DEVELOPMENT OF ISCHEMIC STROKE MODEL BY RIGHT UNILATERAL COMMON CAROTID ARTERY OCCLUSION (RUCCAO) METHOD

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ABSTRACT

This study was designed to examine motor and cognitive changes, infarct lesion and neurohistological changes, involving histologic staining and immunohistochemical expression of caspase-3 after induction by right unilateral common carotid artery occlusion (RUCCAO) for 90 minutes. The animals were divided into two groups: sham group and stroke model group. Cognitive impairment was evaluated by Y maze. Motor function was measured on days 0, 1, 3 and 7 using FUAT paradigm. Infarct area, histological and caspase-3 expressions were evaluated on day 14 after RUCCAO. The results showed that RUCCAO induced cognitive and motor impairment on day 3 and 7. Furthermore, stroke model group induced infarct lesion. Histopathology examination showed body damage of neuron cell in the ipsilateral hemisphere. Moreover, expression of caspase-3 on RUCCAO group was significantly higher than that in sham group. In conclusion, RUCCAO method caused significant cognitive and motor function impairment. Furthermore, RUCCAO also induced infarct lesions and cell death in the thalamus brain area. Thus, RUCCAO can be employed as a method for ischemic stroke model, especially in focal ischemia.

Keywords: Ischemic stroke; right unilateral common carotid artery occlusion; Caspase 3; rats

INTRODUCTION

Ischemic stroke comprises more than 70% of all acute strokes types and is a leading cause of death and disability worldwide (Tsai et al 2015). Ischemic stroke results in inhibition of the oxygen and nutrients supply to the brain, resulting neuronal death. The damage and neuronal death result in the decrease of neurological function. This happens few minutes after the onset of ischemia, leading to the occurrence of tissue damage at the center of the ischemic injury (Mehta & Vemuganti 2014).

In ischemic stroke, there are two-pathway mechanisms of cell death in neuron, the necrosis and apoptosis. In ischemia, necrosis is the major cause of cell death in the intensely ischemic core. The core is surrounded by ischemic penumbra, where neurons primarily die by...
apoptosis, and a highly regulated mechanism of cell death. Penumbra is the term used for the reversibly injured brain tissue around ischemic core. This area is reported as an area with progressive apoptosis process. Thus, penumbra is the target for acute ischemic stroke treatment (Nguyen et al 2014, Liu et al 2010).

Animal stroke model are a necessary tool for some reasons. First, human ischemic stroke is very diverse in its manifestations, causes and anatomic localization. Whereas, an experimental ischemic stroke is highly reproducible and standardized. Furthermore, it produces more precise analysis of stroke pathophysiology. Second, the mechanism of molecular, genetic, biochemical and physiological investigations frequently require invasive direct access to brain tissue. Third, the pathophysiological events during the first minute of an ischemic stroke are most often not detectable by imaging techniques used in human stroke. Therefore, it can be studied only in an animal model (Fluri et al 2015).

Animal models for ischemic stroke can be classified as global, focal and multifocal ischemia. Animal models of focal cerebral ischemia are widely used in experimental studies. In focal ischemia, the obstruction of blood flow occurs in a specific region of the brain called as core of the ischemia, while some amount of blood reaches the core via collateral circulation, called as penumbra region of ischemia. Therefore, this model can be aiming at the elucidation of pathophysiological mechanisms of strokes and for the development of new therapeutic approaches in the treatment of occlusive cerebrovascular diseases (Kumar et al 2016). Numerous animal species have been used to study strokes. Mice and rats are the most commonly used species, with a growing use of larger species, such as rabbits and even non-human primates (Casals et al 2011).

Unilateral common carotid artery (CCA) occlusion is known to induce brain focal ischemia barely enough to cause infarction (Guo et al 2011). This model demonstrates a chronic mild reduction in cerebral blood flow, white matter lesions and delayed memory impairment. Furthermore, the levels of pro-inflammatory cytokines increase and those of anti-inflammatory cytokines decrease in the brain (Yoshizaki et al 2007). Besides, this method offers a simple and inexpensive method.

