine, Universitas Sumatera Utara and Universitas Sumatera Utara Academic Hospital, Medan, Indonesia. The authors declare no conflict of interest regarding the publication of this article.

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JMedSci

Journal of the Medical Sciences
(Berkala Ilmu Kedokteran)

Volume 54, Number 2, 2022; 161-170
http://dx.doi.org/10.19106/JMedSci005402202207

Antibiotic resistance of biofilm-producing bacteria from sepsis patients in Prof. Dr. Margono Soekarjo Hospital, Purwokerto, Central Java

Rani Afifah Nur Hestiyani[1*], Wahyu Siswandari[2], Vitasari Indriani[2], I Dewa Sang Ayu Putu Peramiarti[1], Hermin Prihartini[3], Dwi Utami Anjarwati[1]

[1]Department of Microbiology, Faculty of Medicine, Universitas Jenderal Soedirman, [2]Department of Clinical Pathology, Faculty of Medicine, Universitas Jenderal Soedirman, [3]Department of Anesthesiology and Intensive Therapy, Prof. Dr. Margono Soekarjo General Hospital, Purwokerto, Central Java, Indonesia

Submitted: 2018-11-12
Accepted : 2021-09-13

ABSTRACT

Sepsis is a life-threatening organ dysfunction induced by the body's response to infection and is a significant cause of critical illness and death in hospitals. Bacteria are the most common pathogens that cause sepsis, and their ability to form biofilms increases their resistance to antibiotics. As a result of the failure of antibiotic administration therapy, the severity and pain of sepsis worsen. The study used a descriptive research design to determine the antibiotic resistance pattern of biofilm-producing bacteria from clinical isolates of sepsis patients. Using the BacT/Blood Culture System Alert, all patients suspected of sepsis in the intensive care unit of Prof. Dr. Margono Soekarjo General Hospital Purwokerto were examined for blood cultures between March and July 2018. These were then identified and tested for antibiotic resistance with the Vitek 2 Compact. Biofilm formation was detected utilizing the microtiter plate assay method, and the data were analyzed using a frequency distribution table. The results obtained 12 bacterial isolates, with *Escherichia coli* (41.67%), *Staphylococcus haemolyticus* (33.33%), *Klebsiella pneumoniae ssp pneumoniae*, *Enterobacter cloacae complex*, and *Acinetobacter baumannii complex* (8.33%) as the most common bacteria. All gram-negative bacteria (more than 80%) were resistant to ampicillin, cefoxitin, ceftazidime, ceftriaxone, aztreonam, and trimethoprim but were sensitive to meropenem (100%). Gram-positive bacteria were resistant to cefoxitin, benzylpenicillin, oxacillin, ciprofloxacin, erythromycin, and clindamycin (100% each). However, they were sensitive to tigecycline, nitrofuran, quinupristin, linezolid, vancomycin, and tetracycline (100% each). Gram-negative bacteria formed 50% biofilms, and 50% did not, whereas gram-positive bacteria produced 100% biofilms. In conclusion, bacteria clinical isolates of septic patients from Prof. Dr. Margono Soekarjo General Hospital Purwokerto are multiresistant to more than six types of antibiotics and produce weak to moderate biofilms, which can promote antibiotic resistance.

Keywords:
bacteria;
biofilm;
antibiotic resistance;
sepsis;
epidemiology

ABSTRAK

Sepsis adalah disfungsi organ yang mengancam jiwa yang diakibatkan oleh respon tubuh terhadap infeksi dan diyakini menjadi penyebab utama penyakit kritis dan kematian di rumah sakit. Bakteri sebagai patogen utama penyebab sepsis sering menjadi resisten terhadap antibiotik dan kemampuannya membentuk biofilm dapat meningkatkan resistensinya sehingga meningkatkan keparahan dan kesakitan kejadian sepsis karena kegagalan terapi pemberian antibiotik. Penelitian ini bertujuan untuk mengetahui pola resistensi antibiotik bakteri penghasil biofilm dari isolat klinik pasien sepsis. Desain penelitian yang digunakan deskriptif. Semua pasien terduga sepsis di ruang perawatan intensif RSUD Prof. Dr. Margono Soekarjo Purwokerto selama bulan Maret-Juli 2018 dilakukan pemeriksaan kultur darah dengan alat *BacT/Alert Blood Culture*

*corresponding author: rani.hestiyanti@unsoed.ac.id

System, kemudian diidentifikasi dan diuji resistensi dengan alat Vitek 2 Compact. Pembentukan biofilm dengan metode mikrotiter *plate assay*. Data dianalisis dengan tabel distribusi frekuensi. Hasil didapatkan 12 isolat bakteri dengan bakteri terbanyak, yaitu *Escherichia coli* (41,67%), *Staphylococcus haemolyticus* (33,33%), *Klebsiella pneumoniae ssp pneumoniae*, *Enterobacter cloacae complex*, *Acinetobacter baumannii complex* masing-masing (8,33%). Semua bakteri gram negatif resisten diatas 80% terhadap ampisilin, cefoxitin, ceftazidin, ceftriaxon, aztreonam, dan trimetoprim tapi sensitif terhadap meropenem 100%. Bakteri gram positif resisten terhadap cefoxitin, benzilpenicilin, oxacilin, ciprofloxacin, eritromisin dan clindamisin masing-masing 100% dan sensitif terhadap tigecyclin, nitrofurat, quinupristin, linezoid, vancomycin and tetracyclin masing-masing 100%. Dari ke 12 bakteri, 50% bakteri gram negatif membentuk biofilm dan 50% tidak membentuk biofilm, sedangkan bakteri gram positif penghasil biofilm 100%. Kesimpulan penelitian ini adalah bakteri isolat klinik pasien sepsis dari RSUD Prof Margono Soekarjo Purwokerto multiresisten terhadap lebih dari 6 jenis antibiotik dan menghasilkan biofilm lemah sampai moderat yang dapat meningkatkan resistensinya terhadap antibiotik.

INTRODUCTION

Sepsis is a potentially fatal organ dysfunction induced by the body's immune response to infection and is a vital cause of critical illness and death in hospitals.[1] The incidence is relatively high, affecting millions of individuals each year, and it is rising. According to the World Health Organization (WHO), sepsis affects more than 30 million people each year, with the potential to cause 6 million deaths,[2] which is a heavy burden for developing and impoverished countries. In Indonesia, 35 patients were studied at Prof. Dr. R. D. Kandou Manado Hospital, where 29 patients (82.8%) were diagnosed with sepsis, and 23 patients (65.7%) died.[3]The patients with sepsis experienced organ dysfunction due to irregular host body responses that can lead to septic shock and death.[4] Nearly 15% of sepsis patients are treated in Intensive Care Units (ICUs), with two-thirds developing septic shock. According to reports, sepsis is a leading cause of death among patients treated in these units.[3,5] In addition, patients with sepsis are particularly vulnerable due to low immunity, malnutrition, and exposure to medical treatment, all of which increase mortality and cost of care.[6,7]

Gram-negative bacteria such as *Escherichia coli*, *Klebsiella* spp., *Pseudomonas aeruginosa*, *Enterobacter* spp., *Proteus* spp., *Neisseria* spp., *and Acinetobacter* spp., are known to be the most common pathogens that cause sepsis, other than gram-positive bacteria (*Staphylococcus aureus* and *Coagulase-negative Staphylococci*).[3,8] Bacteria can produce clinical symptoms of sepsis because they share virulence factors with their hosts and induce an inflammatory response. Furthermore, Gram-negative bacteria can infect the host due to the presence of lipopolysaccharide and an endotoxin, but gram-positive bacteria contain an exotoxin that functions as a superantigen.[8]

Bacteria that cause sepsis are often antibiotic-resistant, worsening the condition of the patients.[9] Appropriate antibiotic selection is crucial for lowering sepsis mortality and is administered within 1-2 hours after sepsis diagnosis. In addition, the pattern of resistance differs by hospital and changes over time. A study at Moewardi Hospital in 2016, *E. coli* is resistant to ampicillin, chloramphenicol, and gentamicyn.[10] Lewis *et al.*[11] also reported *Acinetobacter resistance* to ceftriaxone and imipenem. Research conducted in the intensive care room of Fatmawati Hospital, Indonesia, showed that *P. aeruginosa*, *S. epidermidis*, and *E. coli* were resistant to meropenem, gentamicin, and levofloxacin.[12] Furthermore, in Margono Soekarjo General Hospital, the resistance of

Klebsiella to meropenem was reported.[13]

Antibiotic resistance is a primary cause of antibiotic therapy failure in patients with sepsis and septic shock, raising the mortality rate in hospitals. Biofilm formation by sepsis-causing bacteria is another factor that contributes to the ineffectiveness of sepsis patients' treatment.[14] Bacteria can produce biofilms, making them more resistant to antibiotics at least 10-1000 times than planktonic bacteria.[15] This can increase the severity and pain of the incidence of sepsis due to the failure of antibiotic therapy. Biofilms protect bacteria from antibiotics and the immune system by bacterial colonization.[16] For instance, biofilms can tolerate antimicrobial agents at concentrations of 10–1000 times that needed to inactivate genetically equivalent planktonic bacteria.[17]

Sepsis requires fast and precise handling. Proper and prompt administration of antibiotic therapy is needed to reduce sepsis mortality.[17] Information on bacterial antibiotic resistance that causes sepsis and the ability of these bacteria to produce biofilms that can increase the incidence of antibiotic resistance is needed to be used as guidelines for appropriate antibiotic therapy in the treatment of septic patients. Research on the antibiotic resistance of bacteria that causes sepsis and the ability of these bacteria to produce biofilms has never been carried out at the Prof. Dr. Margono Soekarjo General Hospital, Purwokerto. This study aimed to determine the antibiotic resistance pattern of biofilm-producing bacteria from clinical isolates of sepsis patients in Prof. Dr. Margono Soekarjo General Hospital, Purwokerto.

MATERIALS AND METHODS

Subject

The research design used is descriptive. The study sample was all bacteria isolated from adult patients suspected of sepsis treated in intensive care at Prof. Dr. Margono Soekarjo General Hospital, Purwokerto, during the period March - July 2018. Identification of bacteria and antibiotic resistance testing was carried out according to the standards in the Microbiology Division of the Clinical Pathology Laboratory Prof. Dr. Margono Soekarjo General Hospital, Purwokerto using Vitek 2 Compact. Biofilm testing was conducted at the Research Laboratory of the Faculty of Medicine, Universitas Jenderal Soedirman, Purwokerto.

Bacteria isolates

All bacteria were obtained from Prof. Dr. Margono Soekarjo General Hospital and isolated from clinical material of blood samples from sepsis patients in the intensive care unit room. As much as 5 mL blood samples were taken by skilled health workers according to the standards, then put into the BacT/Alert Blood Culture System transport media for review.

Identification and antibiotic susceptibility test

The bacteria were identified and tested for antibiotic resistance with Vitek 2 Compact.

