***Original article:***

# Transient Bilateral Common Carotid Artery Occlusion (tBCCAO) of Rats as a Model of Global Cerebral Ischemia

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**Abstract:**

***Background****:* Transient bilateral common carotid artery occlusion (tBCCAO) has been performed in rats as a model of global ischemia. However, the technique varied between laboratories and produced difficulties in the comparison of results. Variations such as rat strain, age, ischemic and reperfusion duration could affect the results. This review aims to provide a general overview of the variation of animal strains, duration of tBCCAO, reported cerebral ischemic area produced by tBCCAO, use of TTC staining for measurement of volume of brain ischemia and functional neurological tests. ***Method:*** The data of this review were obtained from abstracts in PubMed database and Google Scholars and were not limited by publication time. Keywords used to search the abstracts were (BCCAO OR "*bilateral common carotid artery occlusion*" OR "*stroke*" OR "*cerebral ischemia*" OR "*brain ischemia*") AND (*rat* OR *rats*). The research method of each study was identified from the collected abstracts. The abstracts were chosen for further study on the basis that they met the inclusion criteria which were English language articles; original research article; animal model used were adolescent, adult, and elderly rats; ischemic finding in rats' cerebrum by BCCAO technique was presented; ischemic size were assessed and the result was described; studies that had control group; and studies that induced transient global ischemic to the rats' cerebrum. Data that were extracted to the datasheet were references; animal model strain; ischemic duration; reperfusion duration; ischemic area; 2,3,5 Triphenyltetrazolium Chloride (TTC) staining; Cavalieri method; and rats' neurological functional tests. ***Results and Conclusions:*** There were differences in the ischemic area between Wistar and Sprague-Dawley rats after transient BCCAO. There were differences in the TTC staining solution concentration that was used to identify ischemic area of the brain following transient BCCAO. There was a very limited number of studies using Cavalieri method for the quantification of ischemic volume of rats' brain after transient BCCAO. Neurological functional tests in animal models post transient BCCAO did not include sensory and memory functions tests.

**Keywords:** tBCCAO, rats; global cerebral ischemia

*Bangladesh Journal of Medical Science Vol. 18 No. 03 July'19. Page : 491-500*
*DOI: https://doi.org/10.3329/bjms.v18i3.41616*

***Background***

Translational neuroscience may advance through the application of results of pre-clinical study on clinical studies. However, this advancement faces obstacles in the forms of the discrepancies in the results of studies using animals and humans. Thus, the results of neuroprotection medicine research which use animal model could not be fully implemented in humans. This problem might be derived from the diversity of pre-clinical parameter preferences and

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broad variation of results of pre-clinical study.

A good experimental animal model is important to support the success of research on disease pathogenesis and drug development, including research in the field of neurological diseases. However, several systematic reviews suggest that the successful use of drugs for nervous system protection in the experimental model was not confirmed by the stage III of clinical trials[1-3]. Of 700 types of drugs used in the brain ischemia model, only recombinant tissue plasminogen activator (rt-PA) and aspirin have efficacy[4,5]. The lack of correlation between the results of tests in animal models and in patients underscores the necessity of developing a brain ischemia model in experimental animals that can mimick humans ischemia condition[5-7].

The current models of experimental animal of brain ischemia are focal cerebral ischemia with Middle Carotid Artery Occlusion (MCAO) technique and global cerebral ischemia with Bilateral Common Carotid Artery Occlusion (BCCAO) technique. Meta-analysis study shows that the selection of animal model strain, anesthetic technique, and MCAO technique are the most influencing factors on study results validation[1]. The variance of animal model strains (Sprague-Dawley or Wistar rat) has to be noticed when a researcher investigates new substances/drugs using the MCAO technique[8]. Several studies have demonstrated that the rats strain affect the study results. There is a difference in response of neurogenesis of hippocampus dentate gyrus between Sprague-Dawley (SD) and Lister Hooded (LH) rats[9]. Furthermore, the post-adrenalectomy response of dopaminergic mesencephalon system is different between DBA/2 and C57BL/6 mice[10]. There were also differences in the feedback of brain corticosterone in the tissue regeneration process between MRL/MpJ and C57Bl/6J strains[11], as well as in the differentiation process and cell proliferation of the hippocampus dentate gyrus between C57BL/6N (susceptible to obesity) and C3H/HeN (resistant to obesity) strains[12]. Thus, the proliferation of neuronal progenitor cells is affected by the strains of animal model[13]. Animal model response to neonatal hypoxia is influenced by strains (for example, C57BL/6, 129SVJ, BALB/c, CD1 and FVB strains)[14]. Convulsion response is also affected by the strain of rats[15]. In depression study, a stark contrast response has been found between Wistar-Kyoto (WKY) rats and Sprague-Dawley (SD) as well as Wistar (WIS) rats. There are dissimilarities of dopamine (DA), serotonin (5-HT), and norepinephrine (NE) transporter site densities between WKY, SD, and WIS rats[16].

