Original article:

Effect of BCCAO Duration and Animal Models Sex on Brain Ischemic Volume After 24 Hours Reperfusion

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Abstract:

Background: Literature study shows, there are several variations regarding BCCAO duration and duration of reperfusion after BCCAO that can cause cerebral ischemia. Duration of BCCAO techniques varies between 10 to 30 minutes, while the duration of reperfusion period ranging between 45 minutes to 72 hours. Differences in the duration of occlusion, duration of BCCAO reperfusion and the sex of animal model could lead to different responses to ischemia conditions. **Objective**. This study aims to determine whether the duration BCCAO and sex of the animal models influences the volume of cerebral ischemia after 24 hours reperfusion. ***Method:*** This study uses *post-test only study group* design. The subjects are 20 female and 20 male Wistar rat that being divided into 8 groups which are male rat with occlusion duration of 5 minutes, 10 minutes and 20 minute, also the female rat with occlusion duration of 5 minutes, 10 minutes, 20 minutes respectively. BCCAO occlusion then followed by 24 hour reperfusion. Rat decapitation and brain extraction are done after reperfusion. Brain tissue sliced into 2 mm size and stained with 0.05% TTC solution for 30 minutes. Ischemic brain volume are being observed using Cavalieri method. Statistical data are being analyzed using One Way Anova. ***Result:*** There are significant difference in male rat cerebral ischemia volume between 5 minutes and 10 minutes occlusion ($p<0.006$). Meanwhile, there are no significant difference at cerebral ischemia volume between 10 and 20 minutes occlusion group ($p=0.377$). There are significant differences in the volume of brain ischemia between the 5, 10 and 20 minutes ischemia group ($p<0.05$). Post-hoc test showed no significant differences between the male and female rat ($p>0.05$). ***Conclusion:*** Duration of the bilateral common carotid artery occlusion for 5 and 10 minutes affect the volume of cerebral ischemia in male rat after 24 hour reperfusion. The occlusion of bilateral common carotid artery for 5,10 and 20 minutes also affect the volume of cerebral ischemia in female rat after 24 hour reperfusion. No significant differences of cerebral ischemia volume between the sexes after 5, 10 and 20 minutes occlusion.

Keyword. BCCAO occlusion duration; Animal models sexes; Rat cerebral ischemia volume

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Introduction

Stroke is one of the deadliest disease. Reduction of cerebral bloodflow resulted in ischemia of the brain. Since the discovery of animal models of stroke, the research on brain ischemia grows rapidly. This technique induces ischemia in animal model's brain. One of the technique commonly used is bilateral common carotid artery occlusion (BCCAO). Nandagopal et al (2010), states that the BCCAO techniques can be used to study the mechanism of

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neuroprotectant drugs. This model could identify mechanism of brain tissue injury as well as the basis of the development of stroke therapy in pre-clinical level.[1,2].

BCCAO technique performed by ligating bilateral common carotid artery of rat for a few minutes, followed by the release of the ligature (reperfusion period). This ligating and reperfusion activity of the artery causes damage to rat's brain cells. Literature study shows, there are several variations regarding BCCAO duration and duration of reperfusion after BCCAO that can cause cerebral ischemia. Duration of BCCAO techniques varies between 10 to 30 minutes, while the duration of reperfusion period ranging between 45 minutes to 72 hours. Differences in the duration of occlusion, duration of reperfusion BCCAO and the sex of animal model could lead to different responses to ischemia conditions[2-9].

Ischemic brain region can be observed both macroscopically and microscopically. TTC staining technique can distinguish between the healthy and ischemic brain tissue. This is because TTC staining can detect the presence of lactate dehydrogenase enzyme (LDH) which makes the healthy tissue to appear red. Meanwhile, ischemic tissue will have a reduced amount of LDH enzyme so it will appear white on macroscopic observation[4,6,10,9]. In addition to TTC, ischemic tissue can be observed by Haematoxyline Eosin (HE) and Toulidin Blue (TB) staining[3]. One study proves there is no significant difference when observing ischemic tissue using TTC staining and HE staining. TTC staining is an inexpensive yet accurate method to asses ischemic cells[11,12].

