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Standardization of the Simple Methodology for Experimentally Induced Ischemic Stroke in Rat Models

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ABSTRACT

Stroke is a globally significant and devastating disease that requires prompt treatment. Animal models are commonly used to investigate stroke therapy, often through experimentally induced ischemic stroke (EIIS). However, challenges arise in implementing EIIS in animal models. The current study aimed to present a simple EIIS methodology for animal models. A total of 60 male Sprague-Dawley rats were randomly divided into five groups, namely Group 1 (sham-operated), Groups 2 to 5 (EIIS groups) with different duration of common carotid artery (CCA) ligation, including 1, 2, 4, and 8 hours, respectively. The ligation was performed on the CCA and its branches. Before the experiment, the rats were anesthetized, and the incision area was shaved and disinfected. The sagittal ventral midline was incised, with neck muscles retracted to expose the right CCA. The occlusion was performed on three sides of a carotid artery (common, external, and internal) using a simple interrupted suture. The occlusion of blood flow using ligation was performed at different times depending on the groups. After that, the CCA ligations were re-perfused by cutting the suture knot. The brain and blood were collected on days 1 and 7 after reperfusion. The results indicated that 4 and 8 hours of CCA ligation significantly impacted the general condition and neuro-deficit score. Moreover, 4 and 8 hours of CCA ligation could induce ischemic stroke by its capacity to cause infarction within the brain parenchyma and increase the platelet-to-white blood cell ratio, C-reactive protein, and De Ritis ratio. In contrast, 1 and 2 hours of CCA ligation did not significantly affect the observed parameters. It can be concluded that the EIIS using 4 and 8 hours of CCA ligation can be applied to induce ischemic stroke in rat models with consistent impacts on general conditions, neuro-deficit, hematology, and serology.

Keywords: Common carotid artery, Ischemic stroke, Ligation, Rat model, Standardization

INTRODUCTION

Stroke is a prevalent degenerative disease worldwide caused by the disruption of the circulatory system. The most common type of stroke is ischemic stroke (Yi et al., 2020), characterized by thromboembolism within a cerebral artery (Chugh, 2019). Recognized as a catastrophic disease, stroke significantly impacts human life, posing substantial social and economic challenges. In Indonesia, ischemic stroke has emerged as a leading financial burden, with an annual cost of IDR 2.56 trillion (Venketasubramanian et al., 2022). Hence, the ischemic stroke study is one of the major research topics of the Indonesian national research program.

Experimentally induced ischemic stroke (EIIS) studies on animal models have some shortcomings concerning the methodology and duration of middle cerebral artery occlusion (MCAO). Additionally, the study of EIIS in animal models encounters issues related to research parameters (Bacigaluppi et al., 2010). Several frequent procedures to induce ischemic stroke include craniotomy and middle cerebral artery (MCA) occlusion by inserting a nylon suture (Bertrand et al., 2017). Due to the limited number of veterinary surgeons in animal models, those methods are brutal to be conducted in developing countries, including Indonesia. Further, the MCAO duration impairs the volume of the infarct area (Narayan et al., 2021). Too short occlusion time impacts the minimum infarction area and unclear neurological score. In contrast, too-long occlusion has significant mortalities (Singh et al., 2022).

There is evidence suggesting that artificial induction of ischemic stroke in animals may not consistently result in significant brain damage, as the brain exhibits a certain level of tolerance to ischemic insults (Gidday, 2006). Accordingly, this preliminary study aimed to provide brief information regarding the simple method of EIIS procedure in rat models that could be reliable to support the pre-clinical study of ischemic stroke. This study was supported by several biomarkers for ischemic stroke, including platelet-to-white blood cell ratio, neutrophil/lymphocyte ratio, C-reactive protein, and De Ritis ratio.

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MATERIALS AND METHODS

Ethical statement

This study has been approved by the ethics committee of the Faculty of Veterinary Medicine, University of Wijaya Kusuma Surabaya. The approval number of this study was KKE-34/IX/2022, and it was stated as 2 September 2022. All the experimental procedures were performed in the Laboratory of Pharmacology, Faculty of Veterinary Medicine, University of Wijaya Kusuma Surabaya, Indonesia, from September 2022 until January 2023.

Research design and sample collection

This study used 60 male Sprague-Dawley rats, six months old, weighing 254.25 ± 8.18 grams (Laboratory of Pharmacology, Faculty of Veterinary Medicine, University of Wijaya Kusuma Surabaya). The rats were divided into five groups. Each group was maintained inside the acrylic cage (80 cm × 40 cm × 50 cm), with husk bedding, 12/12 hours light/dark, 25°C, and 60% humidity. The rats were provided water and feed access *ad libitum*.