In BCCAO procedure (as a model for global transient brain ischemia), there are variations in the duration of ischemia, duration of reperfusion, damaged brain area, age of experimental animals, concomitant conditions in ischemic models, experimental neurological functional tests, and techniques for assessing volume of ischemic animals. The duration of ischemia is the length of time of the bilateral ligation of the common carotid artery, while the duration of reperfusion is the period between the release of the ligation until the termination of the experimental animal.

The current review aims to provide an overview of the variation of the tBCCAO procedures and the reported cerebral ischemic area produced by the procedures. Furthermore, the quantification of the ischemic volume and neurological functional test of the rat model are evaluated.

## Method

The data of this systematic review were obtained from abstracts in PubMed database, Google Scholars, and were not limited by publication time. Keywords used to search the abstracts were (BCCAO OR "*bilateral common carotid artery occlusion*" OR "*stroke*" OR "*cerebral ischemia*" OR "*brain ischemia*") AND (*rat* OR *rats*).

The research method of each study was identified from the collected abstracts. The abstracts were chosen for further study on the basis that they met the inclusion criteria which were English language articles; original research article; animal model used were adolescent, adult, and elderly rats; ischemic finding in rats' cerebrum by BCCAO technique was presented; ischemic size were assessed and the result was described; studies that had control group; and studies that induced transient global ischemic to the rat' cerebrum. Data that were extracted to the datasheet were references; animal model strain; ischemic duration; reperfusion duration; ischemic area; 2,3,5 Triphenyltetrazolium Chloride (TTC) staining; Cavalieri method; and rat' neurological functional test (Tables 1 and 2).

***Ethical approval***: The study was approved by ethics Committee of this Institution

## Results

The literature search found 5 articles which were identified for the strains of animal model, BCCAO duration, ischemic area, TTC staining method, and volume measurement using Cavalieri method (Table 1). The results of neurological function article search is shown in Table 2.

Table 1. Animal model strain to BCCAO duration, ischemic area, TTC staining and Cavalieri method.

| No | References | Strain | Ischemic duration (minutes) | Reperfusion duration (minutes) | Ischemic area | TTC | Cavalieri method |
|---|---|---|---|---|---|---|---|
| 1 | Lapi et al. (2012). | Wistar | 30 | 60 | Striatum | Yes | No |
| 2 | Hussein and Shaheed (2015). | SD | 30 | 60 | Cortex Striatum | Yes | No |
| 3 | Barbhuiya et al. (2015). | Albino rat | 15 | 4320 | Striatum | Yes | No |
| 4 | Iwasaki et al. (1995). | SD Wistar | 5 | 10080 | CA1 hippocampus | No | No |
| 5 | Handayani et al. (2018) | Wistar | 5,10,20 | 1440 | Cortex, Striatum Hippocampus | Yes | Yes |

**Table 2. Neurological function test in BCCAO transient model rats.**

| No | References | Ischemic duration (minutes) | Reperfusion duration (minutes) | Ischemic area | Motoric test | Sensory test | Memory test |
|---|---|---|---|---|---|---|---|
| 1 | Lapi et al. (2012). | 30 | 60 | Striatum | No | No | No |
| 2 | Hussein and Shaheed (2015). | 30 | 60 | Striatum, Cortex | No | No | No |
| 3 | Barbhuiya et al. (2015). | 15 | 4320 | Striatum | Torso Twisting Circling Rotarod | No | No |
| 4 | Iwasaki et al. (1995). | 5 | 10080 | CA1 hippocampus | No | No | No |

## Discussion

Transient BCCAO (tBCCAO) ligation technique could cause cerebral ischemia[17]. In the studies examined, transient BCCAO technique was performed for five minutes to half an hour and followed by reperfusion for one hour to seventh days.

