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Research Article

Routine Blood Profiles of Global Ischemic Rats Based on Ischemia Durations

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Abstract: Stroke is the third cause of death and the first cause of disability in the world. It is around 80% of stroke patients in the world are ischemic stroke. According to the development of stroke models in animals, BCCAO is one technique that can induce global cerebral ischemia. An ischemia is known to influence activities of inflammatory cells which can be measured through peripheral blood. This study aims to determine effects of ischemia duration on routine blood profiles of rats (*Rattus norvegicus*) after bilateral common carotid artery occlusion (BCCAO). This study was a quasi-experimental study. Its subjects were adult Wistar rats (*Rattus norvegicus*). The rats were grouped into four treatment groups, and each group consisted of 6 rats. Group A was a group of sham-operated rats, group B was a group of rats with ischemia duration for 5 minutes, group C was a group of rats with ischemia duration for 10 minutes, and group D was a group of rats with ischemia duration for 20 minutes. Its obtained data were analysed by one-way ANOVA test and Post Hoc Tamhane's test. The ischemia duration significantly influenced the neutrophil and lymphocyte count after ischemia, with $p < 0.05$. The ischemia duration could affect the routine blood profiles of the rats after BCCAO, especially the neutrophil and lymphocyte count.

Keywords: Global ischemic rats, ischemia duration, routine blood profile

Introduction

In the last few decades, the ischemia/reperfusion injury (I/R I) in experimental animals has been widely used to study stroke. Ischemia/reperfusion injury (I/R injury) is a brain injury caused by backflow of blood to areas of the brain experiencing ischemia. The ischemia/reperfusion injury consists of three periods; those are ischemia, reperfusion and repair period (1).

One of I/R I models that induce global brain ischemia is Bilateral Common Carotid Artery Occlusion (BCCAO) by ligating the right and left common carotid arteries. In this technique, there are two durations, namely ischemia duration and reperfusion duration. The ischemia duration is a BCCAO duration which varies from 5 min to 60 min. Then, the reperfusion duration is a time when bonds in the common carotid artery are released until the decapitation of termination of experimental animals is conducted. Variation of the reperfusion starts from 30 min to 10,080 min (2),·(3).

Some studies in Europe and America found a relationship between routine blood profiles and clinical degrees of stroke patients. It is widely known that roles of leukocytes and platelets are very important in determining the area of brain tissue experiencing ischemia after stroke (4). Studies on the leukocyte-platelet relationship in pathogenesis stroke have been widely conducted, but studies on global ischemia duration in acute ischemic stroke model have not been widely elaborated. The mechanism of ischemia/reperfusion injury that occurs during stroke is known to be mediated by various mechanisms of blood cells that result in worsening tissue injury. Therefore, this study aims to determine whether the BCCAO duration can affect the routine blood profiles of the rats after 24 h reperfusion.

Materials and Methods

Materials

This study was a quasi-experimental study using a post-test only control group design. This study obtained ethical clearance with No. 20/Ka.Kom.Et/70/KE/VIII/2017.

This study used healthy male Wistar rats (*Rattus norvegicus*) aged 3-4 months old with body weight 175-250 g. The rats were divided into four groups. Group A was a group of sham-operated rats. Group B, C, and D were a group of ischemic rats induced by BCCAO for 5, 10, and 20 min and reperfusion for 24 h.

Method

The BCCAO duration was the length of BCCAO treatment (5, 10, and 20 min) and the reperfusion for 24 h. BCCAO procedure according to previous research (5). Before the ligation, the rats were anesthetized with kethamine 80-100 mg/kgBW IM. The rats were placed on sterile Digital Jumbo Hotplate (LHT-2030D) platform and the rectal temperature of 37±1 ° C was maintained. Next step was disinfection in order to prevent infection using alcohol and betadine solution in the rats' anterior neck surface. Incision is done vertically in anterior median neck. Neck exploration was done without cutting submandibular gland and phrenic nerve. When the common carotid arteries are seen, the ligation was performed using micro-clamp for artery. After ligation, the rats were given an analgesic therapy (bupivacaine 0.25% 0,1 mL local) once per day, for 3 days (Analgetic suggested for rat stroke model). The incision is sutured with silk thread, while the area nearby the incision placed was disinfected by betadine (5).

