A Descriptive Study of Histopathological of Chronic Brain Ischemia of Rat Tissue (Studi Deskriptif Histopatologis Iskemia Otak Kronis Jaringan Tikus)

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ABSTRACT

Worldwide, cerebrovascular accidents (stroke) are the second leading cause of death and the third leading cause of disability. However, not many the histopathological study of progression in chronic stroke has been published so far. This study gives the detail explanation of mechanism of recovery and might give the idea of new timeline when to set up the treatment to regenerate restoration of damaged cells. Fourteen male Wistar rats (15–20 weeks, weighing 250–400 g) were used in this study. Prior to 7 days of adaptation to the laboratory environment, rats were divided into four groups. Sham group (n=2), rats that sacrificed 4th week (n=2), 8th week (n=5), 12th week (n=5). 90 minutes temporary MCAO procedures were performed using the Indonesian modified technique. CD31 and Doublecortin markers were used to evaluate angiogenesis and neurogenesis. The results showed that ventricle size of ipsilateral brain was not so affected as in week 12th compared to 8th week. Gliosis as a response to damage to the central nervous system was more dense in week 12th as oppose to week 4th. Regarding angiogenesis and neurogenesis, there is significant improvement of angiogenesis and neurogenesis within weeks, however 4th week post MCAO shows prominent recovery. We summarized that rat's brain shows spontaneous improvement in chronic phase of stroke ischemia and angiogenesis and neurogenesis still happens until week 12th.

Keywords: MCAO, chronic phase, angiogenesis, neurogenesis

ABSTRAK

Di seluruh dunia, kecelakaan serebrovaskular (stroke) adalah penyebab utama kedua kematian dan penyebab utama ketiga kecacatan. Namun, tidak banyak studi histopatologis perkembangan stroke kronis telah dipublikasikan sejauh ini. Studi ini memberikan penjelasan rinci tentang mekanisme pemulihan dan mungkin dapat memberikan peluang tentang waktu yang tepat untuk memulai terapi perawatan dalam rangka upaya untuk meregenerasi pemulihan sel yang rusak. Empat belas tikus Wistar jantan (15-20 minggu, dengan berat 250-400 g) digunakan dalam penelitian ini. Sebelumnya tikus diadaptasi terhadap lingkungan laboratorium selama 7 hari, tikus dibagi menjadi empat kelompok. Kelompok sham (n = 2), kelompok selanjutnya tikus dikorbankan pada minggu ke-4 (n=2), minggu ke-8 (n=5), minggu ke-12 (n=5). Sembilan puluh menit kemudian dilakukan prosedur MCAO dilakukan dengan teknik yang telah dimodifikasi di Indonesia. Penanda CD31 dan Doublecortin digunakan untuk mengevaluasi angiogenesis dan neurogenesis. Hasil penelitian menunjukkan bahwa ukuran ventrikel otak ipsilateral tidak begitu terpengaruh seperti pada minggu ke-12 dibandingkan dengan minggu ke-8. Gliosis sebagai respons terhadap kerusakan pada sistem saraf pusat lebih padat pada minggu ke-12 dibandingkan dengan minggu ke-4. Mengenai angiogenesis dan neurogenesis dijumpai peningkatan yang signifikan dari angiogenesis dan neurogenesis dalam beberapa minggu, namun minggu ke empat setelah MCAO menunjukkan pemulihan yang menonjol. Kami menyimpulkan bahwa otak tikus yang mengalami stroke fase kronik mengalami perbaikan spontan. Proses angiogenesis dan neurogenesis masih terjadi sampai minggu ke-12.

Kata Kunci: MCAO, fase kronik, angiogenesis, neurogenesis.

INTRODUCTION

The incidence of stroke is increased worldwide especially in low to middle income countries (Feigin *et al.* 2009) and stroke is causing disability and dependency (Lee *et al.* 2020). Focus for the management of stroke has shifted from neurop-protection to neurorestoration nowadays

(Sadewo *et al.* 2020), and there has been massive progression of cell therapy treatment to restore damage cells in the affected brain.

Prior to proceeding the treatment to humans, all trials begin in animal trials. The animals that resemble human's brain vasculature are rodents. There have been many trials that seek the mechanism of cell repair in damaged cells of the

stroke procedure.

Animal stroke procedure that mimics the mechanism of stroke in human is luminal occlusion of middle cerebral artery (MCAO) procedure (Makkiyah & Sadewo 2019). This technique along with the reperfusion method will produce infarct as in humans and rodents. However, not many the histopathological study of progression in chronic stroke has been published so far. This study gives the detail explanation of mechanism of neurorestoration and might give the new timeline when to set up the treatment to regenerate restoration of damaged cells.

MATERIALS AND MTHODS

Fourteen male Wistar rats (15–20 weeks, weighing 250–400 g) were used in this study. Prior to 7 days of adaptation to the laboratory environment, rats were divided into four groups; such as sham group, rats that sacrificed 4th week, 8th week, 12th week (Figure 1).