According to Lapi et al. (2012), and Hussein and Shaheed (2015), an ischemic induction for 30 minutes followed by a reperfusion period for 60 minutes caused an ischemic area of striatum and cerebral cortex in the rats' brain. Lesion in the striatum could be seen in both WIS and SD rats, while lesion in the cerebral cortex could be seen in only in SD rats. A striatum ischemic condition was represented by a clear lesion in the striatum, a decrease in the striatal neuronal number, and pale areas in the Wistar rats striatum post TTC staining[18]. A similar ischemic induction conducted on SD rats has shown necrotic and hemorrhagic areas in the striatum as well as the cerebral cortex. Additionally, TTC staining showed pale areas in the striatum and the cerebral cortex of the SD rats[19]. The necrotic area sites and their extensions in each section were evaluated using an image analysis software (Image-Pro Plus)[18] and Digimizer[19]. Lapi et al. (2012) and Hussen et al.(2015) did not measure the ischemic volume of the rats' brain.

Barbhuiya et al. (2015) argued that rats striatum damage could be induced by tBCCAO with 15 minutes ligation and 4320 minutes reperfusion. Unfortunately this study did not measure the ischemic volume of the rats' brain. The fresh brain sections stained with TTC were examined for the observation of the induction intensity of global ischemia[20].

Hippocampus damage occurred after tBCCAO. Handayani et al. (2018) reported that hippocampus damage occurred in rats undergoing ischemia induction for five minutes and reperfusion for 24 hours[17]. TTC staining showed ischemic areas in the hippocampus. Furthermore, hippocampus damage in

rats was also induced by 5 minutes ligation and 168 hours reperfusion[21]. Iwasaki et al (1995) investigated the effects of ischemic/reperfusion injury on the number of CA1 hippocampus neurons of different rat strains. However, the quantification of CA1 hippocampus neurons after the transient BCCAO did not use unbiased stereology method. It turned out that the number of CA1 hippocampus neurons in the WIS rats was higher than that of the SD rats. However, in the Wistar rats, the number of CA1 hippocampus neurons of the post R/I (reperfusion/ischemia) injury group was not significantly different from that of the control group[21].

Depending on the duration of BCCAO, the SD strain showed a larger ischemic area than the WIS strain. The ischemic area comprised the striatum and cortex. This is also supported by Iwasaki et al (1995) study which found that the number of CA1 hippocampus neurons in WIS rats was higher than that of SD rats following the tBCCAO. Strain differences in the vulnerability of hippocampus neurons to an ischaemic insult were investigated in Sprague-Dawley and Wistar rats. Transient global brain ischaemia was induced for five minutes by a combination of bilateral carotid artery occlusion. The number of viable neurons in the CAl subfield was counted under a light microscope seven days after the ischemic insult. The induction of global brain ischemia reduced the density of viable neurons in the CA1 subfield of WIS and SD rats[21].

Generally, in all types of animal models (mice or rats), the brain structures which were affected by the tBCCAO were striatum, hippocampus, cortex, and thalamus. The duration of occlusion and reperfusion that could affect each of those brain structures were various. The tBCCAO for five minutes to half an hour followed by a reperfusion for one hour to 168 hours has been shown to impair the striatum[11–14,17–19]. In mice, striatum damage could be induced by eight to 18 minutes of ischemia followed by 2880 minutes of reperfusion[22], and 14 to 15 minutes ischemia followed by 1440 to 10080 minutes of reperfusion[23]. In rats, the duration of BCCAO for five minutes followed by 24 hours of reperfusion[17], or 30 minutes of ischemia followed by one hour of reperfusion[19,25], or five minutes of ischemia followed by 4320 minutes of reperfusion[20] has been shown to impair the striatum.