Euthanasia was performed after 24 h of transient bilateral common carotid artery ligation. Rats were anesthetized with ketamine 80-100 mg/kgBW IM. Then, a midline incision was made on the abdominal wall. The incision was continued along the axillary line until the thorax wall was opened and the heart was visible. Blood taking of the rats used a 3 cc spuit. The volume of blood taken ranged from 0.5 to 2.5 cc. The blood then was stored in a 3 mL EDTA tube to avoid clotting and was immediately sent to Animal Hospital Prof. Soeparwi, Faculty of Veterinary Medicine, Universitas Gadjah Mada, for routine blood tests.

Analysis of hematology profiles were performed at Animal Hospital Prof. Soeparwi. The results of hematology profiles examined were erythrocytes (millions/UI), haemoglobins (gd/dL), haematocrits (%), platelets (thousands/UI), leukocytes count (thousands/mm^3), including differential white blood cell count such as neutrophils, lymphocytes, and monocytes (entirely in percent %). Then, there was no analysis for basophils and eosinophils due to the absence of their data.

Differences of routine blood profiles between the groups were tested by a statistical analysis of ANOVA. The results of the analysis of the obtained data were significant ($p < 0.05$), and then a post-hoc test was performed to determine which groups showed differences. Before the ANOVA test, a normality test of the data was performed by using the Shapiro-Wilk test.

Result and Discussion

Result

The results of the One-Way ANOVA test showed that the number of Erythrocytes, Hemoglobins, Leukocytes, Haematocrits, Platelets, Neutrophils, Lymphocytes, and Monocytes respectively indicated p values of 0.190; 0.548; 0.201; 0.424; 0.225; 0.000; 0.000; and 0.480 as listed in table 1. Based on these results, the values of both neutrophils and lymphocytes were $p < 0.05$, so it could be concluded that the results were significant, meaning that there was an effect of ischemia duration on the neutrophil and lymphocyte count of the rats after BCCAO.

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Table 1. Hematological Analysis of Rats

Variable	Ischemia Duration (min)	Mean± SD	p Value
Erythrocytes (million /IU)	*Shame operated*	6.68 ± 3.60	0.190
	5	8.29 ±0.20	
	10	7.67 ±0.82	
	20	8.11 ±0.68	
Haemoglobins (gd/dL)	*Shame operated*	12.63 ±4.70	0.548
	5	15.30 ±1.39	
	10	14.64 ±0.93	
	20	14.40 ±1.52	
Leukocytes (thousand/mm^3)	*Shame operated*	1.76 ±0.35	0.201
	5	2.92 ±1.25	
	10	4.01 ±2.37	
	20	4.50 ±2.56	
Haematocrits (%)	*Shame operated*	35.63 ±11.47	0.424
	5	47.15 ±4.66	
	10	46.74 ±2.65	
	20	43.52 ±4.11	
Platelets (thousand /IU)	*Shame operated*	766.00 ±809.13	0.225
	5	309.50 ±379.73	
	10	1462.00 ±620.34	
	20	1311.00 ±128.18	
Neutrophils (%)	*Shame operated*	22.03 ±2.49	0.000*
	5	47.10 ±29.77	
	10	43.92 ±22.26	
	20	80.02 ±2.51	
Lymphocytes (%)	*Shame operated*	72.46 ±3.50	0.000*
	5	47.85 ±31.78	
	10	50.50 ± 23.54	
	20	13.26 ± 2.24	
Monocytes (%)	*Shame operated*	5.50 ±1.18	0.480
	5	5.05 ±2.29	
	10	5.48 ±1.80	
	20	6.72 ±1.01	

* p value < 0.05, significant

Then, a Post Hoc Tamhane's test was conducted to determine the differences between the groups as shown in table 2.