Group 1 was sham-operated (skin neck incision without CCA ligation). Groups 2 to 5 were EIIS groups, including Group 2 with EIIS using 1 hour of CCA ligation, Group 3 with 2 hours of CCA ligation, Group 4 with 4 hours of CCA ligation, and Group 5 with 8 hours of CCA ligation. After the EIIS procedure, the rats were observed regarding their general condition and neurodeficit score on days 1 and 7 after reperfusion. The scoring system was conducted following a previous study with modifications (Bertrand et al., 2017), and it is embedded in Table 1.

Table 1. A scoring system of general condition and neurodeficit of experimentally induced ischemic stroke in rat models

Category	Parameter	Score			
		1	2	3	4
General condition	Hair condition	Worst	Moderate	Clean	-
	Ear position	Droopy	-	Raised	-
	Hearing	Unresponsive	-	Responsive	-
	Eye condition	Unresponsive	-	Responsive	-
	Posture	Crawling	Leaning	Normal	-
	Spontaneous activity	Unconscious	Low activity	Normal	-
Neurodeficit	Gait	Unconscious	Crawling	Walking to one side	Normal
	Climbing	Unconscious	Crawling	Walking to one side	Normal
	Compulsory circling	Unconscious	Falling to one side	Leaning	Normal
	Front limb symmetry	No grabbing at all	One side grabbing	Both grabbing but continually loose	Normal
	Whisker response	No response	Whisker movement only	Turn trunk	Normal
	Epileptic behavior	General tonic spasm	Transient general tonic spasm	Transient focal tonic spasm	Normal

Experimentally induced ischemic stroke procedure

The ligation of a common carotid artery (CCA) was conducted to induce EIIS in animal models. The ligation was performed on the CCA and its branches. Before the EIIS procedure, the rats were anesthetized using a combination of 50 mg/kg BW ketamine (Agrovet Market, Canada) and 4 mg/kg BW xylazine (Interchemie, Holland). The rats were placed on their back on the operating table. The incision area was shaved and disinfected using 70% alcohol. Further, the sagittal ventral midline incision was performed. The neck muscle and salivary glands were carefully retracted to expose the right CCA. The right CCA was carefully separated from the vagal nerve to prevent transient parasympathetic nerve dysfunction. After that, the CCA was observed to find its bifurcation and both the external carotid artery (ECA) and internal carotid artery (ICA, Figure 1a). The occlusion was performed on three sides of a carotid artery (CCA, ECA, and ICA) using a simple interrupted suture using a 2-0 braided silk suture (Gea Medical, Indonesia; Figure 1b). The occlusion of blood flow using ligation was performed at different times depending on the groups. Furthermore, the ligation was released by cutting the suture knot using a surgical scissor (Figure 1c-d). The reperfusion was performed for seven days.

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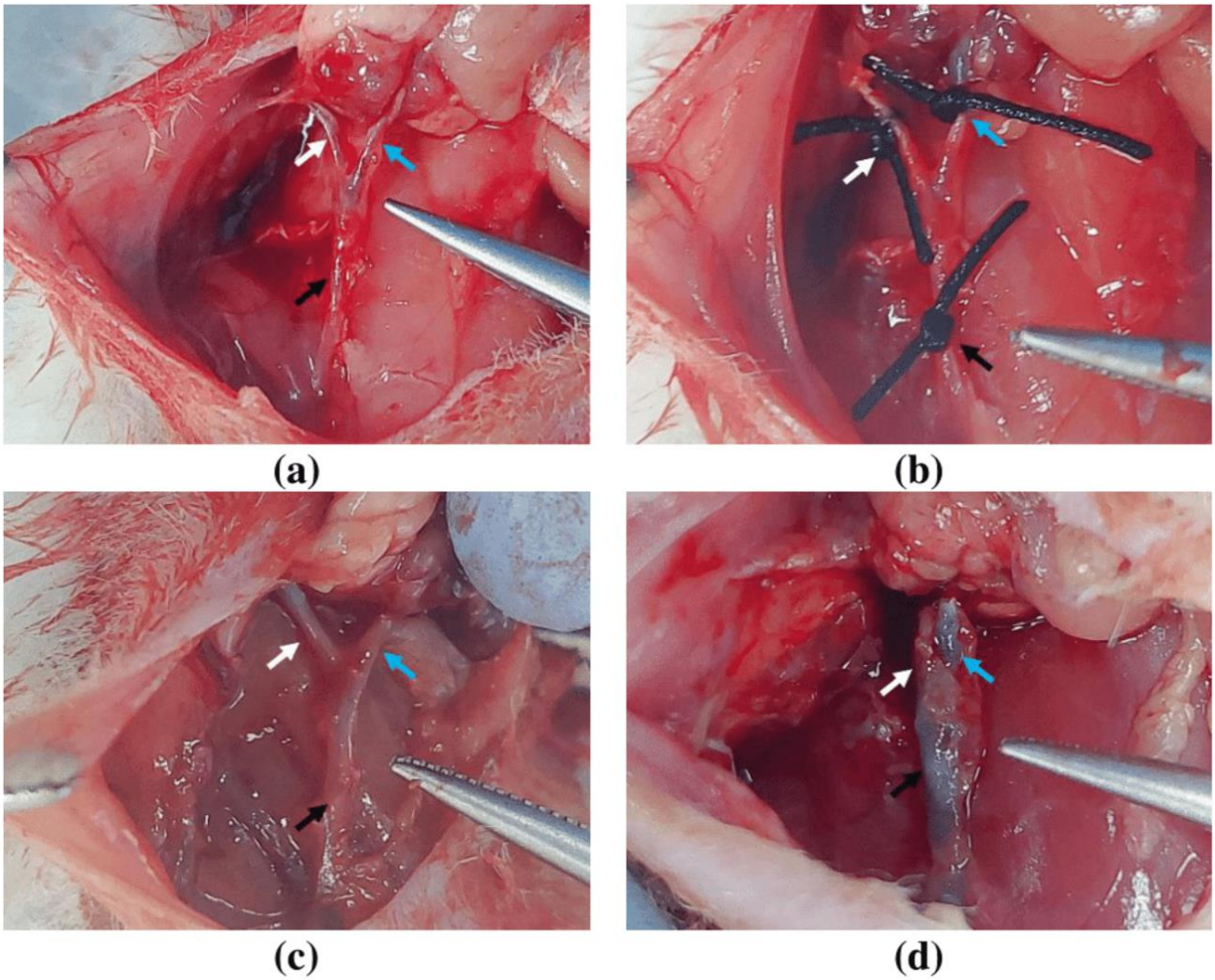