Literature study showed that there are variation of animal models that being used on BCCAO experimental studies, among others are Wistar rat, Spraque-Dawley rat and Mongolian gerbils. These differences of animal models strain will provide different results[13-20]. Genetic differences on animal models experiment will affect the outcome, one example is the *neonatal mouse model of hypoxia-ischemia* which shows there are variation between strains[21]. In addition to strain differences, animal models sexes also lead to differences in research result. An in vitro study showed that the neurons of the female subject is more resistant to ischemia than the male subject[22]. Instead, Sanches *et al*. (2013) found that 3 days old male rat were more resistant to hypoxia and ischemia condition[23].

Variations on BCCAO duration, reperfusion duration, strain and animal models sex could lead to different responses to ischemia condition. That variation shows lack of standardization in BCCAO technique which can lead to difficulties for researchers who want to use BCCAO techniques for developing the theory and therapy of stroke. Besides the use of BCCAO techniques with TTC staining to assess cerebral ischemia is still rare in Indonesia, so Indonesia does not have any technical standard on BCCAO model experiment. With that in mind, we wanted to develop BCCAO techniques with variation in duration and sex of the animal models from the same strain, so we can standardize the BCCAO duration and post BCCAO ischemic area mapping.

Method

Research design

This research applied quasi experimental design using post test for the control group. The study was conducted from November until December 2016 at the Pharmacy Laboratory, Islamic University of Indonesia.

Animal and experimental procedure

The animal used in this study were 20 male rats and 20 female rats (*Rattus norvegicus* of the wistar strain) that had met the inclusion and exclusion criteria. The rats were reared in the Pharmacy Laboratorium, Inslamic University of Indonesia. Inclusion criteria for this study were helthy 3-month old male rats without any defect, of 175-250 g body weight. Determination of healthy rats was based on the physical state of the rats, i.e. those with clean, not wet or sticky bristles, active movements, and appropriate cycle of eating, drinking and sleeping. Exclusion criteria of this research were sick and dying rats during the study.

During the 1st day until the 7th day, the experimental animals were located in cages for adaptation (40x20x20) cm^3 . One cage was filled by 1 rat. The inside temperature was set at room temperature. Lighting was arranged with light-dark cycle for 12 hours. Light cycle was began at 06.00 am and dark cycle was started at 06:00 pm. Pellets were given every day in the morning at 06.00 am. Drinking water was provided ad libitum.

Subjects were divided into eight groups, of which each consisted of 5 rats. The description of the group are as follows:1. Group 1 was sham operated male rats (the same operation without BCCAO), group 2 was 5-minute BCCAO male rats, group 3 was 10-minute BCCAO male rats, group 4 was 20-minute BCCAO male rats., group 5 was sham operated female rats, group 6 was 5-minute BCCAO female rats, group 7 was 10-minute BCCAO female rats, group 8 was

20-minute BCCAO female rats. Brain ischemia was produced by 5, 10 and 20-minute bilateral carotid communis artery occlusion (BCCAO), continued with 24 hour of reperfusion.

BCCAO procedure

BCCAO was performed on the 8th day. Stages occlusion is as follows: a. Anesthesia. During surgery, anaesthetize the rats using 80-100 mg / kg im ketamine. The rat is placed in a sterile heat platform (HeatPlate Lab Tech) and keep the rat rectal temperature at 37 ± 1 ° C. b. Disinfection stage. This stage aims to prevent infection. Swipe surgical are with betadine from center of surgical site to outside (anterior surface of the rat neck). c. Incision stage. Open the anterior neck with midline vertical incision. Dissect the underlying submandibular gland. Dissect the medial of right sternocleidomastoid muscle to expose the common carotid artery (CCA). Separated the arteries carefully from the vagus nerve and connective tissue. d. Occlusion stage. Use a vascular micro klem (Serrefine Small Curved. Q1Y:01No) to make 5, 10, and 20-minute bilateral carotid artery occlusion . e. After occlusion is complete then given analgesic therapy ie 0,1 mL 0.25% bupivacaine, frequency of one time / day (analgesic recommended for rat stroke model).