Microtiter plate assay

The microtiter plate method was conducted as previously described[14] with modification: bacteria were grown in 3 mL of trypticase soy broth (TSB) medium for 24 h at 37°C. The initial inoculum was diluted with a ratio of 1: 100 (which concentration 10µL culture in 1 mL TSB), then put 100 µL TSB with 1.5% glucose and TSB without glucose into each of the 96-well microtiter plate wells and added 10 µL of diluted culture into each well. The plate was covered

with plastic (wrapping) and incubated at 37°C for 20 h. Planktonic bacteria were carefully removed from each well with a 200 µL pipette (gently pipette and leave the biofilm on the bottom of the well until the bottom of the well looks clear. Furthermore, the well was washed with 300 µL PBS (phosphate-buffered saline) to remove non-adherent bacteria. After that, PBS wasis discarded with a 200 µL (2 times) pipette slowly. Then the well was painted with 100 µL 1% crystal violet (try not to touch the bottom of the pipette), then save 30 min at room temperature (on the table). The well was washed again with distilled water then added 5% acid isopropanol (HCl and two propanols). Optical density (OD) was calculated at a wavelength of 450 nm. *Staphylococcus epidermidis* 12228, which is a biofilm-producing strain, was used as a positive control. Bacterial cells are categorized as very strong biofilm producers when > 4 X ODC; 2 X Odc <... ..4X Odc is moderate; or Odc <... .. ≤ 2 X Odc is said to be weak, ≤ Odc includes non-biofilm. Odc = OD negative control, Ods = OD.

Data analysis

The data were analyzed and then presented descriptively with a frequency distribution table. This research has passed the ethical review by the Medical Research Ethics Commission, Faculty of Medicine, Universitas Jenderal Soedirman, Purwokerto.

RESULTS

The patient characteristics

The results showed 12 positive blood cultures from sepsis patients: five men (41.66%) and seven women (58.33%)

Bacterial distribution

This research obtained 12 bacterial isolates from sepsis patients, namely *E. coli* (41.67%), *S. haemolyticus* isolates (33.33%), *K. pneumoniae ssp pneumoniae*, *E. cloacae complex*, and *A. baumannii complex*, respectively (8.33%). The distribution of bacteria that cause sepsis is presented in TABLE 1.

TABLE 1. Distribution of sepsis bacteria in Prof. Dr. Margono Soekarjo General Hospital Purwokerto for the period March-July 2018 (n = 12)

Microorganism	Number of isolate	Percentage (%)
Gram-negative		
▪ *E. coli*	5	41.67
▪ *K. pneumoniae ssp pneumoniae*	1	8.33
▪ *E. cloacae complex*	1	8.33
▪ *A. baumannii complex*	1	8.33
Gram-positive		
▪ *S. haemolyticus*	4	33.33
Total	12	100

Antibiotic resistance

In this study, the antibiotic resistance result, obtained automatically by a Vitek® 2 Compact. Gram-negative bacterial antibiotic resistance test results from sepsis patients are presented in TABLE 2, and the gram-positive bacterial resistance test results are presented in TABLE 3. It was found that all gram-negative bacteria are still sensitive to carbapenem antibiotics, which are meropenem and resistant to beta-class antibiotics lactam, namely ampicillin. *Escherichia coli* is still sensitive to meropenem, amikacin, ertapenem, gentamicin, tigecycline,

and nitrofuran(100%) but resistant to more than 50% beta-lactam class antibiotics. *Klebsiella* is still sensitive to carbapenem, amikacin, gentamicin, ciprofloxacin, tigecycline, and nitrofurat groups (100%) and all resistant to ampicillin, cefoxitin, ceftriaxone, and trimethoprim. *Enterobacter cloacae complex* shows sensitivity to meropenem and amikacin but is resistant to 14 other types of antibiotics. *Acinetobacter baumannii complex* is sensitive to ertapenem, meropenem, and tigecycline respectively by 100% but resistant to 12 different types of antibiotics.

Gram-positive bacteria *S. haemolyticus* is resistant to cefoxitin, benzylpenicillin, oxacillin, ciprofloxacin, erythromycin, and clindamycin 100% and sensitive to tigecycline, nitrofuran, quinupristin, linezolid, vancomycin, and tetracycline 100%. *Staphylococcus* is resistant to cefoxitin (TABLE 3).

TABLE 2. Resistance patterns of septic gram-negative bacteria in Prof. Dr. Margono Soekarjo General Hospital Period March-July 2018.

Antibiotic	Antibiotic resistance [% (n)]			
	E. coli	*K. pneumoniae ssp*	*E. cloacae complex*	*A. baumannii complex*
Ampicillin	80 (4)	100 (1)	100 (1)	100 (1)
AS	60 (3)	100 (1)	100 (1)	0
Piperacillin	20 (1)	0	100 (1)	100 (1)
Cefoxitin	60 (3)	100 (1)	100 (1)	100 (1)
Ceftazidime	60 (3)	100 (1)	100 (1)	100 (1)
Ceftriaxone	60 (3)	100 (1)	100 (1)	100 (1)
Cefepime	40 (2)	100 (1)	100 (1)	100 (1)
Aztreonam	60 (3)	100 (1)	100 (1)	100 (1)
Ertapenem	0	0	100 (1)	0
Meropenem	0	0	0	0
Amikacin	0	0	0	100 (1)
Gentamicin	0	0	100 (1)	100 (1)
Ciprofloxacin	0	0	100 (1)	100 (1)
Tigecycline	0	0	100 (1)	0
Nitrofuran	0	0	100 (1)	100 (1)
Trimethoprim	60 (3)	100 (1)	100 (1)	100 (1)

TABLE 3. The pattern of resistance of gram-positive bacteria to cause sepsis in Prof. Dr. Margono Soekarjo General Hospital Period March-July 2018.

Antibiotic	Antibiotics resistance to *S. haemolyticus* [% (n)]
Cefoxitin	100 (4)
Gentamicin	75 (3)
Tigecycline	0
Nitrofuran	0
Trimethoprim	50 (2)
Benzylpenicillin	100 (4)
Oxacillin	100 (4)
Ciprofloxacin	100 (4)
Levofloxacin	100 (4)
Moxifloxacin	25 (1)
Erythromycin	100 (4)
Clindamycin	100 (4)
Quinupristin	0
Linezolid	0
Vancomycin	0
Tetracycline	0
Rifampicin	75 (3)

Biofilm assay

The biofilm test results using the microtiter plate assay method are presented in TABLE 4. Negative control OD was obtained (Odc = 0.0128). Of the 12 bacteria, 50% of gram-negative bacteria form biofilms, and 50% do not form biofilms, while 100% gram-positive bacteria produce biofilms. *Staphylococcus haemolyticus* and *K. pneumoniae ssp pneumoniae* bacteria show the weak ability to produce biofilms (0.0128 < ODc 00.0256), while *E. coli* and *E. cloacae complex* do not produce biofilms (Odc ≤ 0.0128). Only *A. baumannii* is a moderate biofilms former (0.0256≤Odc 0.0512). The frequency of biofilm-producing bacteria and multi antibiotic resistance of bacterial isolates are presented in TABLE 5.

TABLE 4. Results of biofilm production for bacterial isolates using the microtiter plate method

Bacterial isolates	Biofilm former			
	Non-biofilm [n (%)]	Weak [n (%)]	Moderate [n (%)]	Strong [n (%)]
Gram-positive				
▪ *S. haemolyticus*		4 (33.33)		
Gram-negative				
▪ *E. coli*	5 (41.67)			
▪ *E. cloacae complex*	1 (8.33)			
▪ *A. baumannii complex*			1 (8.33)	
▪ *K. pneumoniae ssp pneumoniae*		1 (8.33)		

Non biofilm: ODc ≤ 0.0128; Weak: 0.0128 < ODc ≤ 0.0256; Moderate 0.0256 < ODc ≤ 0.0512; Strong ODc> 0.0512

TABLE 5. Frequency of biofilm-producing bacteria and antibiotic multi-resistance

Microorganism	Antibiotic resistance	Biofilm former
Gram-positive bacteria		
▪ *S. haemolyticus*	11 multi-resistance	Weak
Gram negative bacteria		
▪ *E. coli*	11 multi-resistance	Non-former
▪ *K. pneumoniae ssp pneumoniae*	8 multi-resistance	Weak
▪ *E. cloacae complex*	14 multi-resistance	Non-former
▪ *A. baumannii complex*	12 multi-resistance	Moderate

DISCUSSION

The results showed that the most common bacterial causes of sepsis in adults were gram-negative bacteria, *E. coli* (41.67%), and gram-positive bacteria *S. haemolyticus* (33.33%). This is consistent with the study in Brazil (53.2%), in Southeast Asia (5%), and in Indonesia (68,8%).[5,18,19] The research conducted in Moewardi General Hospital, Solo, and in Ethiopia shows a different result, where gram-positive bacteria were the most common causes of sepsis by 15.09% and 83.4%, respectively.[8,10] The variations of bacteria that cause sepsis in hospitals in different countries can occur due to geographical location,

epidemiological variants, the nature of the patient population, limited sample size, and limited research time span. The prevalence of infection can be affected by season; this study may have failed to show the actual prevalence of pathogen that causes it.[5,20] In this study, the research time span is only five months (March to July), therefore the pattern of bacteria is different from the other hospital.

Escherichia coli in the study was a gram-negative bacteria that was reported to be the cause of sepsis and other gram-negative bacteria, namely *Klebsiella, Acinetobacter*, and *Enterobacter*. These results are consistent with the study in Ethiopia and Brazil.[5,20] The different results found from Indonesia's research, where the gram-negative bacteria found were *Klebsiella* sp. This can occur because of differences in geographical location, causing a variety of agents causing sepsis.[19]

Gram-positive bacteria *S. haemolyticus* is the second *Staphylococcus negative coagulase* (CoNS) after *S. epidermidis*, most often isolated from clinical cases such as sepsis.[20] However, the results of the study showed *S. haemolyticus* to be the most gram-positive bacteria causing sepsis. This difference is likely due to differences in blood culture systems and media content. The amount of CoNS isolated from blood cultures needs to be considered because it is considered a contaminant in many studies. The current research of CoNS is an important pathogen for nosocomial infections and sepsis.[21,22]

Antibiotic therapy for septic patients is faced with the challenge of the emergence of antibiotic-resistant bacteria and even develops into multi-resistance. The antibiotics recommended for adult sepsis patients for gram-positive bacteria are vancomycin and gentamicin, while for gram-positive bacteria is meropenem.

Echerichia coli isolates in the study were resistant to beta-lactam antibiotics above 50% but sensitive to meropenem, amikacin, ertapenem, gentamicin, tigecycline, and nitrofuran 100%. This is according to study at Riau Hospital and Solo.[9,10] *Klebsiella* is still 100% sensitive to carbapenem, amikacin, gentamicin, ciprofloxacin, tigecycline, and nitrofuran groups, all of which are resistant ampicillin, cefoxitin, ceftriaxone, and trimethoprim. *Enterobacter cloacae complex* shows sensitivity to meropenem and amikacin but is resistant to 14 other antibiotics. *Acinetobacter baumannii complex* is still 100% sensitive to ertapenem, meropenem, and tigecycline, but resistant to 12 different types of antibiotics.

