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An Evaluation of the Alteration in the Number of Neuron and Expression of S100B, MMP-9, and GFAP in Ischemic Rat Brain Tissue Induced by the Left Unilateral Common Carotid Artery Occlusion Method

Aris Widayati[1,2], Fedik Abdul Rantam[3,4], Abdulloh Machin[5,6,*] and Wibi Riawan[7]

[1]*Doctoral Program Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia*

[2]*Department of Physiology, Faculty of Medicine, Universitas Brawijaya/Universitas Brawijaya Academic Hospital, Malang, Indonesia*

[3]*Research Center for Vaccine Technology and Development, Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia*

[4]*Virology and Immunology Laboratory, Department of Microbiology, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia*

[5]*Department of Neurology, Faculty of Medicine, Universitas Airlangga/Dr. Soetomo General Hospital, Surabaya, Indonesia*

[6]*Post Graduate Doctoral Program, Faculty of Medicine, Universitas Airlangga/Universitas Airlangga General Hospital, Surabaya, Indonesia*

[7]*Department of Biomolecular Biochemistry, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia*

([*]Corresponding author's e-mail: abdulloh.m@fk.unair.ac.id)

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Abstract

Background: The middle cerebral artery occlusion (MCAO) model has been widely used as a tool for learning the focal ischemic stroke. However, the MCAO method has several limitations, including a lack of visibility and risk of subarachnoid hemorrhage. In addition, Common carotid artery (CCA) occlusion is another form of ischemic stroke type in human with a prevalence ranging from approximately 0.24 to 5 % among in ischemic stroke patients. Thus, CCA occlusion method can be developed as an alternative for creating ischemic stroke models to learn pathophysiology of ischemic stroke. Aim: The aim of this study is to prove whether an occlusion of the left CCA for 180 min in rat can induce ischemic stroke based on the change number of neuron and biomarker in brain tissue. Methods: The 15 male Wistar rats were randomly divided in to 3 groups. The first and second 5 rats, each of them were occluded on the left CCA for 60 and 180 min, then were categorized as the Group A and Group B respectively. Meanwhile, the remaining 5 rats without occlusion were included in the sham group. The assessment of the motoric dysfunction used LRWT tool to evaluate foot fault scoring (FFS) and foot placement accuracy, while the number and distribution of neuron was evaluated by H and E staining. The expression of MMP9 and S100B were assessed using Elisa technique, while GFAP was evaluated with immunohistochemistry technique. Results: The number of neuron in ischemic stroke groups (A and B) was significantly decreased compared to the sham group. Meanwhile, Expression of MMP9, S100B, GFAP was found to increase significantly in ischemic groups (A and B). Motoric function was found to decrease in ischemic stroke group (A and B) both in RHL and RFL. Conclusions: The occlusion of left unilateral CCA for 180 min yield in the declining number of the neurons, motoric deficit and in increasing of MMP9, S100B, GFAP expression. This finding was expected to give a new venue to develop preclinical research to learn pathophysiology of ischemic stroke.

Keywords: Neuron, GFAP, Neuroinflammation, Ischemic stroke model, Common carotid artery occlusion

Introduction

Stroke is the second leading cause of death and disability worldwide. In 2019, for approximately 11.6% of deaths was caused by stroke. Among the types of stroke, ischemic stroke is the most common type, approximately 62.4% of all stroke cases [1]. Recently, several research have advanced greatly for understanding of ischemic stroke, especially regarding pathophysiology of ischemic stroke [2,3].

The development of ischemic stroke model for preclinical purpose is crucial. Ischemic stroke model is made for several reasons. The first is because stroke in human body has heterogeneous conditions. Study which has controlled and standardized conditions will result greater accuracy in viewing pathophysiology of ischemic stroke. The other reasons are molecular, genetic, and biochemical investigations often require access to brain tissue directly, which can be studied only in animal model. In addition, collateral vascular system that is beneficial in the pathophysiological processes is unable to be seen *in vitro* study [4]. Several ischemic stroke models have been developed including middle cerebral artery occlusion (MCAO) model which represents focal ischemic stroke [2].

The option to use animal model from rat type is based on ethical considerations where the use of rat more acceptable than other types of mammals because the maintenance cost for rat is much cheaper and its vascular anatomy is similar to human. Moreover, although the MCAO is the most proper method to create ischemic stroke model and can give good result for outcome achieved Macrae [5], this model has some disadvantages, one of which is the risk of subarachnoid hemorrhage [2].