All surgical procedures were performed in accordance with our institutional guidelines under 8 times surgical loupe magnification. 14 male, 6 months, 250 grams–400 grams wistar rats were taken from Biofarma Pasteur Bandung, held in half day dark and half day bright cage under temperature of 22–24°C and with food and water ad libitum. Animals were not fasted prior to the surgery. The animals were anesthetized by 10 mg/kg i.p Ketamine hydrochloride (Ketamine® 10% Essex Pharma GmbH, Germany) and 5 mg/kg i.p Xylazine hydrochloride (Rompun®, Bayer AG, Germany) given intraperitoneally. The animals were not intubated and blood gases were not monitored during the MCAO. The ethical commission of the Faculty of Medicine University of Indonesia has given ethical permission to perform the described experiments. Prior to one week adaption in Animal Research Facilities in School of Medicine of University of Indonesia, rats were operated by MCAO–well trained–two neurosurgeons and two residents. We followed the Indonesian simple procedure of MCAO that used loupe magnification lens during the procedure (Makkiyah *et al.* 2019). All animals were temporary occluded for 90 minutes duration that

allowed the reperfusion happend. All animals survived the MCAO procedure.

When it is the time sacrifice time the rats in control and all experimental groups were euthanized. Brain tissue was fixed by 10 % formaldehyde for 24 h. The rat's brain were cut in 2 mm each. Slide level 2 and level 3 (Figure 2) were taken into further study. Tissue preparation for H&E staining was performed in three stages, deparaffinization, hydration, and staining. Sections were stained with the filtered Harris's hematoxylin for 1 min, rinsed with tap water, immersed in eosin stain for 1–2 min, and rinsed with tap water again. Then, sections (with 6-µm thickness) were subjected to dehydration in ascending concentration of alcohol solutions (50, 70, 80, 95 % × 2, 100 % × 2). Sections were examined using a light microscope (Nikon, Japan).

To study the neurogenesis and angiogenesis, Doublecortin and CD 31 antigen were purchased from Abcam company, and according blocks to the company protocol (Abcam), first, block parafin were cut 3 µm, then section were at 37° C dried, heat over slide warmer at 60 °C for 60 minutes. Then the sections were deparaffinized with xylol 1,2,3 for about 3 minutes each and hydrated with ethanol from absolute alcohol,

Figure 1. The schema of descriptive study of chronic brain

Figure 2. The rat's brain were cut in 2 mm each. Slide level 2 and level 3 were taken into further study. (OC= optic chiasm, OB= optic block)

96 to 70 % were about 3 minutes each. The following steps are rinsed with water for 3 minutes, blocked with endogen peroxide 3 % (methanol + H₂O₂) for 10 minutes and washed again with water for 5 minutes. Then put in decloacking chamber at 96 °C for 10 minutes with Tris EDTA pH 9 solution. Put the sections in the open air (make them cold) for 25 minutes. Wash in PBS pH 7.4. Put background sniper for 15 minutes.

We added 100–400 µl primary antibody (1:2000) (doublecortin or CD 31) to each section and incubated overnight at 4 °C for 1 hour then, we removed antibody solution and the sections were washed with PBS pH 7.4 incubated for 20 min with secondary antibody (from Trevi universal link) and washed with PBS three times for 2 min each. We applied 100–400 µl DAB to each section 1-2 minutes and monitored closely. One to ten minutes generally provides an acceptable staining intensity with hematoxylin, and we finally repeated it in 100 % ethanol, incubated the sections two times for 10 second each, and mounted the sections with coverslips.

RESULTS

To find out whether MCAO technique in this study produce an infarct, one sample of sham group was coloured with 2,3,5 Triphenyl-tetrazolium chloride (TTC). TTC is a marker of ischemic areas. Figure 3 shows that left cerebral area became white after stained with TTC, means that infarct formed and the procedure of MCAO is succeed.

Haematoxyllin Eosin (H&E) Staining

Examination of H&E stained slides of rat brains (Figure 3) revealed normal brain histology in the control group. The structure of cells were regularly arranged and a clearly situated. The nucleoli were clearly visible and no edema presented. Histopathology of MCAO rat showed shrunken neurons with pyknotic cells. White matter was filled with gliosis and loss of normal architecture. Striatum showed neuronal edema and vacuoles. With high magnification showed the cytoplasm of cells had lost and filled with vacuoles. Brain's rat from week 4th,

week 8th and week 12th post MCAO were compared. The appearance of rat's brain in week 4 shows that the ventricle of ipsilateral brain was not affected as in week 12th (remarkably seen atrophy of the brain). Gliosis as a response to damage to the central nervous system was more dense in week 12th as oppose to week 4th (Figure 4,5).

Immunohistochemistry Staining of CD31.