All gram-negative bacteria are resistant above 80% against ampicillin, cefoxitin, ceftazidime, ceftriaxone, aztreonam, and trimethoprim but are sensitive to 100% meropenem. Empiric antibiotic therapy for sepsis can use meropenem. Meropenem is the choice for the treatment of *Enterobacter* infection because of its immunity to destruction by beta-lactamase produced by this pathogen.[19]

Gram-positive bacteria *S. haemolyticus* are 100% resistant to cefoxitin, benzylpenicillin, oxacillin, ciprofloxacin, erythromycin, and clindamycin, and 100% sensitive to tigecycline, nitrofuran, quinupristin, linezolid, vancomycin, and tetracycline. *Staphylococcus* is resistant to cefoxitin, which means that the isolates include methicillin resistance. Many studies report *S. haemolyticus* is resistant to penicillin, cephalosporin, tetracycline quinolones, aminoglycosides, glycopeptides, and phosphomycines.[23,24] The mechanism of *S. hemolytic* methicillin resistance determines resistance to all beta-lactam antibiotics i.e. penicillin, cephalosporins, carbapenems, and monobactams. This mechanism is related to the presence of the mecA gene, encoding the PBP2a protein modified penicillin transpeptidase, which is responsible for

the synthesis of pentaglycine bridges in peptidoglycan. Another important feature of *Staphylococci* is their ability to survive in a hospital environment.[25] Vancomycin can be an empiric therapy for antibiotics against *S. haemolyticus*. However, many studies have reported cases of *Vancomycin Resistance S. aureus* (VRSA).[26]

All bacterial isolates became multiresistant because they were resistant to more than two or more types of antibiotics. Similar studies also showed the same results.[19,20] Ciprofloxacin, which became an antibiotic for gram-positive and negative bacteria, actually experienced resistance.

In this study, the susceptibility test and species identification were carried out automatically by Vitec compact 2. The advantages of this tool are practical and useful in detecting species.[16]

In addition to being resistant to antibiotics, the ability of bacteria to produce biofilms that can decrease antibiotic efficacy is a serious problem in handling septic patients. The ability of bacteria in surface attachments and the formation of multicellular communities is an important key to the infection stage and becomes a bacterial virulence factor.[14,16] Biofilm assay showed that all 100% gram-positive bacteria were able to produce biofilms even though they were weak. In gram-negative bacteria, 75% did not produce biofilms, while 25% produced weak and moderate biofilms. All isolates were multiresistant to more than six antibiotics, where 50% produced biofilms. This shows that most multi-drug resistance bacteria are biofilm producers.

The antibiotics resistance mechanisms of biofilms and planktonic bacteria are different. The biofilm is more resistant to antibiotics up to 1000 times more than free-bacteria.[16] The prevalence of bacteria that cause sepsis, most of which are multi resistant to antibiotics, needs special attention. The pattern of resistance and sensitivity to antibiotics is necessary as a guideline for empirical antibiotic therapy to treat septic patients whose immune conditions are very vulnerable. In addition, the ability of bacteria to produce biofilms that increase their tolerance in biofilms means that different therapeutic methods are needed, such as topical antibiotics.

CONCLUSION

The clinical bacterial isolates from sepsis patients at Prof. Dr. Margono Soekarjo General Hospital, Purwokerto, Central Java is multiresistant to more than six types of antibiotics and produce weak to moderate biofilms, which can increase their resistance to antibiotics

ACKNOWLEDGMENT

We would like to thank all the staff of the Clinical Pathology laboratory at Prof Dr. Margono Soekarjo General Hospital, Purwokerto, who has helped carry out bacterial culture and technician laboratory in the Research laboratory of the Medical faculty Jenderal Soedirman University who has helped biofilm assay. We also would like to thank the Research and Community Service Institute of Jenderal Soedirman University for providing research funding through the Institutional Research scheme.

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Journal of the Medical Sciences
(Berkala Ilmu Kedokteran)

Volume 54, Number 2, 2022; 171-180
http://dx.doi.org/10.19106/JMedSci005402202208

Challenge in diagnosing tuberculosis on a boy with severely wasted in limited resource area

Marini Siagian[1], Norman Tanyadji[2]

[1]Oksibil District General Hospital, Pegunungan Bintang, Papua, [2]Department of Pediatric, Oksibil District General Hospital, Pegunungan Bintang, Papua

Submitted: 2021-01-04
Accepted : 2021-07-26

ABSTRACT

Hunger and malnutrition are still the leading cause of morbidity and mortality among children around the world. Undernutrition increases the risk of tuberculosis (TB) which in turn could worsen undernutrition. Indonesia is currently the second highest newly diagnosed TB in the world after India. TB in children with severely wasted is difficult to diagnose. This is a case report about a 35-month-old boy with severely wasted complicated with hypoglycemia, severe dehydration, and pneumonia. After proper nutritional management without the expected outcome, patient was then worked up for TB resulting negative result of tuberculin skin test (TST). Nevertheless, he was still treated with antituberculosis and had significant improvement, hence continuation to complete 6 mo period of therapy. This case report describes the challenge of diagnosing TB in children with severely wasted in limited resource areas. The reduced immune responses, due to severely wasted, caused subtle clinical signs of TB and decreased sensitivity to tuberculin testing. The unavailability of radiologic examination added further problem in diagnosis. The diagnosis of TB should be considered among children in areas with a high prevalence of TB, presenting with severely wasted refractory to proper nutritional management.

ABSTRAK

Kelaparan dan malnutrisi masih menjadi penyebab utama morbiditas dan mortalitas anak-anak di seluruh dunia. Kekurangan gizi dapat meningkatkan risiko terjadinya tuberculosis (TB) yang pada gilirannya dapat memperburuk kekurangan gizi. Saat ini Indonesia menduduki peringkat kedua tertinggi penderita TB baru di dunia setelah India. Tuberkulosis pada anak gizi buruk sulit didiagnosis. Pada makalah ini dilaporkan kasus anak laki-laki berusia 35 bulan keadaan gizi buruk dengan komplikasi hipoglikemia, dehidrasi berat, dan pneumonia. Setelah manajemen nutrisi yang tepat tanpa hasil yang diharapkan, pasien kemudian diperiksa untuk TB dengan hasil tes kulit tuberkulin (TST) negatif. Namun demikian, ia masih diobati dengan antituberkulosis dan mengalami peningkatan berat badan yang signifikan, sehingga dilanjutkan untuk menyelesaikan periode terapi 6 bulan. Laporan kasus ini menjelaskan tantangan dalam mendiagnosis TB pada anak dengan kondisi gizi buruk di daerah dengan sumber daya yang terbatas. Berkurangnya kekebalan tubuh, karena kondisi gizi buruk, menyebabkan tanda-tanda klinis TB yang tidak jelas dan penurunan kepekaan terhadap tes tuberkulin. Tidak tersedianya pemeriksaan radiologis menambah masalah lebih lanjut dalam diagnosis. Diagnosis TB harus dipertimbangkan di antara anak-anak di daerah dengan prevalensi TB yang tinggi, dengan gejala yang sangat refrakter terhadap manajemen nutrisi yang tepat.

Keywords:
severely wasted;
tuberculosis;
pneumonia;
malnutrition;
children

*corresponding author: marinisiagian@gmail.com

INTRODUCTION

Hunger and malnutrition are still the leading cause of morbidity and mortality among children around the world. The Sustainable Development Goals (SDGs) seek sustainable solutions to end hunger and all forms of malnutrition by 2030 and achieve food security. Nutritional problems, such as malnutrition and stunting, are still major problems that need to be addressed immediately.[1] In 2019, 47.0 million children under five years around the world were wasted, of which 14.3 million were severely wasted and 10.5 million of severely wasted cases occurred in Asia.[2] According to Basic Health Research (Riset Kesehatan Dasar, Riskesdas) 2018, the proportion of children under five years in Indonesia (0-59 mo) is around 8.8% (23 million), and 3.5% (805,000) children are severely wasted. The prevalence of severely wasted in Papua is 4.8%.[3,4] Based on Statistic Data of Papua 2020, incidence of wasted in Pegunungan Bintang Regency were 110 cases.[5]

Wasting has short and long-term impacts, such as growth and development disorders, including cognitive dysfunction, impairment of the immune system function, risk of degenerative disease later in life, and ultimately death. The risk of death associated with severe wasting is 12-fold higher compared to the well-nourished children.[6,7] Children with undernutrition are at increased risk of death from infectious diseases and, conversely, severe infectious diseases in early childhood can affect nutritional status. Undernutrition is also known to increase the risk of tuberculosis (TB) and TB can cause or worsen undernutrition.[8]

There were estimated 10.0 million new cases of TB disease in 2019 globally (130 cases per 100,000 population, and about 845,000 new TB cases in Indonesia (312 per 100,000 population). The number of new TB cases in Indonesia was the second highest after India. Prevalence of TB in Papua based on Basic Health Research 2018 was 0.77%. This number was higher than national average. TB affects all countries and age groups whereas 12% were children.[4,9,10]

According to Indonesian national guideline of medical service for management of TB 2019, the diagnosis of TB in children based on history, symptoms, physical examination include analysis of child development, tuberculine test or IGRA (interferon gamma release assay), bacteriological confirmation and other relevant investigations (chest X-ray, lumbar puncture, biopsy and others according to the location of the affected organ).[9]

Diagnosis of TB among children utilizes a scoring system that can be carried out in limited health care facilities, both with limited medical personnel and diagnostic tools. If the microscopic examination is negative or there is no access to referrals (radiology/ Xpert MTB/RIF/culture), then broad-spectrum antibiotic therapy (non-OAT and non-quinolones) should be administered for 1-2 wk. If there is no clinical improvement after antibiotic administration, the patient needs to be assessed for TB risk factors. Patients with high risk factors for TB can be diagnosed as clinical TB. Since the higher risk of disseminated TB in children less than 5 y.o., TB therapy should be given as soon as the diagnosis is made.[9]

The bacteriological diagnosis of TB in children is more difficult due to low production of sputum. The diagnosis of TB among children in limited resource area both with limited medical personnel and diagnostic tools is even more difficult. Clinical signs of TB in children are largely non-specific, such as unexplained weight loss or failure to grow normally, chronic cough, unexplained fever, especially when it continues for more than 2 wk.[11] Clinical signs of TB in children, especially those with severely wasted, are often subtle. The diagnosis of TB is even more

difficult when such children present with acute pneumonia.[12] Following below is a case report of Pulmonary TB in a boy with severely wasted presented with pneumonia.

CASE

A 35-mo.o. Papuan boy, presented to the emergency room (ER) of Oksibil General Hospital with chief complaint of difficulty of breathing over the last 48 h. Patient had history of three weeks of watery diarrhea, twice to three times over 24 h, slightly greenish stools with no blood noted. Diarrhea was associated with 3 wk cough and intermittent fever. Patient was noted to get weaker by day and drink poorly. The symptoms persisted until two d prior to admission, he had difficulty of breathing. There was no history of vomiting. His urination was within normal limit. There was no history of pain during urination or bloody urine.

The patient was the youngest of three siblings. One year ago, the patient was admitted to our institution with community-acquired-pneumonia. His weight and height at that time was 8 kg and 82 cm. The patient was discharged fully recovered after seven d of treatment with antibiotics and since then had never come for follow up and/or seeking any treatment at our institution. The patient had never been evaluated for TB, and both parents denied any history of TB, prolonged coughing, or TB treatment. During pregnancy, the mother only ate sweet potatoes and had never done any antenatal care at the health center and/or hospital. The labor occurred spontaneously at home, assisted by non-medically trained family members, and had his umbilical cord cut with razor. Patients had exclusive breastfeeding until the age of 6 mo, then introduced with sweet potatoes for complimentary feeding from 6 mo old until now. For the past month, he preferred more breast milk and was unwilling to eat solid food. Parents can sometimes provide formula milk, but due to economic problems, it was often unavailable. During his present illness, the patient can only consume about 50 mL of formula milk or about 50 g of sweet potatoes. Patient had never been immunized, particularly BCG. The history of growth and development is not clear. Patient took several steps and say a few words besides mama, papa at the age of 1 y, and more fluently at 2 y.o.