Furthermore, the use of imaging both computed tomography (CT) and magnetic resonance imaging (MRI) is extremely needed to diagnose stroke especially to differentiate its types. Unfortunately, not every hospital owns these 2 imaging facilities. Therefore, in these recent years, researchers have developed several biomarkers that can be used to diagnose stroke Rozanski and Audebert [6], 2 of which are matrix metalloproteinase-9 (MMP9) and S100 calcium-binding protein B (S100B) that are released during ischemic stroke. MMP9 is a biomarker which will be activated during neuroinflammation, while S100B will be released when astrocyte is activated. The level of S100B in peripheral will increase after the ischemic stroke was found both in human and animal body Huang *et al.* [7] and can be used to determine infarct size and stroke severity [8]. In addition, the other biomarker that can be utilized to detect ischemic stroke is glial fibrillary acidic protein (GFAP), which will be released in 2 till 6 h after stroke onset both in the ischemic and in hemorrhage stroke [9].

Histologically, neuron and astrocyte are structurally changed in 4 to 6 h after stroke, while many neurons will be lost with in high variable, ranging from < 35.000 to > 27.000.000 cells per min during in ischemic stroke [10]. Study conducted by Szymankiewicz-Szukala *et al*. [11] conveyed that temporary 5-min lasting occlusion of common carotid arteries does not induce total inhibition in corticospinal tract neurons [11]. This means that it takes more than 5 min to yield reperfusion injury in brain. Ischaemia-Reperfusion injury causes further damage, threatening function and viability of the brain in order to yield of great ischemic in rat model [12]. The study conducted by Mentari *et al.* [13] which used the right unilateral CCA occlusion (RUCCAO) method for 90 min showed presence of cognitive disfunction and motoric deficit but this neurological deficit arose in 3 until 7 days after the ischemic induction. Meanwhile, RUCCAO method for 60 min demonstrated that there was no significant difference between the sham and stroke model in the cognitive, motor function and in infarct lesion [13]. An occlusion for 180 min was also observed by Machin *et al.* [14] in the right internal carotid artery but it was not mentioned if there was any motoric deficit in the study [14].

Based on the molecular concept following ischemic stroke and previous studies, the goal of this research was to prove that occlusion of left CCA for 180 min in rat can induce ischemic stroke based on the alteration in the number of neuron and some biomarkers in the brain tissue. This period of occlusion was expected to create a great reperfusion injury in the brain, that can result in more pronounced cerebral ischemia than it was in 60 or 90 min occlusion. The result of this study is expected to give simpler method to create brain ischemia and can develop next research particularly for preclinical purpose.

Materials and methods

Animal preparation

Fifteen male wistar rats aged 3 - 4 months, weighing 200 - 260 g were obtained from food security and agriculture department, Bandung, West Java, Indonesia. Before the stroke model was established, these rats were conditioned for one week. The rats were placed in cages, with 5 rats per cage, under a 12-h dark-light cycle. The food and water was available ad libitum, at a temperature of 22 - 24 °C, with constant humidity (55 ± 5%). The rats were then randomly assigned into 3 groups: Sham Group (n = 5) with no occlusion, Group A for ischemic stroke model in 60 min occlusion (n = 5) and Group B for ischemic stroke model in 180 min occlusion (n = 5). The experiment is performed in pharmacology laboratory of the Faculty of Medicine, Brawijaya University, Indonesia. The experimental protocol was approved by animal ethics commission of Faculty of Veterinary, Brawijaya University, under protocol numbers 219-KEP-UB-2023.

Induction of ischemic stroke by left common carotid artery occlusion for 180 min

Wistar rats were anesthetized using ketamine 80 mg/kg BW and xylazine 10 mg/kg BW intraperitoneally. Then, these animals were turned to supine position and fixed to the surgical table using the adhesive tape. In the next process, a small incision was made in the neck midline for approximately 3 - 4 cm and an exploration was done till the trachea can be seen. Subsequently, a gently exploration was we explore gently to find left common carotid artery, which accompanies with the vagal nerve. The left common carotid artery was isolated from the vagal nerve and connective tissue gently, then followed by the occlusion of left common carotid artery using small bulldog clamp for 180 min before the bulldog clamp was removed Machin *et al.* [14] to achieve reperfusion injury. After that, the process was going back to the step of the incision on the neck of the rats. When it was done, a checking on the motoric function using ladder rung walking test (LRWT) was conducted to confirm the motor impairment in the ischemic stroke.