Preparation of the brain

The rat brain tissue were taken at day 9, twenthy four hours post BCCAO. Decapitation of rats were performed with a trancardial perfusion technique. Brain tissues were stained with TTC (2,3,5-triphenyltetrazolium chloride) (Sigma Aldrich Catalog T8877-10G). TTC staining procedure is as follow : a. Make 0.05% TTC in 1x PBS, b. Cut the brain coronally plane at 2 mm thickness, c. Incubate the sliced brain in 0,05% TTC in the black boxes for 30 min, d. Carefully aspirate TTC solution and add fresh 10% PFA solution. TTC solution should be protect from light and kept in Room temperature. The ischemia volume of rats brain that were studied with Cavalieri method [24,25].

Statistical analysis

Ischemic area was analyzed using Cavalieri Method. Statistical analysis was performed by using One Way ANOVA test .

Ethical clearance

This study was reviewed by the ethical clearance committee for preclinical research, Faculty of Medicine, Islamic University of Indonesia.

Result

This study has been approved from UII Faculty of Medicine Ethical Committee. Registry Number: 29/Ka.Kom.Et/70/KE/V/2016. This study uses 40 rats, 20 male and 20 female rat of Wistar strain which has fulfilled the inclusion and exclusion criteria.

Animal models mortality rate and routine blood profile

Some supporting data such as mortality rate and routine blood profile results are presented in this study (Tabel 1).

TTC staining description after BCCAO

Rat decapitation performed after 24 hours post-ligation. After that, the cerebral extraction and TTC staining are being done. TTC staining observation results of each group are shown in Fig. 1

Ischemic brain volume analysis using Cavalieri method

With the TTC staining, ischemic areas of the brain are less stained, whereas non-ischemic area appear red in color. The stereology principle by Cavalieri method is used to analyze ischemic cerebral volume in each group. Result of ischemic volume are being measured in mm^3 units. TTC staining photograph was observed using Microsoft Word with pointed grid. Brain slice thickness (t) is 2 mm, while the distance between the grid point is 2 mm. The area represented by a single point (a/p) is by multiplying 2mm x 2mm, so the value of a/p equal to 4 mm^2. Number of point which fall in the ischemic area expressed in $\sum P$. Ischemic brain volume of each sample was measured using formula $V=t.(a/p).\sum P$. Volume calculation processing efficiency in this study was expressed in Coefficient Error (CE). CE formula is Noise = S2, while S2 formula is 0.0724 x shapefactor x(root of n x $\sum P$). n (number of slice). CE value in this study was 0.1 (Table 2).

Mean ischemic percentage results based on occlusion duration and animal models sexes

Analysis shows there are variation of cerebral ischemia mean volume between group A (12.8 mm^3), group B (120.0 mm^3), group C (214.4 mm^3), group D (243.2 mm^3), group E (14.4 mm^3), group F (120 mm^3), group G (225 mm^3) and group H (294.4 mm^3) (*p value* 0,000) (Tab 3).

Post-hoc test showed there were significant differences in cerebral ischemia volume in male rat between 5 minute occlusion group and 10 minute occlusion group. While there were no significant differences between the 10 minute occlusion and 20 minute occlusion male rat groups (Table 3). Post-hoc test in female rate showed significantly different cerebral ischemia volume between the 5, 10, and 20 minutes ischemia groups (Table 3). This study also reveals there are no significant cerebral ischemia volume differences between sexes (Table 4).

Discussion

Effect of ischemia duration to ischemic brain volume

Transient bilateral common carotid artery occlusion (BCCAO) can cause cerebral ischemia. Study that induce cerebral ischemia in rat using BCCAO varied in duration, ranging from 5 minutes to 30 minutes followed by reperfusion for 60 minutes to 10080 minutes. This study induces transient cerebral ischemia in Wisat rat using BCCAO technique with duration ranging from 5 minutes, 10 minutes and 20 minutes, followed by 24 hours of reperfusion.