Upon admission at the ER, the patient was found to be somnolent, looked severely ill, with pulse rate 136 beats/min, respiration rate (RR) 64 breaths/min, axillary temperature (t) 37,6°C, peripheral capillary oxygen saturation (SpO2) 93% at room air. His anthropometric measurement upon admission as follow: weight 8 kg, height 86 cm, and head circumference 48 cm, with mid-upper arm circumference (MUAC) 10.5 cm. He had sunken eyes, anemic conjunctivae, but no icteric sclerae. He had dry oral mucosa, with no thrush or redness. He had no enlarged lymph nodes nor abscesses on his neck. On chest examination, we found intercostal, subcostal retraction, with bilateral rhonchi on both lung fields, with no wheezing noted. He had flat and supple stomach, with normal bowel sounds, slow returning turgor, without hepatosplenomegaly. He also had baggy pants with cold extremities and capillary refill time of 2 sec.

The laboratory test results upon admission were hemoglobin of 9.4 g/dL, leukocytes of 7,500 µL, platelets of 169,000µL, lymphocytes 22.1%, granulocytes 68.0%, MCV 68.4 fL, MCH 24.9 pg, MCHC 36.4 g/dL, non-reactive anti-HIV, GDS of 45 mg/dL. There was no x-ray examination performed due to unavailability of the service in our institution.

Patient was initially diagnosed as severely wasted, short stature, hypoglycemia, severe dehydration, persistent diarrhea probably bacterial, and community pneumonia.

The patient were then hooked to O2 at 2 LPM by nasal cannula, given of D10% 40 mL (5 mL/kg) intravenous bolus to treat hypoglycemia, and administered ringer lactate 80 mL/hour (10 mL/kg/h) for 2 h then slowly reduced within 6 h to his maintenance rate using D5 ½ NS. To treat the infection, the patient received ampicillin 250 mg/6 h (125 mg/kg /day) IV, and gentamicin 40 mg/24 h (5 mg/kg/day) IV. We started the enteral diet immediately after resuscitation via the nasogastric tube using the F-100 from the hospital kitchen. Administration of F-100 started with 50 mL (his normally tolerated volume of milk) which was then increased gradually every 4-6 h to reach 60% of the calorie requirement per day. Apart from F-100, we also provided zinc supplementation and vitamin A through the nasogastric tube.

The patients' weight gain was only 300 g over 13 d of admission, even after proper nutritional management. We considered an ongoing untreated TB infection, hence the tuberculin test which shown no induration after 48 h. Nevertheless, we decided to start oral anti-tuberculosis drugs (OAT). After the initiation of OAT, he had weight gain of 100 g/d for the next 13 d and was able to achieve full oral intake by the 23rd hospital day. The patient was then discharged after undergoing treatment for 26 d with weight of 9.5 kg and height of 86 cm.

DISCUSSION

Nutrition for infants, children, and adolescents should maintain current weight and support normal growth and development. Dietary intake should provide energy requirements as well as the essential macronutrient and micronutrient needed in sustaining the function of multiple vital processes. Nutrient deficiencies can limit growth, impair immune function, affect neurodevelopment, and increase morbidity and mortality. Worldwide, malnutrition and undernutrition are the leading causes of acquired immunodeficiency, and a major factor underlying morbidity and mortality in children <5 y.o.[13] Immediate determinants of nutritional status are maternal age, occupation and educational status, number of family members, family income, psychosocial factors in the mother-child interactions, childcare practices, child dietary intake and health status, access to clean water and sanitation.[14,15] Malnutrition among children 6-59 mo is influenced more by external factors such as dietary intake and immunity to infection.[3]

In this case, the patient had persistent diarrhea for the past 3 wk and a medical history of community-acquired-pneumonia a year prior. The patient was exclusively breastfed for 6 mo and was further weaned with sweet potatoes from 6 mo old until now. Since the last one month, the patient preferred breast milk compared to solid food, formula milk was often unavailable. The patient was only given sweet potatoes daily, a source of carbohydrates without any protein, fat, and other micronutrients. This showed continuously insufficient food intake and repeated suffered infectious disease.[16] When a child's intake is insufficient to meet his daily needs, physiologic and metabolic changes take place in an orderly progression to conserve energy and prolong life, called reductive adaptation. Energy is conserved by reducing physical activity and growth, reducing basal metabolism and the functional reserve of organs, and reducing inflammatory and immune responses.[7,13]

This patient was a 35-mo.o. boy weighing 8 kg, with height of 86 cm, MUAC 10.5 cm. Based on the WHO classification in this patient weight-for-height Z score was at Z < -3SD, height-for-age was -3 <Z <-2, weight-for-age Z <-3SD, so that this patient diagnosed with

severely wasted, severely underweight, and short stature. The complications were severe dehydration, hypoglycemia, and acute pneumonia, made us take the decision the patient to be hospitalized. Based on the Indonesian guidelines for the prevention and management of severely wasted for under-five children, hospitalization for severely wasted 6-59 mo children with complications and/or comorbidities that lead to malnutrition, such as TB and HIV, poor appetite, and inability of the family to give proper care.[3]

Children with undernutrition are at increased risk of death from infectious diseases and, conversely, severe infectious diseases in early childhood can affect nutritional status.[8] The main effects observed in severe acute malnutrition occur in the T-lymphocytes and the complement system. The number of lymphocytes originating in the thymus gland drastically decreases and the gland atrophies.[16] Lower serum leptin levels in malnutrition children lead to atrophy of thymus cells, lymph nodes, and tonsils leading to impaired cellular immunity. There are reduced differentiation of CD4 cells with normal CD8-T lymphocytes as well as loss of delayed hypersensitivity, impaired phagocytosis and reduced secretory IgA susceptibility to invasive gastrointestinal infection.[17-19] The resultant changes lead to increased susceptibility to infections and severe complications.[16] Repeated exposure to pathogens leads to colonization of bacteria in the intestine with accumulation of inflammatory cells in the small intestinal mucosa, damages the intestinal villi and leads to malabsorption of nutrients which ultimately leads to malnutrition.[19,20] The systemic circulating leptin deficiency in malnutrition is also correlated with several other bacterial, viral and parasitic infections such as TB, pneumonia, sepsis, amoebiasis, malaria and other infection due to defective cytokine production.[18]

Children with fast breathing and/or chest indrawing are classified as having pneumonia.[21] The most commonly isolated organisms in severely malnourished children with pneumonia were *Klebsiella* species, *Staphylococcus aureus, Streptococcus pneumonia* (Pneumococcus), *Escherichia coli, Haemophilus influenzae*, and *Salmonella* species. Impaired of immune function, including lower leptin levels cause poorer clearance of *S. aureus* from the lungs increases the spread of this pathogen to the lower respiratory tract.[19,20,22] Arpitha *et al*.[23] reported, severity of malnutrition was a significant risk factor for increased severity of pneumonia. Artawan[24] concluded that nutritional status was related to severity of pneumonia in children. Diagnosis of pneumonia optimally includes a combination of history, clinical signs, and chest X-ray. Adequate laboratory and radiological services are frequently absent in primary healthcare facilities, hence WHO recommends basing the diagnosis of pneumonia primarily on visible clinical parameters, including respiratory rate and chest indrawing.[22] Among well-nourished children, most of the clinical signs have acceptable sensitivity and specificity. In contrast, among children with severe acute malnutrition (SAM), the predictive power of most clinical signs is lower. When fast breathing and lower chest wall indrawing are caused by pneumonia, they persist even after full rehydration, thus allowing for diagnosis of pneumonia after full rehydration.[22]

Based on WHO recommendations, under-five severe malnutrition with complications (hypoglycemia, hypothermia, decreased consciousness/ lethargy, or looking sick) need to receive parenteral antibiotics (IM/IV). The selection of ampicillin and gentamicin has considered the coverage of the cause of infection among malnutrition accompanied by complications, both pneumonia and persistent diarrhea.[3]

We monitored our therapy by weighing and recording body weight after the transition phase in g/kg BW/d. The increase in body weight on the 13th d of treatment when compared to when it was admitted was still less than 5 g/kg BW/d. Evaluation of patients to find out the root of the problem of insufficient weight gain includes giving F-100 in the form of correct administration, correct frequency and correct volume spent by the patient, and there are comorbidities that have not been resolved.

Tuberculin test on day 13th showed no induration after 48 h. The sensitivity of the tuberculin test decreases in severe malnutrition children, and increasing the dose is necessary to increase the sensitivity,[25] so that in these patients the tuberculin dose is increased to 0.1 mL, but still no induration produced. Kumbhojkar *et al.*[26] reported that more than 70% of malnourished children with tuberculin showed negative results, and more positive results in well-nourished children.

Tuberculosis can mimic many common childhood diseases, including pneumonia, generalized bacterial and viral infections, malnutrition, and HIV infection.[27] Undernutrition increases the risk of TB and TB can cause or worsen undernutrition.[8] This is why clinicians in such places rely mostly on a combination of epidemiology, history of exposure, clinical features, chest X-rays, and tuberculin skin test (TST) following WHO criteria in making a diagnosis and treating childhood TB. The Xpert MTB/RIF assay, a highly sensitive real-time polymerase chain reaction (RT-PCR) test, is specific for TB. However, it requires high-quality samples, is expensive, and is not readily available in resource-poor and TB-endemic.[12] National guidelines for the management of TB on children in limited resource area in Bangladesh use symptom-based screening as a good tool in case detection in resource-limited area.[28]

The 1999 WHO manual on the management of severe malnutrition suggested a screening of TB contacts as part of the initial history among children with malnutrition.[8] Routine TB risk assessment among acutely malnourished children, combined with improved linkages with TB services, would help increase TB case finding and improve outcomes for children with TB and undernutrition. Guidelines from South Africa recommended considering TB on children with moderate acute malnutrition (MAM) and SAM, whereas Bangladesh recommended TB screening in the context of specific signs or symptoms such as cough for >2 wk, chest infection that fails to respond to antibiotics, or history of contact with a TB case, which could already count as TB risk assessment.

The diagnosis of TB in children relies on thorough assessment of all the evidence derived from a careful history of exposure, clinical examination and relevant investigations.[26] Clinical signs of TB in children are largely non-specific, such as unexplained weight loss or failure to grow normally, chronic cough, unexplained fever, especially when it continues for more than 2 wk.[11] Systemic signs and symptoms may appear early or late in the disease course. Daily fever, intermittent or persistent throughout the day, and usually lasts more than one week. The cough is usually unremitting for > 2 wk. Night sweats are uncommon, subjective and nonspecific, and are significant only when they drench the child's clothes and bedding. Chills and rigors are rare, except in disseminated disease. Anorexia and associated wasting or failure to thrive during the past 3–6 mo or having lost >10% of body weight over any interval of time.[27]

Peripheral lymphadenopathy from TB typically consists of a unilateral, enlarged, non-painful, rubbery lymph node, sometimes becoming fluctuant, with or without spontaneous drainage in

the form of a sinus tract. Respiratory signs and symptoms depend on the site, and degree of involvement.[27] The diagnosis of TB can be made with confidence in most children using careful clinical assessment.[28]

Global laboratory initiative (GLI) model TB diagnostic algorithms 2018 recommend if no MTB detected by Xpert MTB/RIF or no test available is the evaluation of the clinical response after 3–5 d of antibiotic treatment. The presumptive TB treatment should start among patient with serious illness, danger signs, worsening conditions, or minimal improvement.[29] However, clinical signs of TB on our patient with severe malnutrition was subtle, and the diagnosis of TB were even more difficult due to the presentation of acute pneumonia, negative TST and unavailability of radiological examination in our institution. After the initiation of antituberculosis, the patient showed weight gain of 10 g/kg BW/d for the next 13 d. Patients were also able to tolerate full oral intake by the 23rd d of treatment. The patient was then discharged after 26 d of treatment with no medical complications, good appetite, and good clinical condition. The patient was routinely followed up to pediatric outpatient in our institution for continuous monitoring of weight gain and TB treatment.