Furthermore, a tBCCAO for five to 17 minutes followed by a reperfusion for one day to seven days injured the rats/mice hippocampus[11,18,19,20]. In mice, ischemia duration for 10 minutes followed by 2880 minutes of reperfusion[24], or 15 minutes of ischemia followed by 1440 to 10080 minutes of reperfusion[24], or 17 minutes of ischemia followed by 10080 minutes of reperfusion[26], has been shown to damage the hippocampus. The number of hippocampus CA1 uninjured neurons was assessed using a stereological approach[27]. In rats, the ischemia duration for five minutes and reperfusion for 24 hours has been shown to impair hippocampus[17].

Generally, tBCCAO for ten minutes to half an hour followed by a reperfusion for one hour to seven days destroyed the cerebral cortex[19,23,26,27]. In mice, the duration of ischemia for ten minutes followed by 2880 minutes of reperfusion[27], or 14 minutes of ischemia followed by 1440 minutes of reperfusion[23], or 17 minutes of ischemia followed by 10080 minutes of reperfusion[26], has been shown to damage cerebral cortex. In addition, in mice, tBCCAO for 14 minutes followed by a reperfusion for one day injured the thalamus[23].

Up to present, there is only one study that investigated the volume of hippocampus and cerebral cortex of mice following tBCCAO[26]. For the assessment of the volume of hippocampus and cortex, slices of brains were selected every 200 μm from bregma −1.43 to bregma −2.43 and stained with Nissl staining/ toulidine blue. Areas of the bilateral hippocampus and cortex in each slice were calculated separately, added together, and multiplied by slice thickness to give the volume [26]

One of the methods to macroscopically assess ischemia is TTC staining. The TTC technique is simple, fast and can be completed in less than 60 minutes without using microscopic observations[28]. The principle of this staining is based on the nature of TTC which is easily oxidized by the mitochondrial dehydrogenase enzyme (Lactate dehydrogenase / LDH). This oxidation produces formazam compounds. Formazam compounds stains red on healthy tissues. The mitochondria in ischemic brain is damaged and LDH exits the cell into the plasma. Damaged cells will be white because of the decline or even disappearance of intracellular LDH enzymes[29,30] The time of observation of ischemic tissue using the TTC method varied greatly, starting from 60 minutes after the induction of ischemia up to 12-24 hours after the induction[31] Rekabi et al. (2015) showed that tBCCAO for 30 minutes followed by 1-hour of reperfusion gave rise to infarct areas in TTC staining. The duration of observation of permanent brain damage using the TTC method varied, at the latest two – three hours after the occurrence of ischemia,

while other studies reported a period of around 12-24 hours. The observation of the ischemic area after six hours of induction of ischemia showed variable results. This was probably due to the intervention of macrophages and glia cells in the ischemic areas. Glia cells will surround damaged tissue. Macrophages that have mitochondria will infiltrate the ischemic areas. This caused the areas of ischemia, which were supposed to be white, to be red[31].

In the TTC staining procedure, the brain tissue is cut coronally with a thickness of 1 to 2 mm. The brain slices are incubated in 0,05 %; 1% or 2% of TTC solution for 20 to 30 minutes in 37°C, and subsequently incubated in 10% of formaldehyde for a night [18]. A similar technique was conducted by Hussen & Shaheed (2015) to evaluate the ischemic area of the brain. The difference was that the incubation in 2% of TTC was performed for 30 minutes[19]. Ischemic areas of the striatum were also discerned after the brain tissue was stained by 1% TTC for 30 minutes[20]. A different technique was conducted by Handayani et al. (2018) to evaluate the ischemic volume of the brain. The differences were that the incubation was in 0.05 % TTC, the sections thickness was 2 mm, and the incubation of TTC in the black box was 30 minutes. The ischemia volume of the rats brain that was examined using Cavalieri method[17]. Handayani et al. (2018) reported that the TTC staining on the brain tissue varied in color. The non ischemic brain appeared red, while the ischemic brain appeared pale to white[17].

