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 Wibisono1, Nur Arfian2, Handoyo Pramusinto1, Fauziyatul Munawaroh3, Yeshua Putra Krisnugraha2, Daniel Agriva Tamba1, Dwi Cahyani Ratna Sari2

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

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 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. 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.

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*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).

**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 CGL7 group.

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 H2O2 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® (Toyobo Cat. No. TRT-101, Osaka,
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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: GGCACAGTCAAGGAGTGAATG 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](image)

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. 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.

Moreover, inflammatory response post-ischemia is characterized by morphological alterations and increased mobility of microglia. 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. It is activated by proinflammatory cytokines and Reactive Oxygen Species. 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. Microglia, which are glial cells like macrophages, are rapidly activated and differentiate into M1 or M2. M1 phenotype are the proinflammatory state and will secrete proinflammatory cytokines that induce tissue damage. Several proinflammatory cytokines produced include IL1β, IL6, TNFα, and iNOS. Related to the expression of proinflammatory cytokines, it is known that VCAM1 was dramatically increased by IL1 β and TNFα. In contrast, the M2 phenotype has anti-inflammatory properties that mainly work in debris clearance, extracellular matrix deposition, and angiogenesis. 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. 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.

Due to its unique structural architecture, BBB and its permeability have a significant role in brain impairment. Failure of BBB function could induce seizure activity through brain inflammation and BBB permeability. Epilepsy could be the result of the inflammatory response and the endothelium impairments. 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.\textsuperscript{31,33,34} The expression of cytokines and inflammatory chemokines and matrix metalloproteinases in activated microglial cells contribute directly or indirectly to BBB damage.\textsuperscript{35} The M1-type microglial cells produce TNF-\(\alpha\) and IL-1\(\beta\), and affect the localization of VE-cadherin, occludin, and claudin-5, and therefore contribute to BBB disruption.\textsuperscript{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.

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