Motoric disfunction assessment using LRWT to confirm the occurrence of ischemic stroke

In this study, motoric dysfunction is assessed with ladder rung walking test (LRWT). The LRWT has shown a high sensitivity to measure motoric dysfunction [15]. It also provides qualitative and quantitative measurements of motor function [6]. The horizontal ladder rung apparatus consisted of side walls made of clear plexiglas and metal rungs. The rung will be inserted to make a 3 mm diameter floor whose diameter of each rung is 3 mm. The length both clear plexiglass side walls are 100 cm with 20 cm high [16,17]. The distance between rung was varied from 1 to 5 cm for rats. The ladders were elevated horizontally in 30 cm above the ground, with a neutral cage placed in the starting position and the animal's home cage placed at the opposite end of the ladder [16]. In this study, there were 2 parameters are evaluated using LWRT, which are foot fault scoring (FFS) and foot placement accuracy analysis (number of errors) [17].

Foot fault scoring (FFS)

Foot fault scoring (FFS) is a qualitative examination used to assess forelimb and hindlimb placement on rung [15]. FFS is assessed using 7 categories, which are (1) Score 0/Total miss: This points were given when the limb did not completely touch the rung and the rat falls. A fall was defined as limb fell between rung and body posture and balance were disturbed. (2) Score 1/Deep slip: The limb was initially placed on a rung, then the limb was slipped off rung, and the rat fell. (3) Score 2/Slight slip: The limb was placed on a rung than slipped off during weight bearing, but did not result in a fall. The rat can maintain balance and continue a coordinated gait. (4) Score 3/Replacement: The limb was placed on a rung, but before weight bearing the limb on the rung, the rat quickly lifted and placed on another rung. (5) Score 4/Correction: The limb posited toward for 1 rung, then it placed on another rung without touching the first one. Score 4 is also given when the limb is placed on a rung, but the animal removes the foot and repositions it on the same rung. (6) Score 5/Partial placement: The limb was placed on a rung with either wrist or digits of the forelimb or heel or toes of the hindlimb. (7) Score 6/Correct placement: The midportion of the palm of a limb was placed on the rung

with full weight support. Error scores of the 5 trials were averaged for an analysis [16,17].

Foot placement accuracy analysis (number of errors)

Foot placement accuracy analysis is a quantitative examination carried out using average ratio of the number of errors per step. The number of errors and the number of steps for each crossing were counted for each limb separately and represented as ratio of errors. Error was defined based on the foot fault scoring system (rated with 0, 1, 2 until 6 points as described above). The ratio of errors per step was averaged for 5 trials [15,17].

Evaluation of morphology and distribution of neuron

The identification of the morphology and distribution of the neuron was evaluated through H and E staining. The rats were dissected upon a 180-min occlusion of the left unilateral CCA and the assessment of the motoric dysfucntion. The sham group was also dissected after conclusion LRWT experiment. After the rats were sacrificed, the rat's chest is opened and the rat's blood is sucked out with a syringe so that the blood in the bloodstream comes out. Its head was then removed and the brain was extracted and fixed in 4% formalin [14]. Subsequently, it was then fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) (pH 7.4) and encapsulated in paraffin. The paraffin-embedded brains were cut at 4 μm thick in coronal section.

Measurement of GFAP expression was performed using immunohistochemistry technique

The expression of GFAP was assessed exert immunohistochemistry on the cerebral cortex of left hemisphere. Paraffin-embedded brain tissue blocks from rats were sectioned using a microtome at a point 1.5 cm anterior to the bregma. The blocks were sliced into 4 μm-thick in coronal sections. The tissue sections underwent deparaffinization and rehydration by immersion in xylene twice for 3 min each, followed by 100% ethanol for 3 min, and then washed in PBS for 5 min. Area around the tissue sections was wiped clean and the slides were incubated in a 3% hydrogen peroxide solution (v/v in absolute methanol) for 15 min at room temperature, before it was washed in PBS for 5 min, repeated 3 times. After cleaning the surrounding area of the sections, the primary antibody GFAP (2E1) Sc-33,673, Lot # 1022 Mouse monoclonal IgG2b (Santa Cruz Biotechnology) was applied and incubated overnight at 4 °C followed by washing in PBS for 5 min, 3 times. The Avidin-Biotin Complex reagent (Sc-516,216, Santa Cruz Biotechnology) was applied and incubated at room temperature for 30 min, followed by another round of washing in PBS for 5 min, 3 times. After that, 3,3'-diaminobenzidine (DAB) chromogen (Nichirei Biosciences) was added and incubated at room temperature for 10 min. The slides were washed in distilled water for 5 min, repeated 3 times, then immersed in a counterstain solution and washed with tap water. Finally, the sections were mounted on glass slides and covered with permanent mounting medium.