Figure 1. The ligation procedure of experimentally induced ischemic stroke in a Sprague-Dawley rat model. The common carotid artery has been visible (**a**), a simple interrupted suture was applied on the common carotid artery, external carotid artery, and internal carotid artery (**b**); the reperfusion of common carotid artery after occlusion (**c**); the common carotid artery condition after seven days of occlusion (**d**). Black arrow: Common carotid artery, White arrow: External carotid artery, Blue arrow: Internal carotid artery.

Laboratory test

The blood of rats was collected via the jugular vein (2 mL) on days 1 and 7 after the EIIS procedure. The blood was separated into two parts, one for hematology and the rest for serology tests. After that, the rats were euthanized using lethal doses of ketamine (150 mg/kg BW, Agrovet Market, Canada) combined with xylazine (10 mg/kg BW, Interchemie, Holland). The skull was opened to collect their brain. The blood and serum were tested against several biomarkers for ischemic strokes, such as platelet-to-white blood cell ratio (PWR, Amalia and Dalimonthe, 2020), neutrophil/lymphocytes ratio (NLR, Sharma et al., 2021), C-reactive protein (CRP, den Hertog et al., 2009), and De Ritis ratio (Gao et al., 2017).

Before brain cutting, the brain was incubated at -20°C for 15 minutes to prevent the brain from being crushed during sectioning. The brain sample was cut (2-3 mm) using a surgical blade and stained using 2% 2,3,5-triphenyl tetrazolium chloride (TTC, Sigma-Aldrich, Indonesia) at 37°C for 10 minutes. The stained brain section was then analyzed regarding its ischemic area using ImageJ software. Further, the organ was fixed using 10% neutral buffer formalin for routine histopathology (H&E staining) and thionine staining (Ramírez et al., 2020).

Statistical analysis

The normal and homogenous data were analyzed using a parametric test, including ANOVA and post hoc tests using Bonferroni's test. In contrast, the abnormal and non-homogenous data were analyzed using a non-parametric test, including the Kruskal Wallis and Mann Whitney-U test. This study used a significance level of 0.05. The statistical analysis was performed using SPSS version 26.

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RESULTS AND DISCUSSION

The result indicated that EIIS in rats significantly impacts the score of the general condition until seven days after reperfusion ($p \leq 0.05$). Group 2, with 1 hour of CCA ligation, did not show differences compared to Group 1 ($p \geq 0.05$). This finding indicated that 1 hour of CCA ligation does not impact general conditions in rat models. Moreover, Group 3 showed a significant difference in general condition parameters compared to Group 1, especially in hair condition parameters ($p \leq 0.05$). The severe general condition score was shown by Groups 4 and 5 in all parameters ($p \leq 0.05$), except for ear position ($p \geq 0.05$) compared to the others. However, both groups 4 and 5 did not show any differences regarding the general condition ($p \geq 0.05$, Figure 2). The neurodeficit score of EIIS in rat models indicated similar statistical results to the general condition score. The neurodeficit score in Group 2 has no differences compared to Group 1. Group 3 showed significant differences in gait and whisker response compared to the control ($p \leq 0.05$) but not regarding the other parameters ($p \geq 0.05$). Furthermore, groups 4 and 5 showed severe neurodeficit scores compared to the other groups ($p \leq 0.05$, Figure 2).