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Table 2. Results of the Post Hoc Tamhane's Test

Dependent variable	Treatment Group (I)	Treatment Group (J)	p Value
Neutrophils	*sham operated*	5 min	0.431
		10 min	0.186
		20 min	0.000*
	5 min	*sham operated*	0.431
		10 min	1.000
		20 min	0.218
	10 min	*sham operated*	0.186
		5 min	1.000
		20 min	0.034*
	20 min	*sham operated*	0.000*
		5 min	0.218
		10 min	0.034*
Lymphocytes	*sham operated*	5 min	0.457
		10 min	0.184
		20 min	0.000*
	5 min	*sham operated*	0.457
		10 min	1.000
		20 min	0.222
	10 min	*sham operated*	0.184
		5 min	1.000
		20 min	0.038*
	20 min	*sham operated*	0.000*
		5 min	0.222
		10 min	0.038*

* p value < 0.05, significant

The results of the Post Hoc Tamhane's test demonstrated significant values in the neutrophil count (Group A and Group D p = 0.000, Group C and Group D p = 0.034) and in the lymphocyte count (Group A and Geoup D p = 0.000, Group C and Group D p = 0.038).

Discussion

The results of this study indicated a significant effect of BCCAO duration on neutrophil and lymphocyte counts in the rats after 24 h reperfusion. The results of this study also demonstrated no significant effect of BCCAO duration on erythrocytes, leukocytes, haemoglobins, haematocrits, platelets, and monocyte counts in the rats after 24 h reperfusion.

Leukocyte Count

The results of this study revealed no significant effect of BCCAO duration on the leukocyte count in the rats after 24 h reperfusion. The results of this study are the same as previous studies. There was no an increase in leukocytes count in ischemic stroke patients (6). The less significant results between the number of leukocytes and the ischemia duration which could be due to differences in the

blood vessel occlusion methods, shorter duration of ischemia and longer reperfusion compared to the previous studies. There was an increase of leukocyte count but still within normal ranges. The increase of the leukocyte count within normal ranges is in accordance with other studies. In a study by Sienel *et al*. (2022) reported that rats induced by medial cerebral artery occlusion (MCAO) for 2 hours with a reperfusion duration of 1 hour showed an increase of leukocytes in capillaries and arteriolar adhesion, but the increase was not significant (7).

The meaninglessness of the leukocyte count in the blood might be due to the migration of leukocytes to the cerebral venules and cerebral tissue. Sienel *et al.* (2022) mentioned that rats induced by MCAO for 2 hours with 1 h reperfusion showed a significant increase of leukocyte and platelet-leukocyte aggregation in cerebral venules, whereas leukocytes in capillaries and arteriolar adhesion tended to increase, but their increase was not significant (7). Another factor might be that the rats experienced stress during treatment as stress could lower the body's defence mechanisms (8).

Neutrophil Count

The results of this study found a significant effect of BCCAO duration on the neutrophil count in the rats after 24 h reperfusion. There was an increase of neutrophils in the 20 min group compared to sham-operated rats and 10 min group.

Neutrophils are the first leukocyte subtype which is recruited into plasma. Neutrophils will increase in some hours to a maximum of 3-4 d from stroke onset and will decrease on the 2nd to 4th d (9–11). When the neutrophils in the blood decreased, neutrophils in the brain tissue increased. The Neutrophils would migrate to the brain parenchyma tissue. In the first 24 hours of ischemia, few neutrophils were found in the parenchyma cortex and striatum of the rat brain (11).