The WHO recommends the use of standard-dose combination anti-TB therapy that includes rifampicin (R), isoniazid (H), pyrazinamide (Z) and ethambutol (E) in all children.[30] Children with malnutrition have altered drug metabolism, however, based on a systematic review of the efficacy, safety, and pharmacokinetics of antibiotics in children with SAM, further research is needed to guide optimal antibiotic treatment for these children.[8] There was no significant difference in the half-life of H among the underweight compared to the well-nourished control children.[31] Roy *et al.*[32] reported no significant difference in the serum concentrations of H in moderately malnourished and well-nourished children. Among children treated for TB with thrice-weekly Z or E, the maximum concentration for Z was significantly reduced in severely malnourished compared with their well-nourished counterparts.[33] However, the maximum concentration for ethambutol showed no significant difference when compared for both groups of children.[30] Malnutrition was not associated with low plasma concentrations of isoniazid, rifampicin and ethambutol.[34] It may be safe to use doses lower than the WHO recommended doses of H, R and Z for acutely malnourished children with TB. However, doses should be increased gradually following nutritional recovery, according to the change in BMI, or weight-for-age and weight-for-height z-scores.[30]

CONCLUSION

This case report describes the challenge of diagnosing TB in children with severely wasted in limited resource areas. The reduced immune responses due to severely wasted caused subtle clinical signs of TB and decreased sensitivity to tuberculin testing. The unavailability of radiologic examination added further problem in diagnosis. The diagnosis of TB should be considered among children in areas with a high prevalence of TB presenting with severely wasted refractory to proper nutritional management.

ACKNOWLEDGEMENT

No conflict of interest was declared.

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JMedSci

Journal of the Medical Sciences
(Berkala Ilmu Kedokteran)

Volume 54, Number 2, 2022; 181-201
http://dx.doi.org/10.19106/JMedSci005402202209

Obesity and the role of genetic polymorphism: A review of genes as the risk of obesity

Pramudji Hastuti

Department of Biochemistry, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia

Submitted: 2021-04-26
Accepted : 2021-11-24

ABSTRACT

Obesity can be caused by environmental factors, which are higher energy input or lower energy expenditure. Environmental factors supported by genetic factors cause a person to have a different risk for developing obesity from to another. Genetics factors cause obesity through several pathways, which are impaired regulation in the hypothalamus and the regulation of energy expenditure. Obesity may be caused by one gene as monogenic-associated obesity, however, commonly caused by several genes together with environmental factors as the main multi-factorial risk of obesity. Obesity causes inflammation which leads to metabolic diseases. Many efforts are performed to prevent or treat obesity through the intervention to environmental and or genetic factors. Many attempts to reduce the prevalence of obesity were performed by influencing the environmental risk factors or the genetic risk factors. The genetic risks of obesity may be different from one to another country or between ethnic groups. In this review, we identified the main genes which influence obesity. Therefore, a better treatment approach should consider the differences role of genes in obesity rather than only changes in lifestyle. Nutrigenetic approach by considering the difference role of genes in responding to nutrients or drugs is recommended in individualized treatment plans.

ABSTRAK

Obesitas dapat disebabkan faktor lingkungan, yaitu masukan energi yang tinggi atau pengeluaran energi yang rendah. Faktor lingkungan yang didukung faktor genetik menyebabkan seseorang memiliki risiko obesitas yang berbeda antara satu orang dengan orang lain. Gena mempengaruhi terjadinya obesitas melalui beberapa jalur, yaitu terganggunya regulasi dalam hipotalamus dan pengaturan pengeluaran energi. Obesitas mungkin disebabkan oleh gena tunggal, tetapi umumnya beberapa gena bersama faktor lingkungan sebagai penyebab obesitas. Obesitas menyebabkan peradangan yang dikaitkan dengan timbulnya penyakit metabolik. Berbagai usaha dilakukan untuk mencegah atau mengobati obesitas dengan mempengaruhi faktor lingkungan atau genetik. Banyak upaya untuk menurunkan prevalensi obesitas dilakukan dengan mempengaruhi risiko faktor lingkungan dan genetik. Risiko genetik obesitas mungkin berbeda di satu antar negara atau antar kelompok etnik. Oleh karena itu, pendekatan pengobatan yang lebih baik harus mempertimbangkan perbedaan peran gena dalam obisetas daripada hanya perubahan gaya hidup. Pendekatan nutrigenomik dengan mempertimbangkan perbedaan peran gena dalam merespon nutrien atau obat disarankan dalam perencanaan pengobatan secara individu.

Keywords:
energy expenditure;
genetic;
nutrigenetic;
obesity;
polymorphism

*corresponding author: pramudji.has@ugm.ac.id

INTRODUCTION

Obesity is defined as abnormal or excessive fat accumulation that may impair health. The main cause of obesity is excess energy intake with long-term and low-calorie use. Obesity increases the risk of metabolic diseases such as type 2 diabetes mellitus, dyslipidemia, hypertension, musculoskeletal disease, and various types of cancer. Since 1975, the prevalence of obesity is increasing worldwide. A rapid increase in obesity is recorded in South East Asia, including in Indonesia.[1]

Obesity is caused by the environment and genetic factors. Some individuals are more susceptible to becoming obese than others even in the same environments. When the population is in the same condition as an obesogenic environment, there are obese and normal-weight persons. This means that some individuals are more susceptible to becoming obese than others. These differences are influenced by the genetic variation in individuals. Over the past two decades and with the development of molecular technology, some studies have proven that several genes are associated with obesity. More than a hundred loci related to the polygenic of obesity in these genes have been identified.[2]

Based on genetic criteria, the causes of obesity are classified into monogenic, syndromic, and polygenic. Monogenic obesity is caused by a single gene mutation that affects increased input of food and reduces energy use.[3] This is related to polymorphism or variation in genes associated with the hypothalamic system in the control of energy balance, which includes the leptin-melanocortin system. The result of this mutation leads to changes in the activity of hormones, enzymes, and receptors, which causes hyperphagia with the onset of obesity. Sometimes, these mutations are also correlated with endocrine abnormalities. Some mutation genes correlated with monogenic obesity are *LEP* (leptin), *LEPR* (leptin receptor), *POMC* (pro-opiomelanocortin), *MC4R* (melanocortin-4-receptor), and *PCSK1* (preprotein convertase subtilisin/kexin type 1).[4]

Obesity as a syndrome is caused by a group of genes. Usually, this condition happens in obese patients with cognitive delays, hyperphagia, hypothalamic dysfunction, and organ abnormalities. Obesity is associated with intellectual disorders, dysmorphic disorders, organ-specific abnormalities, and hypothalamic disorders. Some examples of related syndromes are Prader-Willi, Bardet-Biedl, Cohen, Alstrom, and X-fragile syndrome.[5]

Polygenic obesity is found in 95% of cases of obesity and many related genes cause this type of obesity while they are also influenced by environmental factors such as the obesogenic environment. Certain individuals can be susceptible to variations in obesity-causing genes through various pathways: i) appetite control (NPY, POMC, MC4R, etc.), ii) energy expenditure (uncoupling protein/UCP), or iii) inflammatory (adiponectin/ADIPOQ, tumor necrosis factor-α/TNFα, interleukon-6/IL6, Resistin/RETN, etc.).[5]

This review outlines some of the genetic factors that play a role in the occurrence of obesity through appetite control by the hypothalamic system, energy expenditure, and inflammation compared with other studies.

DISCUSSION

Genes associated with energy and appetite regulation in the hypothalamus

Obesity occurs due to a disorder in the regulation of energy metabolism. The central nervous system (CNS) plays an important key in controlling energy homeostasis, and the hypothalamus has a role in integrating and regulating

the entire balance of the body. The hypothalamus is the part of the brain involved in the main controls of the intake of food and energy expenditure. In particular, the arcuate nucleus (ARC) located near the median eminence (ME) in the hypothalamus is essential in regulating metabolism. This ME organ facilitates the transport of peripheral hormones and nutritional signals by the ARC nerve. Thus, the ARC integrates hormonal metabolic signals and nutrients from peripheral circulation.[6]

There are two different types of functional antagonist neurons in ARC, which are orexigenic (appetite-stimulating) neuropeptide Y (NPY) and agouti-related protein-neuropeptide Y (AgRP/NPY) and the anorexigenic pro-opiomelanocortin (POMC). Neuropeptide Y is an appetite stimulator that directly sends signals to the PVN (paraventricular nucleus) to increase appetite and adiposity in humans. AgRP (agouti-related protein) is an appetite-stimulating neurotransmitter that involves the antagonists MC3R (melanocortin-3-receptor) and MC4R (melanocortin-4-receptor) in the hypothalamus. Pro-opiomelanocortin is an appetite-inhibiting molecule that produces α, β, γ-MSH (melanocyte-stimulating hormone) and plays a role through MC4R and MC3R.[7]

When nutrients are enough, POMC undergoes hydrolysis into α-MSH to activate MC3R and MC4R and provide satiety. A disorder in MC4R causes the failure to give an appropriate response to the full condition which causes obesity due to hyperphagia.[6]

Pro-opiomelanocortin

The melanocortin system of the central nerve is an important point to control nutritional conditions, controlling appetite, and metabolic response. Some research shows the molecular pathways of melanocortin as central control of energy homeostasis and appetite to maintain body weight. Melanocortin is highly expressed in the pituitary and ARC in the hypothalamus and released into the blood circulation through the sympathetic nervous system. Melanocortin signal pathways are regulated in two ways i.e. leptin related signals and G protein-couple receptor-related signals.

Leptin-related signals

In this signaling pathway, leptin is produced by white adipose tissue (WAT) and after passing through the blood-brain barrier it will be bound to specific leptin receptors in the hypothalamus. Leptin, through two groups of neurons POMC/CART (Cocaine and amphetamine-regulated transcripts) and NPY/AgRP, increases POMC/CART and produces α, β, γ-MSH in post-translation, which inhibits NPY/AgRP.

G protein-coupled receptor (GPCR)-related signals

At this signal, POMC peptides are activated by cAMP when it is bound to the G-proteins in MCR. In cell membranes, MCR is activated by ligand bonds resulting in changes in the conformation and translating of extracellular signals into biological responses. The MCR joins the Gs family of G-proteins and stimulates the cAMP/ERK1/2 path. The Gs family forwards the signal from the MCR to adenylate cyclase converts ATP into cAMP to activate PKA which will involve the phosphorylated CREB family. This phosphorylated CREB will induce or inhibit the expression of genes containing CRE sequences (cAMP-responsive elements) in the promoter. The Gs subunit complex will modulate the ERK 1/2 path. This modulation facilitates the different biological functions of MCR[7]. Energy homeostasis involves chemical and neuronal signals

in the human body to maintain the amount of energy expenses and regulate the input of energy through the sensation of hunger. MC3R and MC4R peptides and molecular signals of the central melanocortin pathway regulate energy balance and homeostasis by activating or inhibiting leptin and its receptors by MC3R and MC4R in the hypothalamus.