Induction of ischemia in this study leads to ischemia in some areas of the brain depending on the duration. Ischemic area that being observed in Wistar rat's brain among others are the forebrain, striatum, hippocampus and cortex. This study shows that Wistar rat brain can have an ischemia in cortex area 24 hours after reperfusion.

Striatum and cortex ischemia has been seen after 5 minutes of ischemia induction with 24 hours reperfusion. Shorter duration of ischemia followed by longer reperfusion can lead to striatum and cortex damage. This results differ from research by Lapi et al. (2012), which shows striatum ischemia after 30 minutes of induction followed by 60 minute reperfusion in Wistar rat.[4]. Thirty minutes ischemia induction which followed by 60 minutes reperfusion on SD strain animal models show damage in form of necrosis and hemorrhage on striatum and cortex area[26].

In this study, minimal hippocampus damage was found after 5 minutes of ischemia. Indicating that the hippocampus can be induced to ischemia by occluding the bilateral common carotid artery for 5 minutes followed with 24 hours reperfusion. This result differ from previous studies in which the damage occurs in animal models hippocampus after 5 minutes ischemia and 4320 minutes (3 days) reperfusion[10]. Furthermore, damage of the hippocampus can also be induced by 30 minutes ischemia followed by 60 minutes reperfusion in rats, which will show edema, necrosis and neutrophils infiltration to the rat's hippocampus[27].

In general, on all kind of animal models such as mice and rats, the brain structure that can be damaged after transient BCCAO are striatum, hippocampus, cortex, caudoputamen, thalamus, cerebellum, brain stain, white matter, corpus callosum and the internal capsule. Occlusion duration that can induce damage to each of these brain structures still vary. TBCCAO duration of 8 to 30 minutes followed by 60 to 10,080 minutes reperfusion can damage striatum [4,26,10,28-30]. TBCCAO for 10 to 17 minutes followed by 1,440 to 10,080 minutes reperfusion can lead to a damaged hippocampus[30,31]. TBCCAO for 10 minutes followed by 2,880 minutes reperfusion will damage caudoputamen[32]. TBCCAO for 10 to 30 minutes followed by 60 to 10,080 minutes of reperfusion can damage the cerebral cortex[26,29,31,32]. While 14 minutes of TBCCAO followed by 1,440 minutes reperfusion will damage thalamus[29]. Only one study that assess mice hippocampus and cortex volume post transient bilateral common carotid artery ligation[31].

TTC staining method can be used to observe tissue ischemia macroscopically. The coloring principle is to detect the presence of LDH in the brain tissue. Bilateral carotid artery ligation leads to cerebral ischemia, cells that undergone necrosis will swell, while intracellular organelle and plasma membrane will break, releasing some enzyme toward the plasma, one of which is Lactate Dehydrogenase (LDH) This enzyme is more sensitive to describe cerebral ischemic events and can be used to assess the incidence of stroke[33-35].

There are variations to the enzyme level enhancement time. Lampl *et al*. (1990) states that the enhancement of the enzyme level varies from 8 hours to several days after the onset of stroke. Lactate dehydrogenase level in brain tissue will peak at 48-120 hours post stroke. In the first hours of stroke there is a difference between the levels of LDH at the cortex and the subcortical area (34).

Shcherback *et al* (2013) shows LDH activity in Mongolian gerbils hippocampus and II, III, V layer of the cortex after 7 minutes of ischemia. LDH levels fluctuate from 7th minute of ligation and decrease in 2 hour post ischemia, LDH level will be back to normal 7 days after ischemia (reperfusion period)[36].

Ischemic tissue will experience a decrease of LDH enzyme inside their cell cytoplasm which will be observed as pale color under TTC staining. Brain tissue are sliced using vibratome (Campden Instrument, 752M), at 1 mm thickness. These slices then incubated in 2% TTC for 20 minutes at 37^{o}C temperature, followed by incubation inside 10% formalin for 1 night. The same technique are being used by Hussen & Shaheed (2015) to assess ischemic cerebral area[26]. Striatum area ischemia can also be observed after 30 minutes of 1% TTC staining[10].