Thus, we examined the infarct lesion, behavioral and neurohistological changes after induced by RUCCAO. RUCCAO was performed in Wistar rats and characterized by ischemic insult, involving cognitive and motor impairment, neuronal death indicated by infarct lesion, and the expression of caspase-3 as the marker of apoptosis.

MATERIAL AND METHODS

The materials used in the experiment were normal saline, TTC 0.5% (2,3,5 triphenyltetrazoliun Chloride) (Sigma Aldrich), aquadest, buffered-formalin 10%, hematoxylin-eosin (Sigma Aldrich), xylazine (Interchemie), ketamine (Guardian pharma), and caspase 3 antibody (Sigma Aldrich).

Animal preparation

Healthy adult Wistar male rats aged 8-10 months weights ranging between 200-240 gram were used in this study. All animals had free access to food and water ad libitum. They were maintained under standard laboratory condition that was a well-aerated room with alternating light and dark cycle of 12 h each and at room temperature of 25°C. The experimental protocol was approved by the Animal Ethics Committee, Faculty of Veterinary, Universitas Airlangga, Surabaya, Indonesia.

Experimental protocol

The rats were allocated randomly and divided into two groups i.e sham (n=12), stroke model group (n=12). Xylazine (10 mg/kg) and ketamine (80 mg/kg) were administered for anesthesia. The animals were turned to supine position and fixed to the surgical table using adhesive tape. Through a small incision approximately 2-3 cm in the neck midline, the right common carotid artery was isolated from the vagal nerve and connective tissue by blunt dissection. The right common carotid artery (CCA) was blocked by bulldog clamp for 90 mins. After 90 mins bulldog clamp was removed (n=12). As controls (n=12), the rats were subjected to the same surgical procedure without carotid block. The incision was then closed with sutures. For pain relief and post-operative discomfort, lidocaine gel was applied to the wound and 0.5 ml of saline was given intraperitoneally.

Behavioral cognitive assessment using Y-maze

Y maze protocol was performed in accordance to previous studies (Onaolopo et al 2012, Wahl et al 1992). Briefly, Y maze test was performed on day 0 (before surgery), 1, 3 and 7 (after surgery). This spontaneous alternation behavior was used to evaluate the working memory of rats placed in a new environment. Each rat was placed in one of the arm compartements and was allowed to move freely until its tail completely entered another arm. The sequence of arm entries was manually recorded. The arms were labelled A, B or C. Three possibilities were offered to the rats for their first choice: staying in arm A, moving into arm B, or moving into arm C. An alternation was considered as correct if
Infarct area assessment protocol was performed in accordance to previous study (Rupadevi et al 2011). Briefly, the animals were placed in a acrylic cylinder for 3 minutes. FUAT was performed on day 0 (before surgery), 1, 3 and 7 (after surgery). A video camera recorded the animals’ movements and mirrors were placed on the rear lateral sides of the cylinder as an angle to ease identification of forepaws when the animal was turned away from the video camera. We counted the number of times the ipsilateral (right) forepaw and contralateral (left) forepaw touched the side of the cylinder as the animal was exploring. A paw sliding on the side of the cylinder only counted as one touch. The paw had to be lifted in order for another touch to be counted.

Forelimb use asymmetry score was calculated as follows: \[ \frac{[I/(I+C+B)]/[C/(I+C+B)]} \]. [I] was the occasions when the unimpaired (ipsilateral) forelimb was used as a percentage of total number of limb use observations on the wall, [C] was the occasions when the impaired forelimb (contralateral) was used as a percentage of total number of limb use observations on the wall, while [B] was the occasions when both forelimbs were used simultaneously or nearly simultaneously during lateral side-stepping movements as a percentage of total number of limb use observations on the wall.

Hispatology evaluation

The animals were sacrificed at day 14 after RUCCAO and sham operation, and brain sliced were post-fixed with 4% paraformaldehyde in PBS (pH 7.4). The paraffin-embedded brains were sectioned at 10 μm. Hematoxylin and eosin (HE) staining was performed to identify the cellular morphology after RUCCAO.