In the incidence of stroke due to blockage of middle cerebral artery (MCA), 60-67% of capillaries were located distal to the Middle Cerebral Artery (MCA). The blockage was caused by the accumulation of neutrophils in the capillary lumen, thereby increasing blood flow barriers in the cerebral cortex area (12). When stroke occurred, the neutrophils would migrate to the brain within a few minutes. The migration was through blood vessels to areas of ischemic brain. The neutrophils would release various types of endothelial adhesion molecules during the first 15 min of stroke. A study using experimental animals stated that neutrophil infiltration occurred on the 1st d of stroke, peaked on the 3rd d, and decreased on the 4th, 5th and 6th d (13). The neutrophils were still detected on the 7th and 15th d after stroke (14). High neutrophil and leukocyte counts were closely related to a larger degree of ischemic tissue volume (15). The neutrophil activity would weaken on the 2nd to 4th day after ischemia and would decrease further (16). Neutrophil infiltration into the area of damaged neurons would occur more rapidly and more intensively in reperfused tissue than in permanent ischemic tissue. Neutrophil activity would weaken on the 2nd to the 4th d after ischemia occurred and would decrease further after that (16). It was reported that netrophilia occurred on the 7th d after unilateral common carotid artery occlusion (UCCAO) (17).

Neutrophils contributes to secretion of toxic substances and other inflammatory mediators. There is a significant positive correlation between increased levels of IL-8 cytokines and the number of neutrophils in acute ischemia stroke patient (18,19). Pathogenesis of stroke ischemia involves inflammatory processes that play a role in brain damage and the process of brain regeneration. This process is characterized by the production and release of cytokine pro inflammatory mediators, such as Tumor Necrosis Factor α (TNF-α), Interleukin 1β (IL-1β), IL-6, IL-8, IL-12, IL-15, IL-16, IL 17 and IL 20 during brain ischemia. IL-6 activates the JAK/STAT pathway to trigger pro-cytokine expressions. IL-12 triggers the expression of chemokines and pro-inflammatory mediators, causing apoptosis, giving a pro-inflammatory effect. Pro inflammatory cytokines can induce the expression of adesi molecules, such as Intercellular Adhesion Molecule-1 (ICAM-1), Vascular Adhesion Molecules (VCAMs), selectins (P-selectin and E-selectin) and integrins (Mac-1, LFA-1) in endothelial cells, Leukocytes and platelets (19–21).

Lymphocyte Count

This study indicated a significant effect of 20 min duration of ischemic on the lymphocyte count in the rats induced by cerebral ischemia. There was a decrease of lymphocyte in the 20 min group compared to sham-operated rats and 10 min group. On the first day of reperfusion there is a decrease in lymphocytes in the 20-minute ischemia group. This is in accordance with previous studies where an increase in lymphocytes occurred on the third to sixth day of ischemia. The increase of lymphocytes occurred on the 3rd to 6th d after ischemia (22). Lymphocyticenia can occur in brain ischemia models induced with unilateral Common Carotid Artery Occlusion (UCCAO) technique for 7 d (17).

Compared to B lymphocytes, T lymphocytes were a centre of the development of the inflammatory process in stroke models (23). The lymphocytes play an active role in the mechanism of brain ischemia. The ischemic brain increases pro-inflammatory IL-18. The function of this compound will affect the polarization of macrophage M2 into M1. The compounds IL 23 and IL 17 increase the expression of Th17 cells and T cells. These conditions will aggravate ischemia (19). T lymphocyte cells cause attachment of platelets and leukocytes to the vascular endothelial wall. Thromboinflamation of the blood vessels activates proinflammatory pathways (13).

Monocyte Counts

This study revealed no significant effect of BCCAO duration on monocyte count in the rats after 24 h reperfusion. This is in line with a study by Kraft *et al.* (2015) reporting that patients diagnosed with acute ischemic stroke did not experience significant changes of monocyte count (24). In contrast to neutrophils, which respond within hours of brain damage, another study found that monocyte activity was seen on the 3rd to 7th d from the ischemia onset (10). In other words, the time of monocyte observation in this study might not have reached an optimal time, and the results could be different if observed again on the 3rd until 7th d.

A study on rats divided monocytes into two types based on chemokine receptors and expression levels of Ly-6C. The first was low Ly-6C having a long-life span and being responsible for patrolling in the vessel lumen and maintaining homeostasis, and the second was high Ly-6C having a short life and playing a role in the inflammatory response; high Ly-6C increased on the 3rd. d after stroke, and low Ly-6C increased on the 6th d (9). The results of previous studies on stroke in rat models presented that monocyte activity after ischemia could be observed as early as 2 h afterward, while its activity in the brain could be seen after 10 h and from 24 to 48 h after damage; macrophage and microglia activity would be seen in all lesions and could be seen in all lesions in 1 week or longer (23).