Based on several studies before there are variation on TTC staining techniques, TTC liquid concentration (1% and 2%), and also the duration of tissue incubation inside TTC liquid (20 minutes and 30 minutes) Most

optimal TTC solution concentration are at 0.05% in brain tissue post MCAO technique and followed by 30 minutes incubation. The concentration of 0.05% TTC on PBS solution is able to distinguish between ischemic and non-ischemic area more clearly than the 1% and 0.1% concentration[37].

This study uses TTC solution at 0.05% concentration. Brain tissue is being incubated in TTC solution for 30 minutes at 37^{O}C. TTC staining on brain tissue result show variation of color. Healthy brain tissue will appear red, while the unhealthy tissue will appear paler than the healthy tissue.

Cerebral ischemia volume measurements at this study are being done using the Cavalieri method. Stereology is a non-bias quantification method that being used as a histopathology parameter. This method had a great accuracy and precision to measure the volume, surface area, length and particle count[38-40]. Several studies have not been using this method to quantify the observed result, instead this studies analyze the observed necrosis area using Image Analysis Software such as Image-Pro Plus[4] and Digimizer[26].

Effect of animal models sexes to ischemic brain volume

Neuroprotectant preclinical studies should consider animal models sexes, this is because lots of empirical data and literature show the effect of gender on stroke incidence. Empirical data show that the incidence of stroke is influenced by gender, which means that men experienced more strokes than women. Research shows that there is less brain damage due to stroke in women. Steroid Hormone will protect the female brain[41].

An in vitro study showed that the neurons of the female subject is more resistant to ischemia when compared with male subjects[19]. Instead, Sanches et al. (2013) found that 3 days old male mice were more resistant to conditions of hypoxia and ischemia[20].

The results of this study differ from previous studies which found no difference in ischemic volume between male and female rats.

Conclusion

Duration of the bilateral common carotid artery occlusion for 5 and 10 minutes affect the volume of cerebral ischemia in male rat after 24 hour reperfusion. The occlusion of bilateral common carotid artery for 5,10 and 20 minutes also affect the volume of cerebral ischemia in female rat after 24 hour reperfusion. No significant differences of cerebral ischemia volume between the sexes after 5, 10 and 20 minutes occlusion.

Conflict of interest

Conflict of interests: No Relevant disclosures

Acknowledgement

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Picture 1. TTC staining result. A. Sham operated male rats group (the same operation without BCCAO), B-B1. 5-minute BCCAO male rats group, C. 10-minute BCCAO male rats group, D. 20-minute BCCAO male rats group, E. sham operated female rats group, F. 5-minute BCCAO female rats group, G-G1. 10-minute BCCAO female rats group, H. 20-minute BCCAO female rats group

Table 1. Mortality Rate and Routine Blood Profile after 24 hours Reperfusion

Group	A	B	C	D	E	F	G	H
Mortality rate (%)	0	36	36	16	0	0	45	28
Hb (gr/dL)	13,64	16,52	13,516	14,4	16	13,5	13,7	14,12
Hmt (%)	40,16	5056	43,18	43,52	46,46	39,825	42,2	43,3
AL (thousand/ mm^3)	2,54	5,58	4,36	4,5	2,41	3,7	2,85	3,36
AT (thousand/UI)	77,84	1235,2	1235,2	1311	1592	893,125	1539,5	1150,6
AE (million/UI)	7,05	8,56	7,051	7,45	8,26	6,591	7,255	7,45