Immunohistochemistry

Immunohistochemistry protocol was performed in accordance to a previous study by Kaushal et al (2014). The immunohistochemistry of caspase-3 was performed using primary rabbit anti-rat caspase-3 antibody. The deparaffinized sections were incubated in 1% H₂O₂ in PBS containing 0.1% sodium azide for 10 mins. The endogenous peroxidase activity was quenched by incubation with H₂O₂. The sections were incubated with blocking buffer at room temperature for 30 mins. The sections were incubated with caspase-3 antibody at appropriate dilution (at least 1:100) in antibody dilution buffer in a humidified chamber for 1 h at room temperature or overnight at 4°C. To assess non-specific staining or verify the binding specificities of primary antibodies, the sections were incubated with antibody dilution buffer or equal amounts of non-specific mouse, rabbit, or goat immunoglobulins without the primary antibody for negative controls. The sections were washed with PBS-T for 5 mins twice. The sections were incubated with HRP-conjugated secondary antibody at appropriate dilution in antibody dilution buffer at room temperature in the dark for 1 h. The sections were washed with PBS-T for 5 mins, repeated two times. The sections were incubated in dark with HRP substrate solution. The slides were washed with PBS-T for 5 mins twice to remove chromogen excess. The sections were dehydrated with ascending graded alcohols (for 20 s each in 35, 70 and 95% ethanol, and 2 min in 100 % ethanol) and cleared in xylene. The slides were mounted with mounting medium.

Statistical analysis

Statistical analysis was performed using Graph-Pad Prism software version 6.0. Data were presented as mean values ± SEM. All behavioral data were analyzed using two-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test. Infarction size and caspase 3 expression data were compared between groups by independent t test. The difference was considered significant if p<0.05.
RESULTS

Cognitive change in rats after RUCCAO

Fig. 1 shows the cognitive change in the number of alternation as compared to total alternation for 5 minutes in Y maze paradigm. The result showed that rats after RUCCAO had significant decrease in alternation percentage as compared to sham group. Furthermore, the stroke model group showed that there was no improvement in cognitive function until the end of the experiment (F (1, 11) = 24.73, p = 0.0004; Fig. 1).

![Cognitive function after RUCCAO](image)

Motor change in rat after RUCCAO

Fig. 2 shows the motor change in the number of contralateral forepaw compared to ipsilateral forepaw in FUAT test for 3 minutes. The results showed that rats after RUCCAO mostly used ipsilateral forepaw, which indicated that the rats had functional deficit in contralateral forepaw as compared to sham group. Furthermore, the stroke model group showed that there was no improvement in motor function until the end of the experiment (F (1, 22) = 44.13, p < 0.0001; Fig. 2).

![Motor function after RUCCAO](image)

Brain infarct lesion after RUCCAO

Fig. 3 shows brain infarct lesion after RUCCAO on infarct area in thalamus of the brain. Furthermore, Fig. 4 shows the size of infarct area in brain using independent t test, showing difference among groups with p value <0.0001. Moreover, stroke model group demonstrated a significant increase in infarct area as compared to sham group.

![Brain infarct lesion after RUCCAO](image)

Hisopathological and Immunohistological in brain after RUCCAO

Fig. 5 shows hisopathological examination perfomed to observe damage cell of body neurons in brain after 14 days RUCCAO in the ipsilateral hemisphere. Damaged cell of body neurons was characterized by irregular shape and diminished size, dissimilar to the living neuron cells which was characterized by regular shape.
and cells appear brighter than the damaged cells. Furthermore, the immunoreactive cells were clearly illustrated as cells that absorb the brown color with moderate to strong intensity in the stroke model group. Semi-quantitative result expression of caspase-3 after RUCCAO showed that stroke model group had increased expression of caspase-3 (p value 0.0002) as compared to sham group.