Further, the general condition and neurodeficit measurement were confirmed by hematological and serological biomarkers for ischemic stroke. Several markers were PWR, NLR, CRP, and De Ritis ratio. This study obtained an elevated PWR and De Ritis ratio in Group 5 in 1 and 7 days after the EIIS procedure compared to the others ($p \leq 0.05$). However, the PWR and De Ritis ratio in Group 4 increased on day seven only. Groups 4 and 5 showed consistent increases in NLR and CRP in 1 and 7 days after the EIIS procedure compared to the other groups ($p \leq 0.05$, Table 2).

Table 2. Hematology and serology biomarkers of experimentally induced ischemic stroke in rat models

Parameter	Day	Group 1	Group 2	Group 3	Group 4	Group 5
PWR	1	96.17 ± 4.15^{a}	98.16 ± 1.63^{a}	111.30 ± 5.59^{b}	129.56 ± 1.55^{c}	130.19 ± 1.23^{c}
	7	99.66 ± 1.05^{a}	99.57 ± 4.69^{a}	128.23 ± 4.20^{b}	134.43 ± 4.33^{c}	131.86 ± 2.08^{c}
NLR	1	0.27 ± 0.00^{a}	0.25 ± 0.02^{a}	0.32 ± 0.03^{a}	0.42 ± 0.07^{b}	0.36 ± 0.01^{b}
	7	0.27 ± 0.00^{a}	0.31 ± 0.02^{a}	0.31 ± 0.02^{a}	0.38 ± 0.03^{b}	0.37 ± 0.02^{b}
CRP (mg/dL)	1	34.73 ± 0.75^{a}	34.86 ± 1.51^{a}	44.00 ± 4.45^{b}	54.61 ± 0.63^{c}	55.93 ± 0.46^{c}
	7	34.36 ± 0.80^{a}	35.83 ± 0.90^{a}	36.46 ± 1.02^{a}	56.20 ± 0.95^{c}	55.46 ± 2.28^{c}
De Ritis ratio	1	1.24 ± 0.11^{a}	1.30 ± 0.08^{a}	1.32 ± 0.02^{a}	1.22 ± 0.04^{a}	1.29 ± 0.01^{a}
	7	1.26 ± 0.11^{a}	1.23 ± 0.07^{a}	1.24 ± 0.04^{a}	1.43 ± 0.20^{b}	1.42 ± 0.04^{b}

PWR: Platelet-to-white blood cell ratio, NLR: Neutrophil lymphocytes ratio, CRP: C-reactive protein, $^{a, b,c}$ different superscript letters in the same column indicated significant differences ($p \leq 0.05$).

The TTC staining showed that normal brain tissue has deep red stains after staining (Figure 3a). In contrast, the brain infarct was shown by a pale color of the brain after one day (Figure 3b) and an unstained (white) area of the brain after seven days of reperfusion (Figure 3c). The percentage of infarct area of Group 5 was more significant than the other groups ($p \leq 0.05$). The appearance of an infarct area within the brain parenchyma was consistently shown by Group 5 on days 1 and 7. However, the infarct area of Group 4 is similar to that of Group 5 on day 7 ($p \geq 0.05$). In addition, Groups 1, 2, and 3 did not show any differences in the infarct parameter ($p \geq 0.05$, Figure 3d).

The score of brain histopathology showed that necrosis neurons, microgliosis, secondary hemorrhages, and perivascular edema in Groups 4 and 5 were significantly different compared to the other groups ($p \leq 0.05$, Figure 4). Groups 2 and 3 showed an increase in perivascular edema and secondary hemorrhage score on day one after the EIIS procedure; however, the score decreased on day 7, compared to Group 1 ($p \leq 0.05$). It indicated a decrease in severity within the brain parenchyma in groups 2 and 3. Furthermore, the Nissl bodies were distinctly demonstrated on days 1 and 7 in groups 1, 2, and 3; however, groups 4 and 5 showed the Nissl bodies with unclear morphologies and disappeared in all observation periods (Figure 4).