Table 2. CE Measurement

C E componen	Noise/ S2	VARSURS	Total Var	CE
value	4,658	1,1543	5,81235	0.1

Table 3. Anova Analytical Study

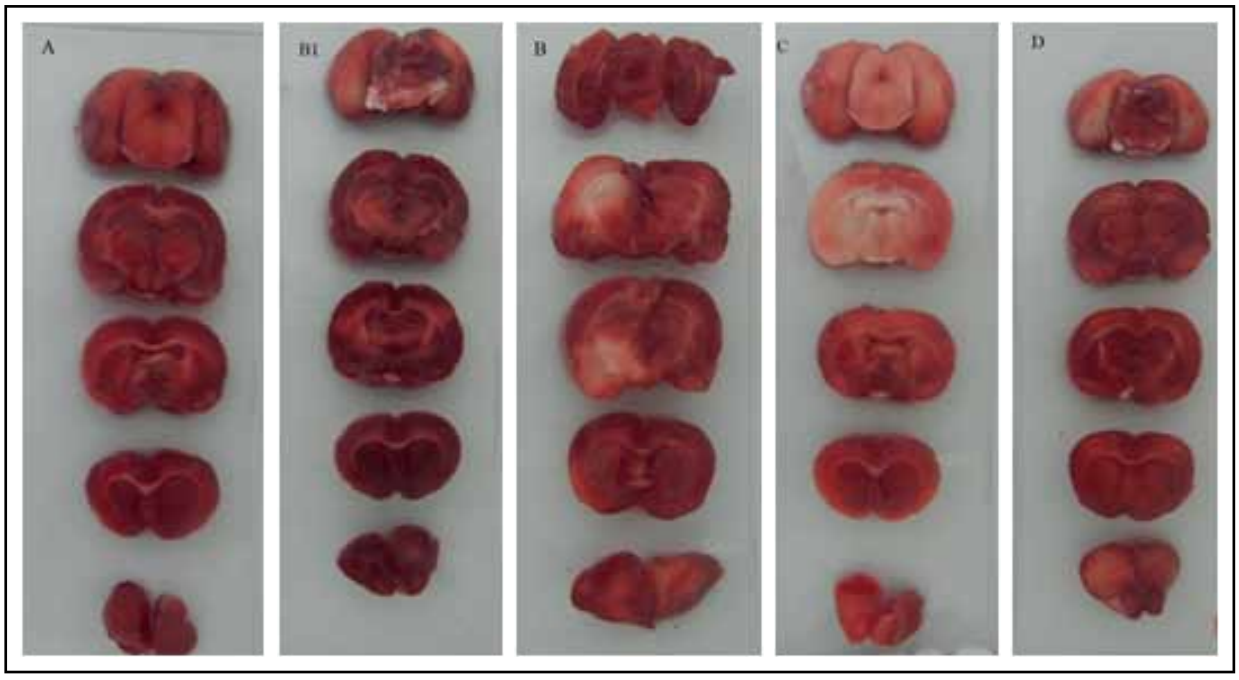
Group	Mean (mm^3)	SD	P Value ANOVA
Sham male	12.8000	16.58915	0,000*
5 minute	120.0000	37.52333	
10 minute	214.4000	94.72486	
20 minute	243.2000	75.81029	
Sham female	14.4000	19.91984	
5 minute	120.0000	28.84441	
10 minute	225.6000	35.50775	
20 minute	294.4000	41.72290	

Table 4. *Post Hoc* test on Rat Cerebral Ischemia Volume after 24 hours Reperfusion

Male group		Sig.	Female group		Sig.
sham	5 minute	.002*	Sham	5 minute	.002*
	10 minute	.000*		10 minute	.000*
	20 minute	.000*		20 minute	.000*
5 minute	sham	.002*	5 minute	Sham	.002*
	10 minute	.006*		10 minute	.002*
	20 minute	.001*		20 minute	.000*
10 minute	Sham	.000*	10 minute	sham	.000*
	5 minute	.006*		5 minute	.002*
	20 minute	.377		20 minute	.040*
20 minute	sham	.000*	20 minute	Sham	.000*
	5 minute	.001*		5 minute	.000*
	20 minute	.377		10 minute	.040*

Table 5. Post Hoc test Based on Sexes

	Sham (mm^3)	p	5 minute (mm^3)	p	10 minute (mm^3)	p	20 minute (mm^3)	p
Male	12.8	.961	120	1.000	214.4	0.730	243.2	0.121
Female	14.4		120		225.6		294.4	



Picture 1. TTC staining result. A. Sham operated male rats group (the same operation without BCCAO), B-B1. 5-minute BCCAO male rats group, C. 10-minute BCCAO male rats group, D. 20-minute BCCAO male rats group, E. sham operated female rats group, F. 5-minute BCCAO female rats group, G-G1. 10-minute BCCAO female rats group, H. 20-minute BCCAO female rats group

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