Erythrocytes, Haemoglobin, Platelets, Haematocrits Count

This study revealed no significant effect of BCCAO duration on erythrocytes, haemoglobin, platelets, and haematocrits count in the rats after 24 h reperfusion. Some studies mention that the number of erythrocytes does not have a major influence on clinical levels of stroke. Erythrocytes count differ in stroke patients. Erythrocytes count may increase, decrease, or normal. Decreased plasma erythrocyte concentrations are known to occur from 48 to 72 h after disease onset. There was a decrease in erythrocytes count in ischemic stroke patients (25). Ischemic stroke patients have normal erythrocytes count (6). Another study said that Erytrocyte Sedimentation Rate (ESR) would increase on the 1th after the ischemia onset on ischemic stroke patients, but not occur in transient ischemic attact (26). However, this will be different if there are abnormalities in the quality of the erythrocytes themselves, which will interfere with oxygen delivery, thereby exacerbating brain ischemia. This risk can happen in certain conditions such as polycythaemia vera, sickle cell anaemia, and paroxysmal nocturnal haemoglobinuria (27). The results of this current study indicated that there was no effect of the number of erythrocytes on the ischemia duration which might be caused by the measurement of the number of erythrocytes in this study performed at 24 h after ischemia; the results could be different or could be significant if the measurements were at 48 h or even 7 d after ischemia.

In this study, the statistical test of haemoglobin of the rats on the ischemia duration showed no significant results, and this is in accordance with a cohort study showing no relationship between

haemoglobin and stroke in male patients. In ischemic stroke patients there are 79.3% of patients have normal haemoglobin levels. Only 17.9% of patients have anaemia (6,28). The results of this study are also different from other studies, where there is anemia in rats induced brain ischemia with the Unilateral Common Carotid Artery Occlusion technique for 7 days (17). There was a decrease in haemoglobins count in ischemic stroke patients (25,29).

Haemoglobin is a component that binds oxygen and carries it to tissues including the brain during stroke. Low haemoglobin levels will decrease the brain oxygenase process, so this can cause acidotic conditions that can result in cerebral edema and microcirculation. This is because there are an increase in vascular resistance and a decrease in perfusion pressure that will affect the expansion of the ischemic area, and high haemoglobin is associated with increased blood viscosity which will affect cerebral blood flow and increase the incidence of cerebral atherosclerosis which may be a risk factor of ischemic stroke, but this is still very rare and inconclusive (30).

This study revealed no significant effect of BCCAO duration on haematocrits count in the rats after 24 h reperfusion. The haematocrit counts are very dynamic because they are influenced by an individual's hemodynamic conditions; stroke subtypes may also affect the relationship between first haematocrit count and stroke outcomes. Other studies reported no consistent relationship between haematrocits and stroke in male patients (31). A study found the relationship between higher and lower haematocrit count in patients with ischemic stroke (32). The haematocrits count were significantly higher in ischemic stroke patients (25).

In this study, There was no difference in platelet count between groups.. The results of this study are different from other studies. Induction of ischemic stroke by 7 d of UCCAO will cause thrombocytopenia (17). Platelets count increased in ischemic stroke patients (25). Platelets have a synergistic role with leukocytes in reperfusion injury. Platelet activation and platelet-leukocyte aggregation in acute and chronic stroke phases can produce brain microcirculation disorders which will contribute to injury and tissue repair mechanisms. The interaction between platelets and leukocytes via selectin-P/selectin P GP-ligand 1 also induces the release of inflammatory cytokines (33).

Conclusion

This study concluded that the 20 min duration of BCCAO (bilateral common carotid artery occlusion) could affect the neutrophil and lymphocyte count of the rats after 24 h reperfusion.

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