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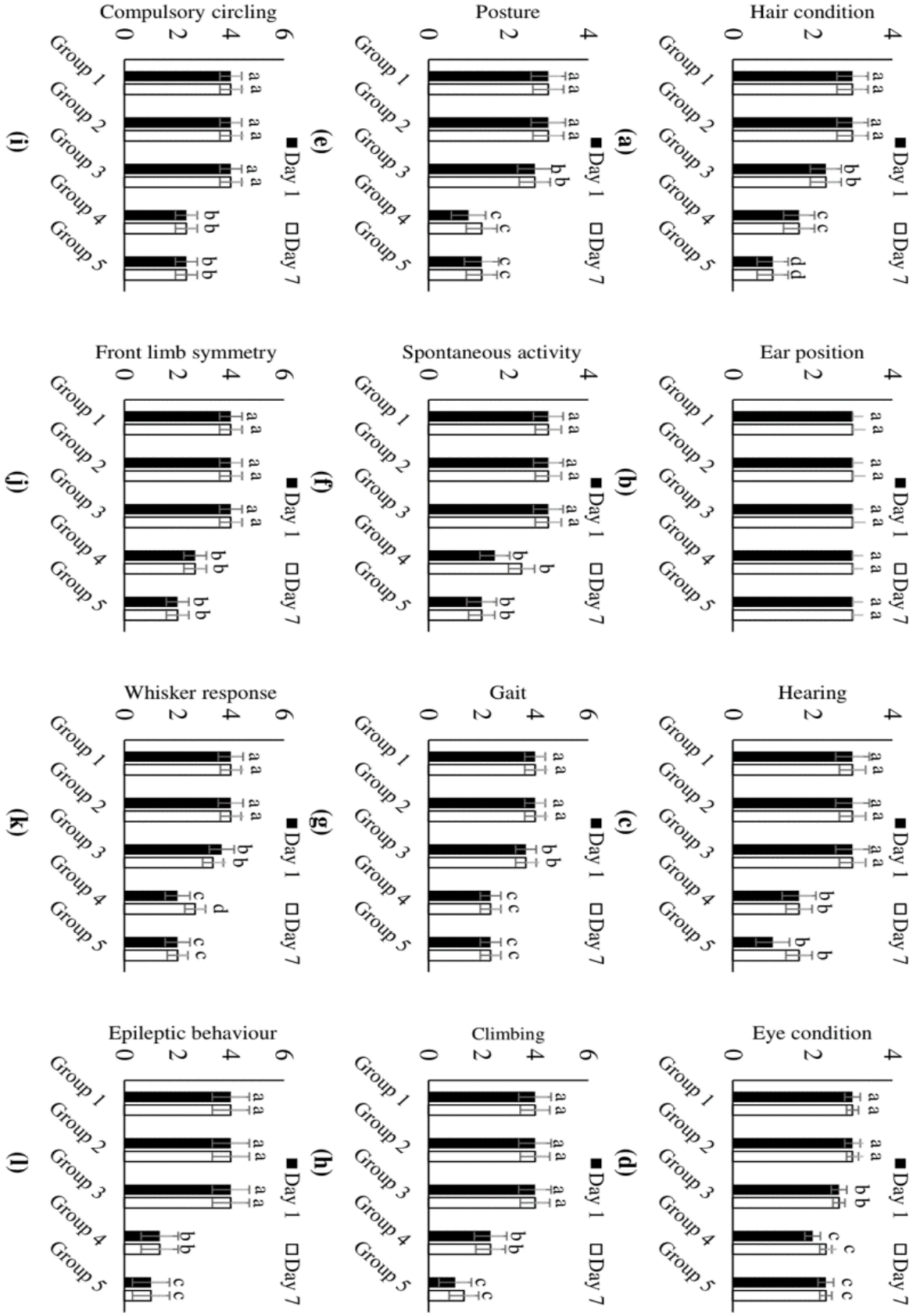

Figure 2. A score of general condition and neurodeficit of the rat after the EIIS procedure. A part of the general condition score consisted of hair condition (**a**), ear position (**b**), hearing (**c**), eye condition (**d**), posture (**e**), and spontaneous activity (**f**). However, the neurodeficit score consisted of gait (g), climbing (**h**), compulsory circling (**i**), front limb symmetry (**j**), whisker response (**k**), and epileptic behavior (l). [a,b,c] Means different superscript letters differ significantly ($p \leq 0.05$).