Measurement of S100B and MMP9 concentration were performed using ELISA technique

Samples for ELISA were obtained from blood plasma. The samples were added to the micro-ELISA plate wells which has been pre-coated with an antibody specific to Rat S100B (Elabscience Catalog No: E-EL-R0868) and antibody specific to rat MMP9 (Elabscience Catalog No: E-EL-R3021). Then, a biotinylated detection antibody specific for Rat S100B and Rat MMP9 (100 μL) is added to each microplate well and incubated for 1 h at 37 °C. Decant the solution from each well, add 350 μL of wash buffer to each well. More than that, soak for 1 min and aspirate or decant the solution from each well. This step was repeated for 3 times. Then, 100 μL of HRP conjugate working solution is added to each well. It was needed to incubate for 30 min at 37 °C. Decant the solution from each well, repeat the wash process for 5 times. The next step is added Substrate Reagent **(**90 μL) to each well. Incubate for about 15 min at 37 °C. Add 50 μL of Stop Solution to each well. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of Rat S100B.

Statistical analysis

Statistical analysis was performed using the software package SPSS (version 24.0). To determine the statistical analysis for data normality and data

homogeneity, the shapiro-wilk test and levene test was conducted. It was also done to analyze the difference between 3 independent groups using the ANOVA test. Data were presented as mean ± standard deviation.

Results

The motoric function significantly decreases in the ischemic stroke model

The final result assessment of LRWT is scored from the average points from all rats walking sessions cross the rung. In this study, an evaluation of motoric function was carried out on the right side extremities of rat both RHL and RFL. According the common carotid artery which was occluded, that was left side. The results of the LRWT assessment showed that FFS of RHL appeared to be lower in both Group A and B than the Sham Group (**Table 1**). For placement accuracy (number of error) of RHL, the lowest result was found in group B namely 0.561 ± 0.32, that means 56 % steps in group B were error with degree of error /FFS was 1.41 ± 1.543. The same result also was found in RFL. The lowest result was in group B, namely 0.44 ± 0.709, that means 44 % steps in group B were error with degree of error /FFS was 2.41 ± 1.698 (**Table 1**).

Table 1 Analysis of foot fault scoring (FFS) and number of error of RHL and RFL.

Parameter	RHL				RFL			
	Sham	**A group**	**B group**	**p-value**	**Sham**	**A group**	**B group**	**p-value**
FFS	6.00 ± 0.224	2.71 ± 2.054	1.41 ± 1.543	0.000*	5.80 ± 0.894	3.18 ± 1.976	2.41 ± 1.698	0.000*
Number of error	0.957 ± 0.190	0.44 ± 0.261	0.561 ± 0.32	0.000*	0.96 ± 0.179	0.420 ± 0.275	0.44 ± 0.709	0.000*

RHL, right hindlimb; RFL, right forelimb; FFS, foot fault scoring. Data were presented in Mean ± SD. *$p < 0.05$ is considered to be significant, analyzed with t- test analysis. SD: Standart deviation.

MMP9 and S100B level significantly increase in the ischemic stroke model

A biomarker is substance that can be measured objectively and can be used to diagnose the disease. MMP9 is one of biomarkers that indicates neuroinflammation process, while S100B is biomarker that indicates activation of astrocyte [7]. The result of this study was MMP9 level increases significantly in both Group A and B, rather than the sham group. Similarly, the result of S100B level shows a significant increase in A and B group than sham. Meanwhile, the highest result was found in group B (**Table 2**).

Table 2 Level expression of MMP9 and S100B in peripheral blood in sham and ischemic stroke group.

Biomarker	Group			*p*-value
	Sham	A group	B group	
MMP9 (ng/mL)	0.5908 ± 0.17496	1.24700 ± 1.769865	3.2494 ± 0.910	0.009*
S100B (pg/mL)	194.0000 ± 52.512	634.00000 ± 199.079130	1482.0000 ± 282.18788	0.000*

MMP9: matrix metalloproteinase-9; S100B: S100 calcium-binding protein B. Data were presented as mean ± SD. *$p < 0.05$ is considered to be significant, analyzed with ANOVA test. SD: Standart deviation.