**Fig. 5.** Histopathological features of penumbra area of the brain in 14 days after RUCCAO, using Hematoxylin Eosin staining in infarct lesions ipsilateral (right) hemisphere. Black arrows in sham model (A) indicate normal cells, meanwhile blue arrow in stroke model (B) indicate damaged cell by shrunken neurons.

**Fig. 6.** Caspase-3 expression staining of infarct lesions in stroke model (B) after RUCCAO and sham model (A). Immunoreactivity of caspase-3 in brain was examined by immunohistological staining with caspase-3 antibody (sigma aldrich). The arrow line indicates the expression of caspase-3 in stroke model.

**DISCUSSION**

Our study aimed to establish an animal model of stroke ischemia exhibiting cognitive impairment and motor function, brain infarct lesions, histological and immunohistological evaluation induced by RUCCAO. Previous study reported that RUCCAO induced memory impairment in mice by used object recognition test.

In our results RUCCAO induction for 90 minutes did not cause mortality in all animals. We also did the RUCCAO for 90 minutes and we did the occlusion for 60 minutes. Our results showed there were no significant difference between sham and stroke model in cognitive, motor function and in infarct lesion (unpublished data).

**Fig. 7.** Mean number of caspase-3 expression in the infarct lesion. Data represent the mean ± S.E.M *p < 0.05 vs sham model; n = 6.

In the present study, for cognitive and motor function there were significances between the two groups in day 3 and 7 after RUCCAO. Additionally, our study reported that motor impairment indicated by higher trend of the rats in using ipsilateral forelimb than contralateral forelimb, which we can suggest that the rats had functional deficit in contralateral forepaw. Furthermore, cognitive impairment was indicated by the decrease in the percentage of alternation. Therefore, the present result indicated that RUCCAO induced cognitive and motor impairment.

In the present study infarct area was found in thalamus area, one of structures in the limbic system besides the amygdala, hippocampus, parahippocampus gyrus, cinguli gyrus, fornix, hypothalamus, dentatus genius and entorhinal cortex, the area that affect cognitive and motor function (Waxman 2007, Devinsky & D’Esposito 2004). The result of TTC staining showed that stroke model significantly increased area of infarct in the brain. Furthermore, infarct area is marked by white color. In living tissue TTC is enzymatically reduced by dehydrogenases to 1,3,5-triphenylformazan (TPF), which is in red color, while in death area it remains white due to absence such enzymatic activity (Chiang et al 2011).

Furthermore, hematoxylin and eosin staining was performed to examine brain histological profile after being induced by RUCCAO. The result showed cell damage of body neurons after 14 days in the ipsilateral hemisphere. RUCCAO leads to the reduction of oxygen and glucose in brain tissue followed by the formation of reactive oxygen species, release of glutamate, accumulation of intracellular calcium, and induction of inflammatory processes (Fluri et al 2015), which in turn
resulted in neuronal death. Therefore, we examined the expression of caspase-3 marker of apoptosis. Apoptosis is one of cell death mechanisms besides necrosis in brain ischemia (Woodruff et al 2011).

In accordance to a previous study by Velier et al (1999), the expression of caspase-3 in neurons and microglia was found between permanent and transient focal ischemia, indicating that the neuronal cell death with characteristics of apoptosis did occur after focal stroke. The present study demonstrated the increasing expression of caspase-3 on infarct lesion area on day 14 after RUCCAO, in which the immunoreactive cells were clearly illustrated as cells that absorbed the brown color with moderate to strong intensity in the stroke model group.

We believed that the unilateral common carotid artery occlusion induced ischemia produced infarct lesion and caused impairment of cognitive and motor function. Thus, RUCCAO method offers a simple, reproducible, and inexpensive method for evaluation of the potential therapeutic agents or to test new drugs against ischemic stroke.

CONCLUSION

Right unilateral common carotid artery occlusion is a useful method for inducing ischemic stroke, especially focal ischemia in rats.

ACKNOWLEDGMENT

This work was supported by research grants “Hibah Riset Tahun 2017” from the Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia.

REFERENCES