Referring to the above-mentioned studies, one can conclude that there was a variation in TTC staining concentration solution (0.05%, 1% and 2%). Moreover, there was also a difference in the tissue incubation duration in TTC solution (20 minutes and 30 minutes). Another study has conducted an optimization of TTC solution concentration and incubation duration after MCAO surgery. It has been found that for the staining of the post MCAO, the most optimal concentration of TTC was 0.05% and the most optimal incubation duration was 30 minutes. In addition, 0.05% TTC in PBS solution could distinguish the ischemic from the non-ischemic area clearer than 1% or 0.1% TTC [17,32]

The quantification method of ischemic volume in studies presented in Table 1 is rather problematic, since only one of them used unbiased stereological method. Stereology is a quantification method that provides a high accuracy and precision in the estimation of histopathological parameters, including the volume, surface area, length, or the number of particles [22-26]. The above-mentioned studies used software in examining ischemic areas of the brain tissue following tBCCAO. The necrotic areas were measured by using Image Analysis Software, including Image-Pro Plus (18) and Digimizer[19]. One may have to ensure that the volume of ischemia is measured by using the Cavalieri principle in order to obtain a more accurate estimate, regardless of using software or not.

Table 2 presents the data of neurological function test that was conducted on rats which underwent tBCCAO. The survival and repair of neurological function in the animal model are the most imperative things to be undertaken. It should be noted that there is a difference in the parameters used for evaluating the neurological output between animal models (pre-clinical study) and human subjects (clinical study). Studies using animal models accentuate more on the brain areas which are characterized with infarct, while studies on human emphasize more on the neurological function repair and the patient quality of life[2]. Additionally, there is a variety in the neurological tests of animal models [3]. Neurological function tests which were performed in several tBCCAO studies ranged from motor, sensory, to cognitive function tests. The tests included rotarod test, wire hang test, forelimb flexion, twisting, vestibulomotor function (beam balance), complex neuromotor function test (beam walk), upper extremity impairment skilled reaching movement analysis, social recognition juvenile recognition test, Morris water maze test, open field activity test, elevated plus-maze test for spatial memory, passive avoidance test, and eight arm radial maze[20,26,38–43]. Nevertheless, the neurological function test that had been undertaken on studies on rats as listed in Table 2 was only motor test comprising torso twisting, circling and rotarod tests.

Generaly, the methods for measuring hippocampus-dependent spatial memory are T maze[44], radial arm maze[45-53], and Morris water maze (MWM). A number of studies have examined the spatial memory of ischemia-induced mice using Morris water maze and assessed the structure of hippocampus. There are four types of ischemia induction model which are used for spatial memory testing. The first model is permanent global ischemia[53–62]. In this model, the commencement of the MWM test varied from five, seven, eight, to 15 days before the end of ischemia induction[58,59,61,63].

The second model is transient global ischemia[46,64–72]. In this model, the examination of the spatial memory using MWM was the most on the seventh day before

the ends of reperfusion period. The latest examination was 3 days before the ends of reperfusion period.

The third model is focal ischemia[73,74]. In this model, the examination of the spatial memory using MWM was at the end of the ischemia/reperfusion period. The last induction model is embolism[48,57,75,76].

**Conclusions**

Following the above-mentioned explanation, there are several points that can be concluded. There were differences in the ischemic area between Wistar and Sprague-Dawley rats after transient BCCAO with the ischemic duration of 30 minutes and reperfusion duration of 60 minutes. There were differences in the TTC staining solution concentration that was used to identify ischemic area of the brain following transient BCCAO. There was a very limited number of studies using Cavalieri method for the quantification of ischemic volume of rats' brain after transient BCCAO. Neurological functional tests in animal models post transient BCCAO did not include sensory and memory function tests.

**Conflict of interests**: No relevant disclosures

**Author's contribution:**

Data gathering and idea owner of this study: Handayani ES, Susilowati R, Setyopranoto I, Partadiredja G.

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Data gathering: Handayani ES, Writing and submitting manuscript: Handayani ES, Susilowati R, Partadiredja G

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