A decreasing number of the neuron in the brain tissue

Histological examination of brain tissue was performed using hematoxylin and eosin staining. In this study, there was a significant difference of number and distribution of neuron between sham and ischemic stroke group. In the **Figure 1(A)**, the H and E staining with magnification 100× showed a paler color and decrease in cell distribution in the ischemic stroke area in both groups A and B (black box). At 400× and 1000× magnifications, there was a decreasing distribution and number of neuron (black arrow) in both groups A and B than sham, while the lowest number of neuron was found in group B (**Table 3**).

Table 3 Average number of neuron cells in sham and ischemic stroke group.

Number of neuron	Group			*p*-value
	Sham	A group	B group	
Neuron	12.00 ± 1.517	9.60 ± 1.140	9.00 ± 1.140	0.016*

Data were presented as mean ± SD. *$p < 0.05$ is considered to be significant, analyzed with ANOVA test. SD: Standart deviation

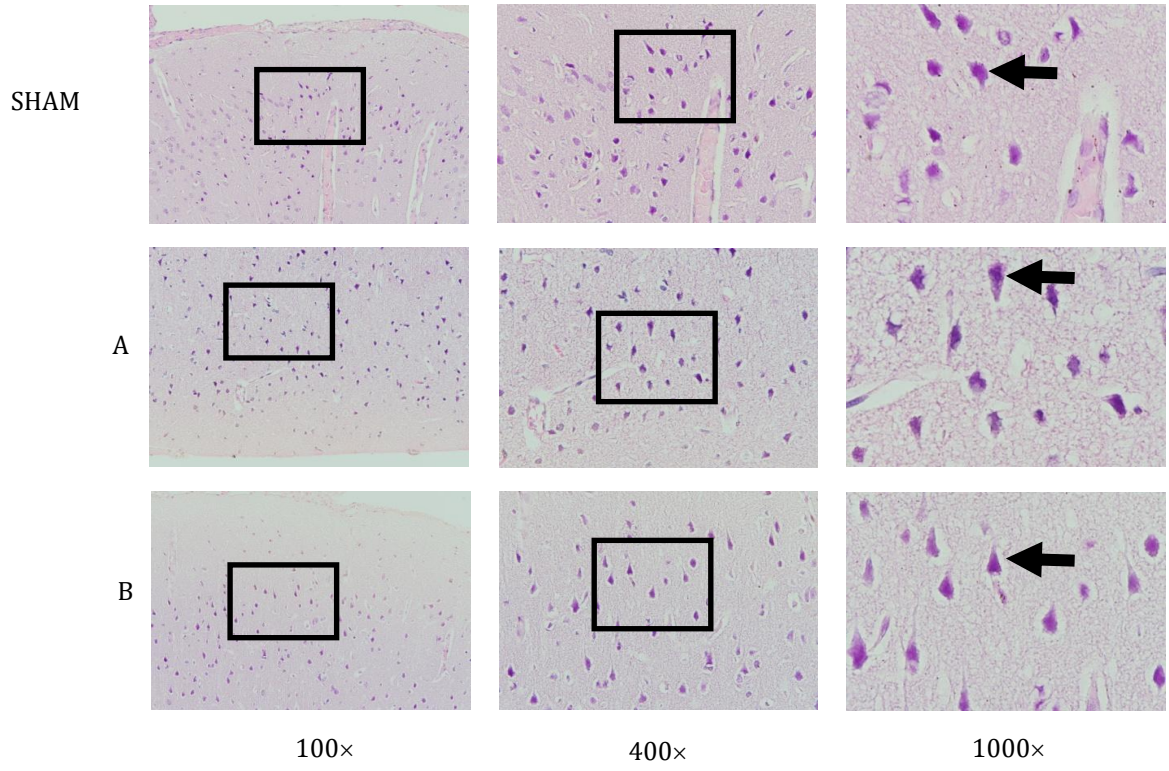

Figure 1 Hematoxylin and eosin (HE) staining of brain tissue. A evaluation of Ischemic stroke area at 100× of magnifications, where in the ischemic group (A and B) there was paler area than sham. At 400× magnifications showed the distribution of neuron in ischemic stroke group (A and B) were decreasing than sham; At 1000× magnifications showed the number of neuron was decrease in ischemic stroke group (A and B) than sham. Black box: Ischemic stroke area; Black arrow: neuron. Black bar: 0.025 mm.

The increase of the expression level of GFAP in the ischemic group

Glial fibrillary acidic protein (GFAP) is one of biomarkers which is related with reactive astrocyte [7]. Reactive astrocyte is an initial process of glial scar formation [18,19]. In this study, from IHC staining with 100×, 400× and 1000× magnification demonstrated that the number and the distribution of GFAP positive neuron (brown color cells) was found to increase significantly in ischemic stroke model both in Group A and B than the sham group (**Figure 2**). Meanwhile, the highest result was found in Group B (**Table 4**).