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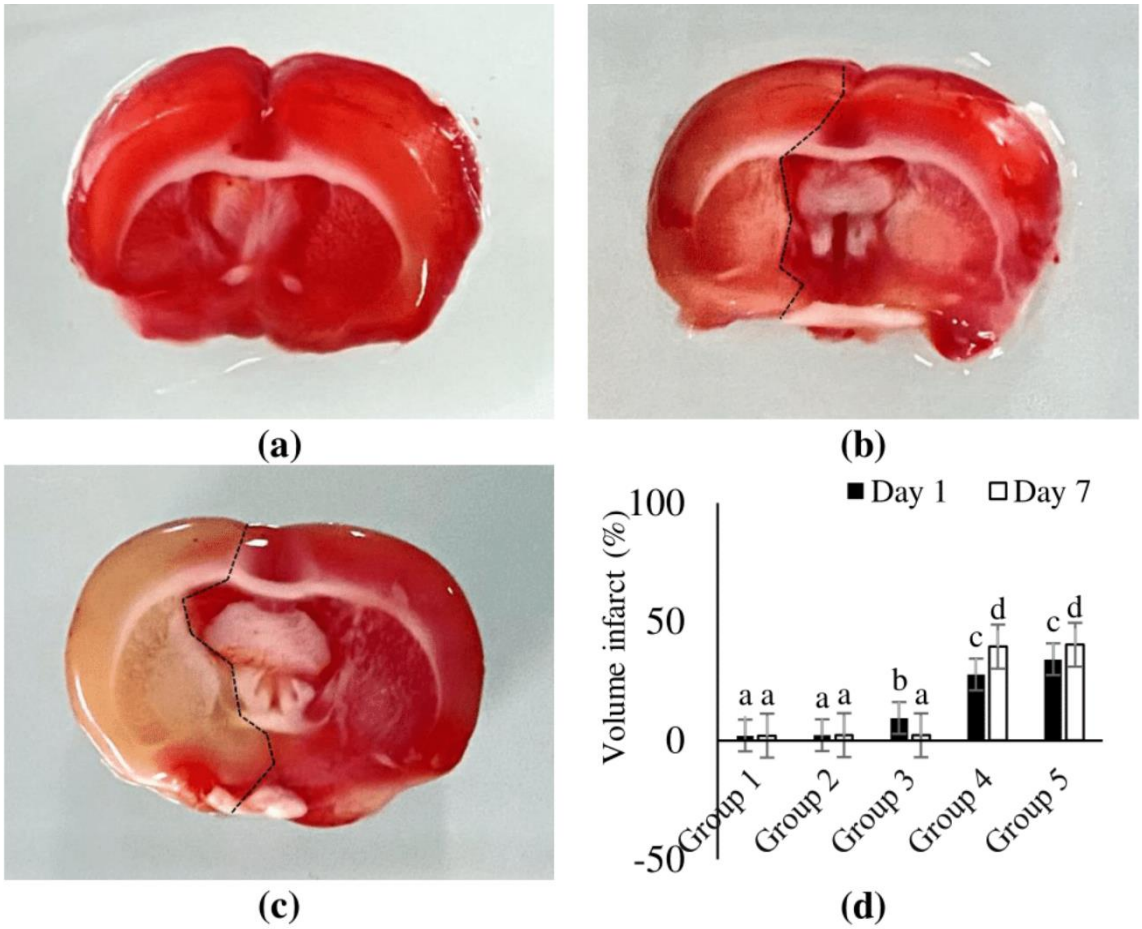

Figure 3. Macro-photograph of the brain after EIIS procedure. Typical appearance of the brain that stained-deep red using TTC staining (**a**), pale coloration (borderline) of the brain after one day of reperfusion (**b**), white coloration (borderline) of the brain after seven days of reperfusion (**c**), the volume of infarct area (%) in days 1 and 7 (**d**). [a,b,c,d] Means different superscript letters in same column and row differ significantly ($p \leq 0.05$).

The CCA is an essential artery that supplies nutrients to brain tissue. The potency of CCA during the physiological aspect makes the researchers utilize it to induce ischemic stroke in rat models. The basic principle to induce ischemic stroke in a rat is the disruption of blood supply to the brain area. One of the standard methods is MCAO. The MCAO can be conducted using two methods. The first method is using inserting the suture within the middle cerebral artery via the CCA, and the second one is using craniotomy. Inserting a sterile operative suture through the CCA can be conducted using a 4/0 and 5/0 nylon suture, and the occlusion can be performed for 30 to 45 minutes (Shvedova et al., 2021). However, this method has a significant limitation, such as a high mortality rate of 71-100% in diver strains of rats (Schulte-Herbrüggen et al., 2006). Another alternative method is using craniotomy (Yeh et al., 2019). A craniotomy is an invasive method that can be applied in rat models. This method correctly exposes the MCA directly, and the occlusion can be performed using coagulation, transection, ligation, and clips (Howells et al., 2010). The previous study described that the modified craniotomy increases the success of ischemic stroke in rat models, proved by the presentation of the infarct area and its inflammatory cytokines (Yeh et al., 2019). However, craniotomy has several difficulties, such as difficulty in finding the precise location of MCA, and cranial bone removal is commonly impeded by a zygomatic arc. It affects the rat's eating ability and physiological function (Theodorsson et al., 2005). Again, all those described methods are brutal to be performed in a research laboratory with minimal veterinary surgery facilities. So, it is necessary to provide a more reliable methodology to induce ischemic stroke in rat models that are simple, cheap, have a low mortality rate, and generate consistent infarction.

This study used a simple interrupted suture to tie up the CCA, ICA, and ECA in the rats. Compared to the other methods, this method provides a more straightforward procedure that can be conducted by veterinarians and researchers with fewer facilities. They need to find the CCA and its bifurcation and ligate it. However, there is a difference between this method compared to the MCAO and craniotomy, including the long ligation time in conducting occlusion. This method requires longer occlusion time compared to the other methods, especially 4 and 8 hours. Using 1 and 2 hours of CCA ligation is not as effective as 4 and 8 hours of CCA ligation because it cannot promote neurological deficit and brain infarction. The failure of infarction forming after 1 and 2 hours of CCA ligation is suspected to be caused by several mechanisms, including the disruption of endothelial cells within CCA and blood crossflow via the anterior communicating artery.