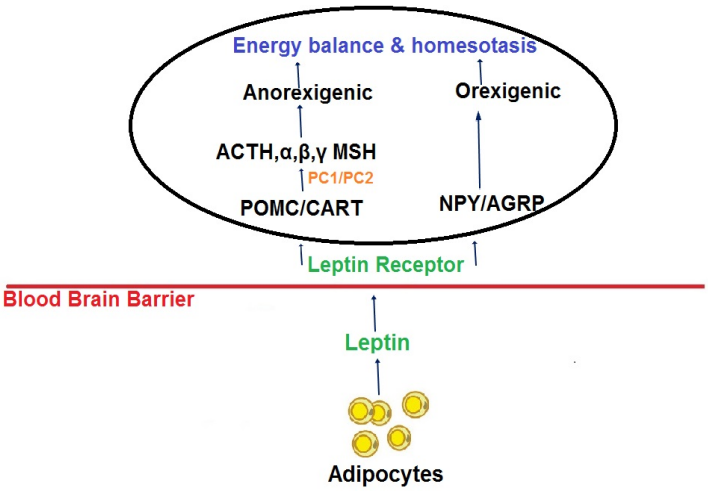

FIGURE 1. Energy balance and homeostasis are regulated by POMC/CART and NPY/AGRP influenced by leptin via leptin receptor (Modified from Singh *et al.*[7]).

Adipocytes secrete leptin polypeptides which pass through the blood vessels of the brain and are bound to specific receptors on two parts of neurons in the hypothalamus ARC. The first part of the neuron is NPY/AgRP with an appetite-enhancing effect as orexigenic, which can be inhibited by leptin receptors. The second is an appetite-lowering POMC/CART neuron as anorexigenic, which can be enhanced by leptin receptors. In this mechanism, POMC/CART by the influence of prohormone convertase 1 and 2 (PC 1 and PC2), is converted into ACTH, and subsequently converted into α-, β-, and γ-MSH. These results can activate the signal to the MCR 1-5 family, which plays an important role in the regulation of energy balance and its homeostasis (FIGURE 1).[7]

POMC is an appetite-suppressing gene, which produces α-, β-, γ-MSH, and ACTH through MC3R and MC4R. As a result of acute POMC and ACTH deficiency in the adrenal cortex, the deficit causes childhood obesity from birth. Research in children with POMC gene mutations led to the occurrence of ACTH disorders with characteristics such as growth delay, mild hypothyroidism, reddish hair, and pale skin as a sign of α-MSH deficiency. In Pakistan, it was found Arg236Gly mutations in the POMC gene affected heterozygous obese subjects by 0.4%.[8] Research in children carrying the Tyr221Cys variant that encodes β-MSH causes impaired MC4R activation thus leading to hyperphagia and obesity conditions.[9] Polymorphism of POMC (C8246T) genes correlated with leptin levels in obese people of the Javanese population in Indonesia, carriers of CC and TC genotypes had higher leptin levels.[10] This result is in accordance with other studies [11-14] but in Caucasian females, this polymorphism is not significantly different[15].

MC3R genetic mutation

This gene has one exon located on

chromosome 20 (20q.13.2-q13.3). MC3R also plays a role as an anti-inflammatory response. There are 15 missense mutations out of 18 mutations reported in the MC3R gene. Mutations in the MC3R gene are associated with obesity in humans. Research in Singapore found 183I/N, 69S/C, 70A/T, 87I/T, 134M/I, 249L/V, 260A/V, 280T/S, 275M/T and 297L/V polymorphisms. There are also reports of 33I/S variants being identified in obese subjects in the USA, Italy, and Poland. Other studies have also shown Thr280Ser mutations that can decrease gene expression resulting in ligand bond failure and intracellular cAMP signal failure.[7,16]

MC4R genetic polymorphism

MC4R is the major MCR in melanocortin pathways. It is also a family of G-protein coupled receptors(GPCR) with seven transmembrane domains expressed in the hypothalamus, brain, muscles, adipocytes, and astrocytes. Single nucleotide polymorphisms (SNP) mutations that occur near the MC4R genes are associated with obesity. The SNP rs1778231 and rs129070134 are reportedly linked to obesity in Europe and India. MC4R mutations are more common in northern Europe compared to Asia. The polymorphism of Val103Ile is associated with abdominal obesity with a minor allele frequency (MAF) of 2-4% in heterozygous (103Val/Ile) compared with homozygous (103Val/Val).[11] There are 376 single-nucleotide variants (SNVs) in the MC4R gene area. These mutations cause failure in ligand bonds and affect the gene expression.[12]

Pro-convertase/ectonucleotide pyrophosphatase pyrodiesterase (PC1/ ENPP1)

PC1/ENPP1 enzyme is an endoprotease serine enzyme that depends on calcium (calcium-dependent serine) which causes the maturation of several nucleotides. In mammals, this PC family consists of several members: PC1/3 (PCSK1)-PCSK9. PC1, and PC3 are widely expressed in neuroendocrine cells, and this enzyme breaks down prohormone and proneuropeptides in secretory granules. It is found with 14 exons and 13 introns in the PCSK1 gene located on chromosome 5 in humans.[17]

PC1/ENPP1 is an enzyme encoded by the PCSK 1 gene that breaks down POMC. Mutations in the PCSK 1 are correlated with obesity, impaired glucose tolerance, low plasma cortisol levels, and hypogonadotropic hypogonadism. This mutation also increased concentrations of proinsulin and POMC in plasma, but plasma insulin concentrations remained at very low levels.[18] Other studies have suggested that there is a positive correlation between PCSK1 deficiency and obesity. In European populations, the prevalence of PCSK1 deficiency as a heterozygous carrier was 0.83% of the population. Other studies have also mentioned that the heterozygous variant PCSK1 is associated with obesity and impaired glucose metabolism in children.[19] Polymorphism of PC-1 K121Q (G > T) (rs10444981) gene had higher leptin levels in GG genotype of the obese group compared to the control group in the Javanese population of Indonesia.[10] This variation of genotype is a risk factor for T2DM in Indonesia[20] and this result is in line with other studies[21-23], but some research found this gene variation was not a significant risk factor for obesity and T2DM[15,24-26].

Leptin

Leptin is an adipokine with the function as a regulator of appetite through its action in the hypothalamus through POMC, AgRP, and MC4R. A homozygous leptin mutation causes leptin deficiency causes hunger and rapid weight gain. Patients with heterozygous

leptin mutations showed low leptin levels and increased weight gain. Mutations in the leptin receptor gene (LEPR) can also cause monogenic obesity which can be treated with the administration of MC4R agonist drugs[26]. Leptin is produced mainly by white adipose tissue (WAT) cells. Biosynthesis and secretion of leptin depend on the amount of WAT and the status of energy storage. Leptin is also known as a hormone that provides satiety. Leptin as an active protein secreted into circulation will be transported to the brain through its receptors in the hypothalamus causing decreasing expression of genes encoded NPY, POMC, and corticoliberin (CRH) with decreased appetite and reduced consumption of food that decrease body weight and increase energy use. Leptin is a factor that protects against hunger and obesity. Leptin may also increase insulin sensitivity in peripheral tissues and increase glucose uptake and oxidation in skeletal muscles. Leptin also has thermogenesis action through the regulation of mitochondrial proteins in brown adipose tissue (BAT).[27]

Leptin and leptin receptors can regulate fat catabolism and energy intake. In the ARC in the hypothalamus, leptin is bound to its receptors and inhibits the NPY/AgRP pathway increasing appetite (orexigenic) and activating the POMC/CART pathway, which decreases appetite (anorexigenic). Leptin deficiency in humans can occur due to frame-shift mutations in the homozygous gene (deletion G133) and result in shorter protein molecules with impaired function.[7] A study showed that two leptin receptor gene polymorphisms in rs1137100 (109K/R) and rs1137101 (223Q/R) were found higher in body weight, waist circumference, and leptin levels in the obesity group than in the control. The frequency of 103R/R homozygous in the obesity group was higher than in the control, while 223Q/R polymorphism was associated with obesity and leptin levels[28] in the Western and Eastern of Indonesia.[29] Additionally, this polymorphism was correlated with metabolic syndrome in the Chinese population[30] and hypertension,[31-32] but this polymorphism was not found to be a risk factor for metabolic syndrome and obesity,[33] in Turkish children nor hypertension in Chinese populations.[34] A summary of mutations of genes that cause obesity through regulation in the hypothalamus is presented in TABLE 1.

TABLE 1. Effect of mutation genes causes obesity in energy balance through the hypothalamus

Genes	Effect of mutation	Some mutations found	References
NPY	Changes in the signal sequence, affect the signal of energy balance, and inhibit lipolysis	rs17149106 (G>T), rs16147 (C>T), rs16139 (T>C), rs5574 (C>T	11,12,13
POMC	Impaired MC4R activation and loss of melanocortin signaling in melanocortin receptor and leptin-melanocortin pathway	Arg236Gly Tyr221Cys C8246T	8,10, 14, 15
MC3/3R	Decreased expression, failure in ligand bond, intracellular cAMP signal failure	183I/N, 69S/C, 70A/T, 87I/T, 134M/I, 249L/V, 260A/V, 280T/S, 275M/T and 297L/V, 33I/S, Thr280Ser	7, 16, 17
MC4/MC4RR	Failure in ligand bonds and affect the expression	rs1778231, rs129070134 Val103Ile	9
Leptin/receptor leptin	Disturbance of the leptin signal causes increases food intake, positive energy balance, and the accumulation of fat.	Lys109Arg, Gln223Arg G133 del	28-34
PC1	Failure in the processing of POMC to some peptides	K121Q (G > T) (rs10444981)	10, 15, 20-26

Genes related to energy expenditure

There are two types of adipose tissue in mammals i.e. white adipose tissue (WAT) and brown adipose tissue (BAT). WAT plays a role in energy homeostasis in the body by storing excess energy as triglycerides and releasing free fatty acids (FFAs) when the energy is needed. BAT specifically regulates energy consumption to give heat production in response to maintaining body temperature. BAT plays an important role in regulating energy balance.[35] Obesity-related genes in maintaining energy expenditure include uncoupling proteins (UCP1, UCP 2, and UCP 3) and adrenoceptor-β (ADRB1, ADRB 2, and ADRB 3).

Uncoupling protein (UCP)

Uncoupling proteins (UCPs) are a group of mitochondrial proteins contained in mitochondrial membranes that serve to transport protons to the mitochondrial matrix. Mitochondrial respiration releases the extrusion of protons (H^+) out of the mitochondria into the intermembrane of mitochondrial, producing potential redox and encouraging ATP synthase. UCP pumps protons from the intermembrane to the mitochondrial matrix and removes proton gradients while reducing ATP production and superoxide production.[36]

UCP1 has been extensively studied and is mostly found in brown adipocyte mitochondria. A total of 8% of UCP1 are found in mitochondrial proteins. UCP1 is characterized as an important transporter for mitochondrial membranes. A Cold environment, increased thyroid hormones, norepinephrine, and stimulation of adrenergic and cAMP may increase UCP1 gene expression. UCP1 plays a role in the regulation of energy consumption, thermogenesis, and reactive oxygen species (ROS). The mechanism is associated with the pathogenesis of type 2 diabetes mellitus and obesity. UCP2 and UCP3 are homologous of UCP1. UCP2 is found in mitochondria of adipose tissue, skeletal muscle, kidneys, liver, lungs, and macrophages. Meanwhile, UCP3 is found in many skeletal muscles. UCP 4 and UCP5 are found commonly in the nerves of the central nervous system (CNS).[36]

Mutations in the UCP genes cause decreased energy consumption and increased risk of weight gain and are associated with metabolic diseases. The UCP1 gene is present on chromosome 4 in q31.1, which consists of 6 exons with 9-kb length. Some studies found SNPs in the noncoding region of the UCP1 gene, such as A3826G (rs1800592), A1766G(rs3811791), and A112C (rs10011540), and coding region Ala64Thr (rs45539933) and Met229Leu (rs2270565). SNP A>G's position at -3826 bp (rs1800592) of 5' noncoding gene is found in Canada with G allele and is associated with increased body weight, and increased risk of diseases correlated with obesity. In another study in Australia, allele G was associated with an increase in body weight and high blood glucose concentrations in women. Another study on women in India found that the GG genotype is linked to obesity and increased blood pressure. Research in Italy found that the G allele is associated with decreased insulin sensitivity in the obese group.