Discussion

Animal stroke models for preclinical purposes have been developed for several reasons. Many methods have been developed such as temporary or permanent middle cerebral artery occlusion (MCAO) model, photothrombosis model and Endothelin-1 model Fluri *et al.* [4], permanent or temporary bilateral and unilateral occlusion of the common carotid artery. A study said

that bilateral and unilateral occlusion of the common carotid artery for 5 min did not result in inhibition of neurons in the corticospinal tract [11]. It revealed that the creation of an animal stroke model using occlusion of the common carotid artery for 5 min has not been able to cause permanent brain injury.

The MCAO model whether permanent or transient is said as a technique that most proper and closer to the condition of human stroke [6]. However, this technique has the own obstacles, the MCAO procedure needs insert intraluminal filament or mechanical device [5]. In this study, the stroke model is made by occlusion of left CCA transiently for 180 min. The transient occlusion for 180 min is aimed to generate great reperfusion injury in brain. Reperfusion injury is restored the blood supply to the organ that is temporarily deprived of blood flow. Reperfusion injury will create the great brain damaged through series of even at cellular and molecular level. [20]. Moreover, in the previous study with RUCCAO for 60 and 90 min, it shows that it did not induce motoric, while the cognitive disfunction increased significantly. This method specially 60 min occlusion did not result in motoric deficit significantly, and 90 min occlusion result in cognitive and motoric disfunction in 3 -7 days after occlusion [13]. This means that there was needed occlusion more than 5, 60 and 90 min to yield reperfusion injury in brain. Based on previous studies, therefore the longer occlusion was made, the better it is, such as more than 90 min. The occlusion for 180 min was expected to induce brain injury more severe than in 90 min.