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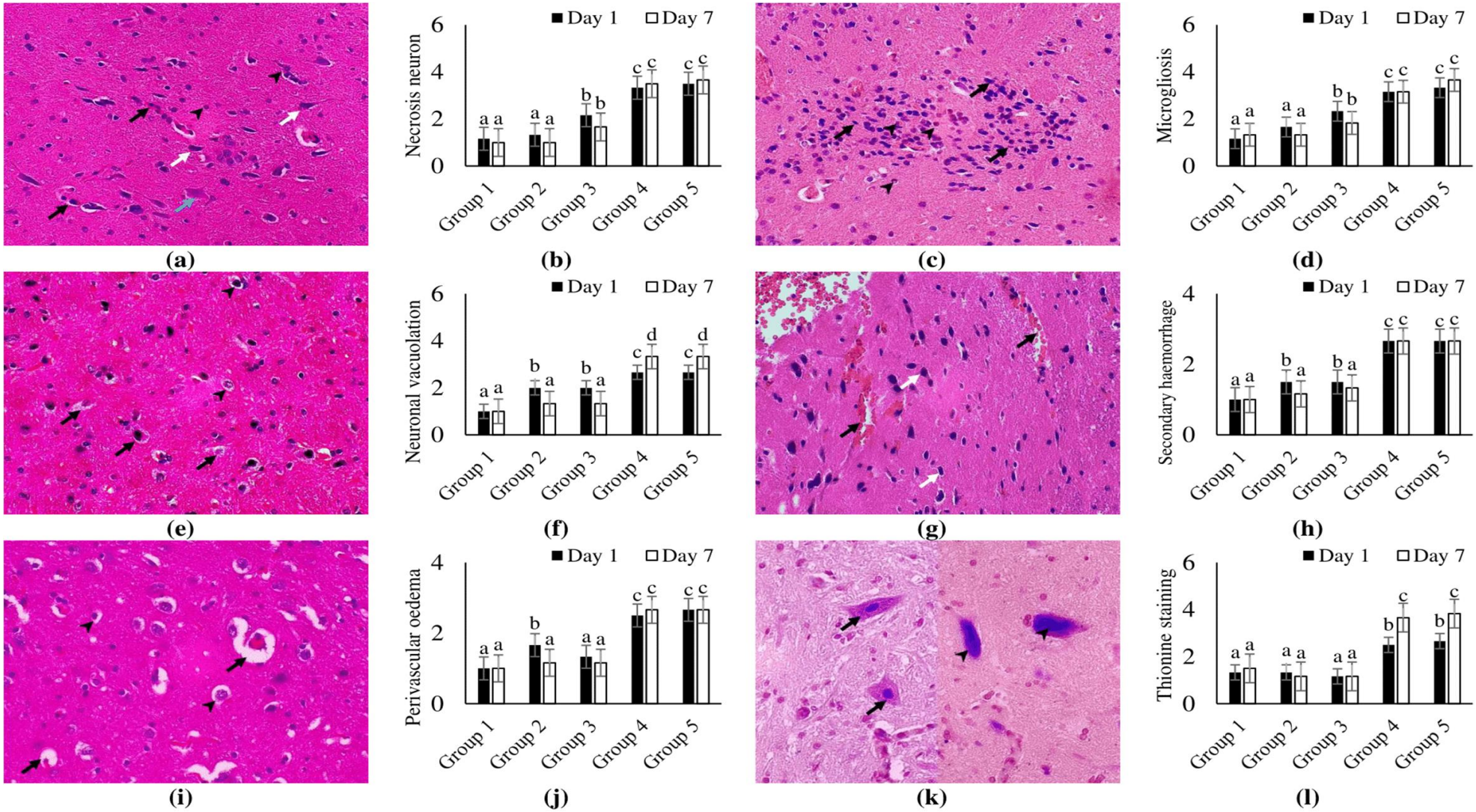

Figure 4. A score of histopathology of the brain after experimentally induced ischemic stroke in rat models. Necrosis neuron that marked by various morphologies, including redness cytoplasm (black arrows), pycnotic nucleus (white arrows), and ghost cell (blue arrow), this area was also surrounded by sateliosis (arrowheads) (**a**), histopathology score of necrosis neuron (**b**), reactive microgliosis (arrows) and glial cells (arrowheads) infiltration within brain parenchyma following the EIIS procedure (**c**), histopathology score of microgliosis (**d**), neuronal vacuolation (arrows) around astroglial (arrowheads) and within the neuronal cytoplasm that indicated by pale cytoplasm (**e**), histopathology score of neuronal vacuolation within the brain parenchyma (**f**), secondary haemorrhage (black arrows) and pycnotic neuron (white arrows) within the infarcted area (**g**), histopathology score of secondary haemorrhage inside the infarcted region (**h**), perivascular edema (arrows) showed the expansion of Virchow Robin space, and its seriously impacts on astroglial (arrowheads) hydropic degeneration (**i**), histopathology score of perivascular edema (**j**), Nissl bodies (arrows) with clear sandy appearance within the neuronal cytoplasm from the control, contrary the infarcted brain showed that the Nissl bodies (arrowheads) disappeared and pycnotic neuron were visible as sharp blue stained (**k**), histopathology score of Nissl bodies using thionine staining (l). $^{a, b, c}$ different superscripts in the same column and row indicated significant differences ($p \leq 0.05$). H&E, 400× **(a, c, e, g, i)**; Thionine staining, 400× (**k**).