Another mutation is substitution A in G in the -1766 (rs3811791) nucleotide of the *UCP1* gene. The allele variant -1766G is associated with severe obesity with waist-hip ratio, body fat percentage, and increased amount of abdominal fat parameters. Studies in Korea found that the carriers of the haplotype polymorphisms A3826G and A1766G have a high percentage of body fat. Another polymorphism at A112C in the UCP1 gene involving the allele C showed decreases in the promoter activity of the UCP1 gene, which were correlated

with insulin response and associated with insulin resistance in patients with T2DM.[37]

UCP2 is highly expressed in skeletal muscle and adipose tissue, involved in energy regulation in the metabolism of lipids. DNA sequencing found there is a substitution of alanine by valine in exon 4 and insertion/deletion 45 bp 3'UTR in exon 8 of UCP2 gene. This polymorphism contributes to the variation in metabolic rate and adiposity in adipose tissue. There is a correlation between UCP2 mRNA in adipose tissue and body weight in humans, in which the UCP2 overexpression is correlated with obesity.[7] Polymorphism of 55A/V occurs in exon 4 UCP2 where substitution C by T nucleotide causes alanine substitution for valine at position 55 amino acid sequence of the UCP2. Some studies found carriers of V/V genotype have a higher risk of type 2 diabetes mellitus and obesity compared with carriers of A/V and A/A genotype. Insertion/deletion of 45 bp at 3'UTR of exon 8 of UCP2 and downstream polymorphism of 158 bp of stop codon increases the risk of obesity. In Indonesia, polymorphism of 55A/V UCP2 genotype in the male group showed that V/V genotype and V allele significantly lower the risk of obesity. Insertion/deletion of 45 bp UCP2 gene in the male group showed that insertion/insertion genotype and insertion allele significantly increase the risk of obesity whereas for females it showed that the insertion/deletion genotype and insertion allele lowered the risk of obesity.[38] The increase of UCP2 expression involves decrease of ATP synthesis lead to decrease of insulin secretion and increase risk of T2DM. G allele polymorphism of G866A is associated with decreased mRNA expression, increased body weight, body fat mass, and risk of height/ body weight. Allele -866A is associated with obesity and high insulin level in the Indian people. In Indonesia, variations of UCP2 (Ala55Val and I/D45 bp) are risk factors for obesity with different stratification of gender.[39] In some populations, this polymorphism is considered as a risk factor for obesity,[40-43] risk of diabetes,[44,45] and chronic kidney disease.[46] However, in Italian[47] and French populations[48] this polymorphism is not found to be a risk factor for obesity.

UCP3 gene polymorphism, i.e. substitution of nucleotide C/T at position 55 occurs in the promoter and near the TATA box. The SNP's in this location has an effect on the transcription of the UCP3 gene and influences the expression of UCP3 mRNA in skeletal muscles which is correlated with metabolic function. One study found that body weight increased in someone carrying 55T allele in Scandinavia.[37]

β2-adrenergic receptors

β-adrenergic receptors are a family of GPCR and catecholamine targets, specifically epinephrine through the sympathetic nervous system (SNS). β-adrenergic receptors play an important role in the risk of excess body weight and are associated with the regulation of inflammatory cytokines. These receptors consist of three classes: β1, β2, and β3-adrenergic receptors (ADRB1, ADRB2, ADRB 3). Molecular signals that occur in the body can trigger activation of β-adrenergic receptors such as β-adrenergic receptors following the PKA/c-AMP pathway through activation in the SNS. Epinephrine stimulates ADRB and then activates the Gs family, especially Gα. Furthermore, Gα binds adenylate cyclase enzyme to catalyze the conversion of ATP into cAMP. After that, cAMP will activate cAMP-dependent kinase which will stop the epinephrine action. This pathway will eventually regulate energy expenditure and lipolysis.[3]

There is evidence that ADRB receptors participate in human weight regulation and some gene polymorphisms in ADRB

receptors affect metabolic complications and increase weight. The ADRB2 gene is located on chromosome 5q31-32 and consists of 2 kb DNA length. ADRB2 gene encodes proteins with a length of 413 amino acids. ADRB2 is found in fat cells, blood vessels, heart, and respiratory tract. These receptors are responsible for stimulating lipolysis activity in adipose tissue and controlling the smooth muscles. The ADRB2 gene includes some polymorphisms in the population. Around 80 polymorphisms have been identified, and some are in SNPs that occur in the 5'-untranslated region (UTR) associated with obesity. The SNPs of 34V/M, 16R/G 27Q/E, and 16T/I. Of the two SNPs, 16R/G and 27Q/E have a minor allele frequency (MAF) of 40-50%, 16T/I with MAF of 1-3%, and the 34V/M with MAF of less than 1%.[49]

Variation in the ADRB2 gene is identified as cause increased body weight and lipid metabolism disorders in females in codon 27 (27Q/E). 27E alleles are associated with increased body weight, subcutaneous fat, and increased levels of leptin and triglycerides in males, while women are associated with increased body weight, body fat mass, and waist-hip ratio.[7] 16R/G polymorphism (rs1042713) and 27Q/E (rs1042714) in the ADRB2 gene are associated with the risk of weight gain, high blood pressure, metabolic syndrome, and asthma. Polymorphism is also often associated with changes in the activity of the SNS and results in lipolysis, metabolic and cardiovascular regulation. There is some correlation between asthma in 16R alleles and 27E alleles in obese individuals in Javanese populations.[50] Polymorphisms of 16R/G shows that carriers of R/G genotype had decreased risk of obesity compared to the R/R genotype. Q/E genotype of 27Q/E increases the risk of obesity. Q/Q and Q/E genotypes had significant differences for plasma insulin in the obese group. The combination of 16R/G and 27Q/E genotypes decreased the risk of obesity compared to 16R/R + 27E/E.[51] This result is in accordance with studies in white[52] and Korean population.[53] However, Kawamura *et al.*[52] reported that polymorphism of ADRB 2 is not a risk factor for obesity in the Japanese-American population. A detailed summary of polymorphisms of the UCP1, 2, 3 and ADRB genes is presented in TABLE 2

TABLE 2. Effect of mutation genes that cause obesity to influence energy expenditure

Genes	Effect of mutation	Some mutation found	Reference
UCP1/3	Decrease UCP protein expression and lower energy expenditure	A3826G, A-1766G, A112C, Ala64Thr, Met229Leu, C55T	37
UCP2	Decrease UCP protein expression and lower energy expenditure	Ala55Val, I/D 45 bp	39-48
β-adrenergic/ receptor	Regulate energy by stimulating lipolysis and thermogenesis through activation of catecholamine induction from adenylate cyclase through the G protein overexpression of adiponectin adipocytes it caused an increase in adipogenesis and lipid storage	34V/M, 16R/G 27Q/E, 16T/I +276G > T	51-54

Genes association of obesity and inflammation

Inflammation is the body's defense mechanism. Obesity is a chronic low-level inflammatory condition. This inflammation is different from general inflammation because there are no signs of inflammation, but it has similarities because this disorder is caused by inflammatory signaling pathways. Obesity that is associated with inflammation is caused by increased adipose tissue, increased production of adipocytokines, and causes inflammation associated with certain pathophysiological processes.

The inflammatory process happens if blood cells (neutrophils, eosinophils, monocytes, and lymphocytes) enter adipose tissue. An increase in the number of adipocyte cells that occurs in obesity leads to an increase in the number of pro-inflammatory molecules such as adipokine/chemokine. Increases of these molecules have an effect on the endothelium to increase the production of intercellular adhesion molecule (ICAM) and vascular adhesion molecules (VCAM**),** polymorphonuclear and mononuclear phagocytes out into the extravascular compartment. Adipocytokines include leptin, which activates endothelial cells and causes the accumulation of macrophages in adipose tissue, will releasing pro-inflammatory molecules. Other adipocytokines, such as resistin induce the expression of adhesion molecules (VCAM-1 and ICAM-1) in endothelial cells and increase the synthesis and secretion of inflammatory cytokines such as TNF-α, and IL-6. and IL-12. In the inflammatory process, macrophages in adipose tissue will release chemoattractants for macrophages, resulting in chronic inflammation. The accumulation of macrophages in adipose tissue plays an important role in the increase of inflammatory mediators (IL-8, IL-6, IL-1, and TNF-α) causes oxidative stress, hypoxia, and lipolysis in the adipose continuing in increasing the production of adipositokin.[55] Some of the genes associated with inflammation and obesity are shown in FIGURE 2.

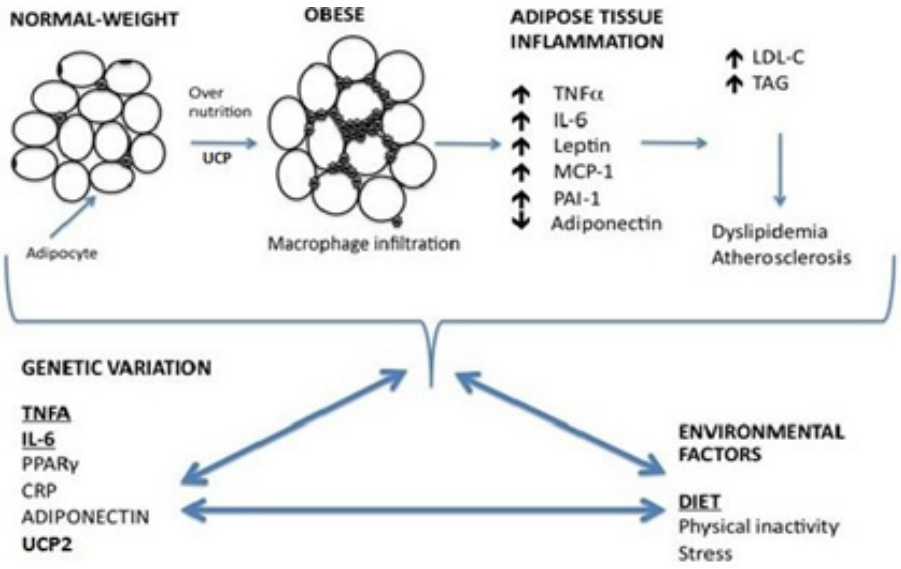

FIGURE 2. Obesity causes an increase in inflammation and is influenced by variations of genes and environment factors.