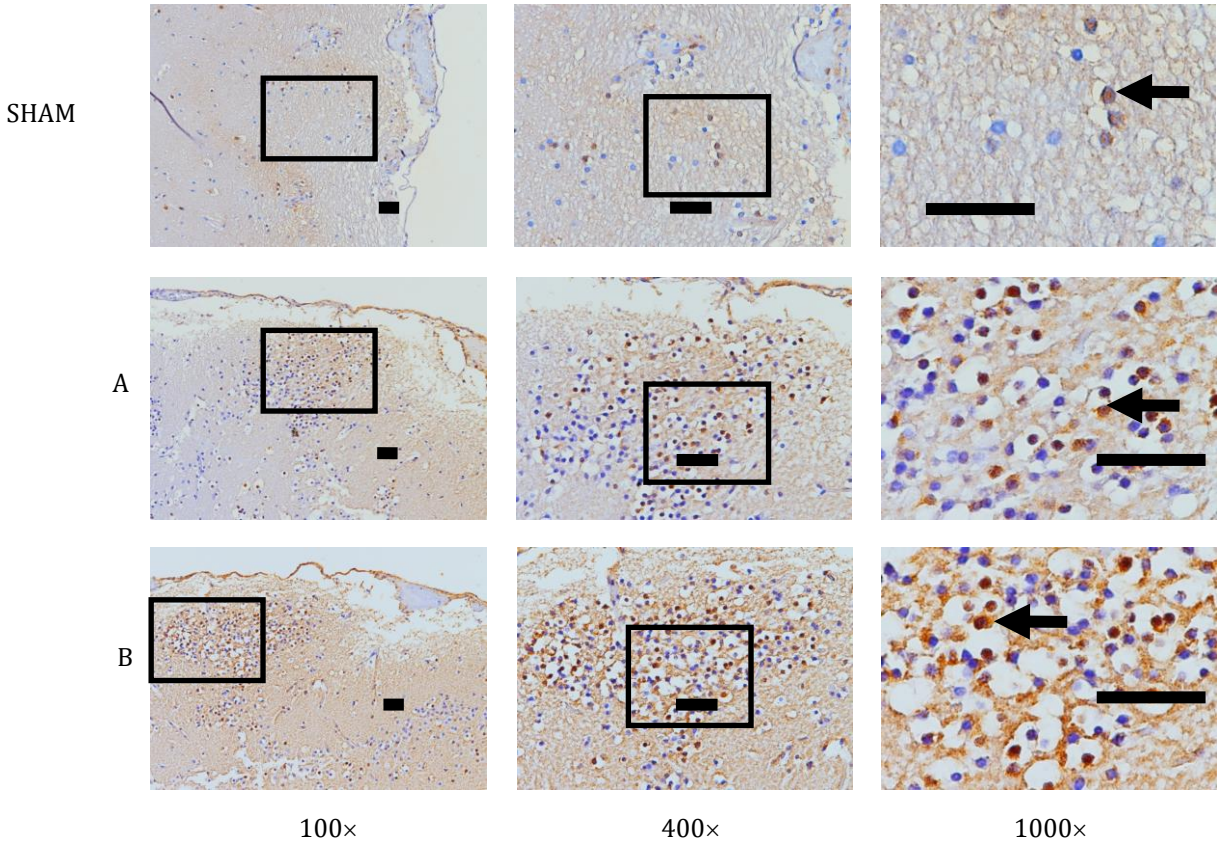

Figure 2 Expression of GFAP in ischemic area was assessed with immunohistochemistry technique. At 100× and 400× magnifications showed the distributions of GFAP positive neuron which was represented by brown color cell were increase in ischemic group in both groups A and B than sham. At 1000× magnifications showed the number of GFAP positive neuron increased in the ischemic stroke group than the sham. Black box: spread of GFAP positive neuron in ischemic area, black arrow: GFAP positive neuron. Black bar: 0.025mm.

Table 4 Expression of GFAP in brain tissue between sham and ischemic stroke group.

Biomarker	Group			*p*-value
	Sham	A group	B group	
GFAP	5.20 ± 1.483	6.60 ± 2.074	9.60 ± 1.517	0.482

GFAP: glial fibrillary acidic protein. Data were presented as mean ±SD. *$p < 0.05$ is considered to be significant, analyzed with ANOVA test. SD: standart deviation

Molecular and cellular mechanisms that are involved in reperfusion injury include the innate and adaptive immune systems as well as the complement systems, platelet, coagulation factors which can trigger nitric oxide synthase, production of nitric oxide that causes cell death through necrosis and apoptosis [20].

An examination of motoric function with LRWT was made based on FFS and foot placement accuracy analysis. FFS is qualitative examination that assesses placement of both forelimb and hindlimb based on the 7 categories. Meanwhile, placement accuracy analysis (number of error) is analyzed by calculating the average number of error (based on 7 categories in FFS) divided by the number of steps [17]. The LRWT is chosen as method to assess the motoric deficit in this study because LRWT is a sensitive method in assessing the presence of motoric deficit for various motoric disfunction models such as unilateral ischemic injury to the motor cortex area [16,21]. Apart from that, the ladder rung walking test is also a method can be used to assesses motoric coordination [22].

This study showed a significant difference in motor deficits between the ischemic stroke groups (A and B) than the sham group, both RHL and RFL. The value of FFS both RHL and RFL were found lower in ischemic stroke model group than sham group. This data shows that there was motoric disfunction in the right side of the extremities in the ischemic stroke model. Foot placement accuracy was also found to be lower in the ischemic stroke model (A and B group) compared to the sham group, meaning that there was a disturbance in walking caused by motor deficits in the right extremity, both RHL and RFL. Meanwhile, the lowest motoric deficit was found in ischemic stroke model for 180 min (Group B). This result revealed that CCA occlusion for 180 min can induce brain ischemic than CCA occlusion for 60 min which was proved through motoric dysfunction.

In this study, ischemic stroke model that was made using occlusion left CCA transiently for 180 and 60 min have been created various morphological changes and elevated certain biomarker such as GFAP, MMP9, and S100B. In addition, the diagnose of the ischemic stroke use biomarker MMP 9 and S100B which is taken from blood stream. The assessment of MMP9 and S100B which was based on the research conducted by Ying Huang *et al.* [7], which showed that several biomarkers will increase their expression in ischemic stroke conditions. The occurrence of neuroinflammation as the effect of brain ischemic is characterized by an increase expression level of MMP9. Likewise with astrocyte, during ischemic stroke, astrocyte will be activated and the biomarker that indicate activated astrocyte is S100B [7]. In this research, the expression level of MMP9 was found to increase in ischemic stroke model (Group A and B) than the sham group. Similar to S100B, there was an increase in ischemic stroke model (Group A and B) than the sham group. Meanwhile, the highest MMP and S100B expression were found in group B. This means that left CCA occlusion for 180 min can induce neuroinflammtion process. This result is in line with research conducted by Zielinka-Turek *et al.* [23] which revealed that MMP 9 and S100B level increased in ischemic stroke patients with carotid artery stenosis than patients with carotid artery stenosis perform endarterectomy without ischemic stroke**.** Furthermore, according to Khandare *et al.* [8] S100B was found to increase concomitant with infarct size. Interaction between S100B and receptor for advanced glycation end products (RAGE) will activate microglia and secrete inflammatory mediator such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and the chemokine 22 (CCL22) that have role in pathophysiology of ischemic stroke [24]. In this study, the number and distributions of neuron appeared extremely different from the sham and ischemic stroke group (A and B). The number and distribution of neuron was decreasing in Group A and B