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The endothelial cell disruption triggers an increase of pro-inflammatory cytokine and glial cells within the brain that causes an increase in vascularization (Mizuma and Yenari, 2017). Further, increased brain vascularization and blood crossflow during 1 and 2 hours of CCA ligation impact a decreased infarct area. It is supported by a previous study that reported that the blood crossflow after MCAO from the other side of Circle of Willis affects the reduction of infractions (Zarow et al., 1997). Further, another study reported that the blood crossflow and brain preconditioning of rat models could generate ischemic tolerance (IT, Speetzen et al., 2013). The occurrence of IT in this study indicated that noxious stimulus applied to the brain using 1 and 2 hours of CCA ligation induces resistance in brain tissue to prevent severe organ damage. However, did those mechanisms not occur in Groups 4 and 5 with 4 and 8 hours of CCA ligation? The answer is yes. However, longer ligation times in Groups 4 and 5 impact the chaos of the cerebral circulatory system to cover the food and oxygen supplies in brain tissue. It means the infarction within the cerebral tissue cannot be avoided.

Cerebral infractions following 4 and 8 hours of CCA ligation significantly affect the score of general condition, neurodeficit, PWR, NLR, CRP, and De Ritis ratio, compared to the others ($p \leq 0.05$). However, one of the general condition parameters, especially ear position, can be excluded from the observing parameter in EIIS. This parameter does not show any differences in all periods of CCA ligation. The changing of general condition and neurodeficit after EIIS in rat models using 4 and 8 hours of CCA ligation have been observed as the compensatory effects of brain injury, such as necrosis neuronal (Baron et al., 2014), microgliosis, and disappearing of Nissl bodies (Zille et al., 2012). Neurons are the essential cells that integrate all physical activity among living things. Neuron pathological changes cause incoordination of the body movement, as proved by this study. Further, microgliosis during stroke promotes severe neurological defects (Sivadas and Broadie, 2020). Microglia have a significant role, both for inflammatory and non-inflammatory responses. As the inflammatory agent, microglia prevent the spreading of pathogenic agents. However, the microglia act to release inflammatory cytokines that increase synapse loss during ischemic stroke (Colonna and Butovsky, 2017). Furthermore, synapse loss seriously impacts the neurodeficit. It is aggravated by the clumping of the Nissl's bodies. A previous study by Liu et al. (2021) described that ischemic stroke induces an increase in disappearing Nissl's bodies that impacts neurological deficits. In advances, cerebral ischemia influences the local and systemic circulatory system, as proved by brain histopathological changes, including secondary hemorrhage, neuronal vacuolation, and perivascular edema (Chen et al., 2021). The ischemic stroke also consistently impacts the PWR, NLR, CRP, and De Ritis ratio level, which are essential as the ischemic stroke biomarkers (Dagonnier et al., 2021).

CONCLUSION

This study indicated that the simple EIIS procedure using ligation of CCA in 4 and 8 hours is reliable for inducing ischemic stroke in rat models that may represent ischemic stroke in humans. The EIIS using 4 and 8 hours of CCA ligation promotes infarct presentation within the brain that can be observed from day one until day 7. Further, the EIIS using 4 and 8 hours of CCA ligation also changes the score of general condition, neurodeficit, histopathology, thionine staining, PWR, NLR, CRP, and De Ritis ratio. An advanced study must be conducted to elucidate the reproducibility of this method compared to the other methods concomitantly. Moreover, if this method is reproducible, it can be used as a simple method for the pre-clinical study of drugs or therapy for an ischemic stroke in animal models.

DECLARATIONS

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Competing interests
The authors declare that they have no conflict of interest.

Ethical consideration
This paper was written originally by the authors. The authors were not submitting this paper to the other journal or publisher.

Authors' contributions
Prakoso YA was the person in charge of this study. Prakoso YA conceptualized, designed, conducted, monitored, and supervised the research. Sigit M conducted the study and analyzed the statistical data. Aliviameita A conducted the hematological test and interpreted the hematology and serology data. All authors have read and approved the final version of the manuscript for publication in the present journal.

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Availability of data and materials
The data of this study are available upon reasonable request.

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