Adiponectin

Adiponectin is a 28 KDa protein consisting of 244 amino acids with a role as an anti-inflammatory cytokine.[56] Adiponectin is secreted by various types of cells including adipocyte and endothelial cells. The anti-inflammatory effects of adiponectin are mediated by two adiponectin receptors (AdipoR1 and AdipoR2). The binding of adiponectin to its receptors will activate several signals such as IRS-1/2, AMPK, and MAPK p38. Activation of IRS-1/2 by adiponectin signals is involved in the sensitization of insulin. In the liver, adiponectin activates glucose transporters and inhibits gluconeogenesis through AMPK, stimulating catabolism of lipids and lowering inflammation through the PPARα pathway. Adiponectin is also found to modulate eating behavior and energy expenditure during fasting through central effects. Adiponectin levels are inversely proportional to obesity, insulin sensitivity, type 2 DM, and metabolic syndrome.[57]

Adiponectin levels in the blood are lower in obese individuals compared to normal weight. There is a gene that encodes adiponectin located on the third chromosome (3q27) close to the locus responsible for the presence of type 2 DM and obesity. Some research on obese subjects found SNP in the adiponectin gene. There is a genetic variation of about 30-70% that affects plasma adiponectin levels. One of the SNP's adiponectin genes is +45T>G (rs2241766) on exon 2 which does not change amino acid sequences.[58] Polymorphism of +276G>T of adiponectin gene in Indonesia is not considered a risk factor for obesity nor DM. Polymorphism of ADRB is a risk factor for obesity with diabetes in the Indian population,[59] adult women,[60] and a risk factor for coronary artery disease,[61] metabolic syndrome,[62] and hypertension.[63]

Resistin

Resistin is produced and released from WAT tissue. It is produced at low levels in preadipocyte, endothelial cells, and vascular skeletal muscle cells, but resistin is mostly found in peripheral mononuclear and spinal cells. Resistin has 11 cysteine residues at the end of the C-terminal. Peptide resistin is rich in cysteine 12.5 kDa and consists of 108 amino acids in humans. The human resistin gene (Retn) is found on chromosome 19. Resistin has a role in activating TNF-α and IL-12 in macrophages and monocytes.[64]

The release of human resistin is mediated by inflammatory factors such as stimulation of lipopolysaccharide or cytokines, IL-1, IL-6, and TNF-α. *In vivo,* resistin can increase cell adhesion molecules in endothelial cells so that there is an increase of ICAM-1, VCAM-1, and Monocyte chemoattractant protein-1 (MCP-1) as opposed to the effect of the adipokine, adiponectin. This increase in resistin levels is correlated with an increase in pro-inflammatory cytokines, especially in patients with metabolic syndrome. Some studies found that increased resistin levels are correlated with increased levels of C-reactive protein and TNF-α. These data stated that the increased level of resistin is associated with increased inflammation.[64]

The relationship between serum resistin, BMI, and body fat was reported that the expression of resistin mRNA and protein levels are detected in subcutaneous and visceral abdominal adipocyte, and serum resistin levels are higher in obesity. Resistin is also a marker in fat distribution because it is specifically associated with abdominal fat deposits. The relationship between resistin and obesity is stronger in the female subjects compared to men. Diet and physical exercise can lower resistin

levels, which is followed by a decrease in BMI and fat mass.[65]

Some SNPs are identified in the Retn gene but only a minor allele frequency of 5% is associated with disease risk. There are several SNP in Retn (-537 and -420) and +299 (IVS2 +181G→A) associated with an increase in BMI. Increased serum resistin is reported in type 2 DM subjects carrying -420G/G genotype. Obesity research in Japan reported SNP −638G>A, −420C>G, and −358G>A although associated with serum resistin, but no significant difference was found in obese subjects and controls and was not correlated with insulin resistance[66]. In research on obese people in Indonesia, polymorphism of +299G > A is associated with insulin resistance and resistin level, negatively correlated with insulin level but -420 C/G and +62G>A polymorphism was not correlated with DM risk[67-69] while resistin is correlated with a marker of inflammation.[70]

TNF-α

TNF-α is a pro-inflammatory cytokine that can provide an immunological response. Activation of the immunologic system during infection or injury causes metabolic changes.[71] TNF-α is expressed on the surface of transmembrane protein cells and is involved in the pathogenesis of various inflammatory diseases. The TNF-α gene is located on chromosome 6.p21.1—21.3. TNF-α is involved in the metabolism of fat and causes a higher level of triglycerides in the blood as a result of decreased activity of lipoprotein lipase and increased synthesis of hepatic fatty acids in the body. Subjects with obesity are associated with high TNF-α expression and correlated with higher insulin levels. In addition, TNF-α is known to regulate the expression and secretion of leptin[72]. In WAT obese individuals, pro-inflammatory M1 macrophages form crown-like structures and surround dead adipocyte cells. Increased macrophages M1 in WAT obesity is a major source of TNF-α and IL-6.[73]

Substitution of guanine by adenine in the promoter (-308G/A TNF-α gene) has been identified to be associated with higher blood pressure, leptin levels, and higher cholesterol level leading to the development of the metabolic syndrome. Research into Caucasian and Chinese populations found a correlation between -308G TNF-α alleles and obesity risk. Other studies have also found that AA and GA genotypes are more common in male obese individuals, whereas in female individuals AG genotype is associated with a higher risk of obesity.[71] In another study in Brazil, -308G/A polymorphism in TNF-α promoters, A allele, GA, and AA genotypes contribute to increased insulin resistance and excess body weight in adult.[73] In the Javanese population, this gene polymorphism showed that GA genotype patients have lipid and TNF-α levels higher than the GG genotype, and variation of -308 G/A TNF-α gene plays a role in a higher risk of obesity,[74,75] risk of breast cancer,[76] hypertension,[77] and inflammatory bowel disease.[78]

Interleukin-6

Interleukin-6 (IL-6) is a pro-inflammatory cytokine that influences the activity of the brain, metabolic regulation, and the development of various cancers. IL-6 cytokines are bound to IL-6R membrane receptors and the complex IL-6 and IL-6R are associated with glycoprotein 130 kDa in which dimerization will initiate signals via Janus kinase (JAK)/signal transducer and activator of transcription (STAT) and phosphoinositide-3 kinase pathways.

IL-6 plays an important role in inflammatory signaling pathways and is associated with obesity as well as visceral adipose tissue. Genetic variants of IL-6 in the promoter of genes affect the function and expression. Polymorphism in functional promoter IL-6-174G/C affects

IL-6 transcription. The human IL-6 gene is located on the 7p21 chromosome, and -174G/C polymorphism is thought to be associated with obesity risk.[79] In the obese group of western Indonesia, carriers of the CC genotype had higher CRP and lower IL-6 levels than the GC and GG genotypes. The frequency of CC genotype in the obese group is 47.2% compared with 28.1% in controls and these genotypes and the allele are considered a risk factor for obesity.[80-84] Effects of gene mutation correlated with obesity and inflammation are summarized in TABLE 3.

TABLE 3. Effects of mutation genes that cause obesity and inflammation

Genes	Effect of mutation	Some mutation found	Reference
Adiponectin	Anti-inflammatory cytokine	+45T>G (rs2241766), +276G>T	20, 57, 58-63
Resistin	Binding of a transcription factor on RETN promoter, activating TNF-α, IL-11, IL-6, IL-12	+62G>A, -420C>G, +299G>A, −638G>A, −358G>A	66-70
TNF-alpha	Modification of the binding sites in certain transcription factors, and affect the regulation of transcription and secretion. TNF-α binds to the receptor will increase the cellular and pro-inflammatory NF-kB and activation of mitogen-activated protein (MAP) kinase	-308 G/A	74-79
Interleukin-6	Affect a transcriptional system, release truncated protein, initiates signals via Janus kinase and phosphoinositide-3 kinase pathways	-174G/C	80-84
Endothelin-1	Affect the synthesis of endothelin-1 and increase levels of endothelin concentration.	T-1370G, 198K/N, G2288T, T-1370G, +138/ins/del	85-87

Endothelin-1

A recent study found there is a positive correlation between higher body weight and endothelin-1 (ET-1) levels. ET-1 is a vasoactive peptide primarily produced and released by endothelial cells. Most ET-1 circulation is derived from vascular endothelial cells and is synthesized by adipocyte tissue. There are ET-2 and ET-3, but ET-1 is the most dominant molecule and has an effect. Active ET-1 is a hormone with 21 amino acids the result of the translation process as preproendothelin with 200 amino acids and hydrolyzed and modified by endothelin converting enzymes (ECEs), resulting in mature and secreted ET-1. Its receptors occur in various tissues such as endothelium, vascular skeletal muscle cells, adipocytes, and hepatocytes. The secretion of ET-1 helps the metabolic function in healthy individuals. When ET-1 levels increase in plasma it can lead to a variety of health problems.

Obese patients have an increase in plasma ET-1 levels when compared to normal-weight individuals. This increase in ET-1 production is mainly in adipose tissues. Adipose in obese patients can release ET-1, which can be 2 to 3 times higher than in normal-weight subjects. The increase in ET-1 contributes to lipid regulation and is a risk factor for insulin resistance in some patients with obesity due to their interactions with ETA and ETB subtype receptors. Stimulation of ET-1 is associated with increased expression of TNF-α by macrophages and increased transcription rate of IL-6, NFκβ, and monocyte chemotactic protein-1.[85] In the Javanese population, variation of 198K/N ET-1 gene, 198N/N genotype is a risk factor of obesity compared to

198K/K genotype. Levels of ET-1 plasma are higher in obese subjects than that of control subjects, and N/N genotype has the highest ET-1 plasma level.[86]

ET-1 or EDN-1 is a polypeptide that has vasoconstriction activity and mitogenic effects, in the heart has positive inotropic and chronotropic characters, stimulates sympathetic and renin-angiotensin-aldosterone systems, as well as homeostasis modification. The human *ET-1* gene consists of 6836 nucleotides located on the 6p23-p24 chromosome, capable of producing pre-pro-ET-1 that can be hydrolyzed into large ET-1s. There are several variants of the *ET-1* gene, including transversion, transition, insertion, and repeated nucleotide polymorphisms, which affect the genetic risk of cardiovascular and other related diseases. Ten polymorphisms including transversions have been found: -1370 (T-1370G), +5665 (198K/N), G2288T polymorphisms (rs2070699), and -974 C>A (rs3087459). Transitional polymorphisms are +3660 (106E/E), G8002A (rs2071942), rs1476046, rs2071943, and rs9296345 polymorphisms. In addition, the polymorphism of insertion/deletion is +138 (+138/ex1ins/delA) (rs1800997). Some are associated with significantly different diseases (phenotypes), especially cardiovascular system-related diseases such as high blood pressure, ischemia, angina, and coroner's syndrome. Some other related diseases are asthma, lung edema, hearing loss, obesity, and sleep apnea.[87]

The differences in results and the limitation of the genetic study in the various populations are due to differences in the gene pool for each population which has different genetic variations, genes associated with obesity are numerous, environmental factors and eating habits in each population/ethnic are different. The pathogenesis of obesity is complex, where one variation of a gene can affect the genes located nearby that influence these diseases. Additionally, the studies of genes and obesity are not able to control all of the confounding factors and differences in criteria or design variables used in the research protocols.

CONCLUSION

There are some genetic factors that contribute to the causes of obesity by acting through control mechanisms in the hypothalamus or changes in energy expenditure. Some genetic variations can influence obesity and may have different risks within some ethnic groups in the world. These results can be used for consideration if treatment will be conducted in different populations, and there may be nutrigenetic or pharmacogenetic variations which could give different results.

ACKNOWLEDGEMENTS

Author would like to thank Maya Uzia B. for the assistance to prepare this manuscript.

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