compare to it in the sham group. Yet, the lowest number of neuron was found in Group B. This means that the left CCA occlusion for 180 min result in functional neuron damage rather than the left CCA occlusion for 60 min. Neuronal death in ischemic stroke determines the clinical outcome in stroke patient. Neurotoxicity mediated by excitatory amino-acids which is called excitotoxicity is the key link between ischemia and neuronal death in strokes. N-methyl-d-aspartate receptors (NMDARs) especially GluN2BR subunits play an important role in permitting excessive Ca^{2+} influx, which in turn leads to neuronal death. Depletion of ATP during ischemic stroke is accompanied by the massive production of ROS, which can directly damage the integrity of plasma membrane and the DNA strand. A damaged DNA will release Cytocrome C from mitochondria into the cytoplasm of neurons, which can in turn lead to apoptosis of neuron [25]. Meanwhile, the declining number of neuron was also caused by the neuroinflammation, particularly the role of microglia in ischemic stroke. Further, in ischemic stroke, microglia will increase phagocytic receptor expression that promote phagocytic process to neuron [26]. Another process which was involved in the neuronal damage that caused a decrease the number of functional neuron is excitotoxicity, which induces inhibition of PERK/eIF2α kinase phosphorylation. This process can cause protein misfolding and subsequently promote autophagy and ribophagy which has contributions in neuron damage [27].

The presence of glutamate excitotoxicity will cause hyperreactivity of astrocytes. This process can result in the structural changes of the astrocytes Amalia [28] such as hypertrophy of astrocytes in 1 to 2 days after injury [29]. The changes of the astrocytes were marked by specific biomarker namely GFAP. Astrocytes are known as constituents of blood brain barrier (BBB), that the presence of damaged astrocytes causes disruption of BBB and subsequently cause leakage GFAP to bloodstream. The common result is also demonstrated in this study, that GFAP level significantly increase in the ischemic stroke model group (both in A and B) than the sham group. The highest expression was found in Group B. This result showed that the left CCA occlusion for 180 min can generate damage of astrocytes which is characterized by an increase in GFAP expression levels than the left CCA occlusion for 60 min. This result also in line with study conducted by Herrmann *et al.* [30], which showed that GFAP and S100β levels in stroke patients were found increasing since the first day until 4 days of hospitalized. S100 calcium bind the protein β and GFAP were also found to correlate with infarct size, neurological deficit, and functional outcome in stroke patients. In addition, GFAP was more sensitive as biomarker for lacunar infarct. This finding indicates that both GFAP and S100β can reflect the level of brain damage which can be seen through the CT imaging [30]. Another study revealed that GFAP has highly diagnostic value in stroke, especially in differentiating ICH and ischemic stroke [9]. Limitation of this research was the measurement of GFAP positive neuron in IHC examination was accounted manually, which can raise bias. Meanwhile, to obtain a better result of the evaluation of the neuron, it is recommended to use a specific neuronal biomarker which assessed using the immunofluorescence techniques.

Conclusions

The occlusion of the left unilateral common carotid artery for 180 min can induce focal brain ischemia which is evaluated from decrease number and distribution of neuron, motoric deficit, and upregulation expression level of MMP9, S100β, and GFAP. These findings provide a simple method for creating ischemic stroke models and is expected to be useful for developing further research in the field of pathophysiology and management of ischemic stroke.

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Declaration of Generative AI in Scientific Writing

The authors declare that no generative AI tools were used in the preparation of this manuscript. All content, including the writing, interpretation of data, and

conclusions, was produced entirely by the authors, who take full responsibility for the accuracy and integrity of the work.

CRediT author statement

Aris Widayati Conceptualization, Methodology, Project administration, Funding acquisition, and Writing – original draft

Fedik Abdul Rantam Supervision, Methodology, Validation, Writing – review & editing, and Resources

Abdulloh Machin Supervision, Formal analysis, Investigation, Validation, and Writing – review & editing

Wibi Riawan Data curation, Formal analysis, Investigation, Visualization, and Writing – review & editing

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