Examination of CD31 stained slides for rat brain revealed positive cells in blood vessels. There difference in blood vessel numbers were most pronounced in week 4th compared to week 8th and week 12th.

Figure 3. TTC colouring of brain rat (post MCAO). Dead tissue in the left cerebral area of rat's brain indicate an infarct region.

Figure 4 Photomicrograph of cerebral cortex of rat brain (A) Control, showing normal structure neuron (N) (B) MCAO rat, showing shrunken neurons with intense neuron eosinophilic homogenization and pyknosis in the pyramidal cell (Pic cell). (C) Control, showing normal whitematter. (D) MCAO rat, gliosis white matter (E) striatum normal (F) striatum MCAO, neuron edema and vacuoles (V) HE 400 x (G) Necrotic cells showing vacuoles due to cytoplasm destruction, nucleus displaced to the periphery (ND) HE 1000x

Immunohistochemistry Staining-Double-cortin (DC) staining.

Examination of DC stained slides for rat brains (Figure 7) revealed doublecortin positive cells in subventricular zone in normal rat with low intensity of positive cells. However, doublecortin positive cells were remarkably found in subventricular zone in week 4, 8 and 12 brain's rat.

DISCUSSION

Histopathology of MCAO rat showed shrunken neurons with pyknotic cells. White matter was filled with gliosis and loss of normal architecture. Striatum showed neuronal edema and vacuoles. With high magnification shows the cytoplasm of cells had lost and filled

with vacuoles (Figure 4). As atrophy happened progressive to week 12th post MCAO, this made the size of ventricle in the ipsilateral of brain was so affected in this periode. Gliosis as a response to damage to the central nervous system was more dense in week 12th as oppose to week 4th post MCAO.

This is consistent with study result from tMCAO rats at 7 and 21 days after reperfusion, a remarkably number of damaged neurons were found in the ipsilateral brain (Sicard *et al* 2006). MCAO produced a variable degree of cerebral ischemia and damaged neuron with ability to recover. Cellular brain were disorganised and loss of neuronal structure, swelling, and necrosis of brain tissue. The recovery might consistent to the improvement of neurological condition and reduced disability

Figure 5. Photomicrograph of cerebral cortex of rat brain Figure A, D, G H&E staining 4th week post MCAO (macrophoto, 40x, 400x) Figure B, E, H H&E staining 8 week post MCAO (macrophoto, 40x, 400x) Picture C, F, I H&E staining 12th week post MCAO (macrophoto, 40x, 400x).

Figure 6. Photomicrograph of CD 31 staining. Figure D CD 31 staining 4th week post MCAO (400x) Figure B, CD 31 staining 8th week post MCAO (400 x) Figure C, CD 31 staining 12th week post MCAO (400x). These differences in blood vessel numbers were most pronounced in week 4 compared to week 8 and week 12 .

state (Zhan *et al.* 2014) The infarct was prominent in Figure 5 is consistent to the pan-necrosis, defined as a complete loss of neurons, astrocytes, microglia and extracellular matrix, with cavitations and loss of volume (Ejaz *et al.* 2013). The cortical infarct in this study happens in the frontal cortex and extended to the striatum, and there is resolution with the cavity starting by the week 4th post MCAO. This result is in line with the formation of the cavity of the infarct from the photothrombotic procedure by the end of the 3rd week post procedure (Yanev *et al.* 2010). Another study in chronic stroke of rat's brain produced area infarct in lateral striatum and near cortex (Guerra-Crespo *et al.* 2009).

Recovery mechanism of stroke includes angiogenesis and neurogenesis. There are many histopathological markers of angiogenesis, that enable an assessment of any restorative treatments (Leung & Jensen. 2013). As what our result showed that CD31 (or marker of endothelial cells) (Cipolla *et al.* 2009) were more prominent in the 4th week post MCAO than 8th and 12th week. The other result from this study showed that angiogenesis still took place even after 12th week post MCAO. This is accordance to the study of Makkiyah *et al.* (2021) administration of bone marrow mononuclear cells to chronic infarct in brain's rat. In that study, the administration of BMMNC did not increased significantly in angiogenesis to control group week 12th.

Angiogenesis induced neurogenesis and vice versa. Neurogenesis still took places until week 12th. As shown in the other study of

chronic stroke that doublecortin was expressed near the lateral wall of the ventricle to the striatum in 72 days post MCAO (Yanev 2010). Even this study still not extend 12th week as in our study, that result showed the neurogenesis still existed in the chronic stroke model.

Limitation of the study was the sample size small, however, from this study we could gain more knowledge about the progress of infarct and mechanism of recovery within weeks. The needs of more imunohistochemistry markers performed such as VEGF, neuron mature, to elucidate the comprehensive angiogenesis and neurogenesis process in chronic brain. The definition of chronic periode, should be defined carefully. Because rat's brain improves faster than human's brain. The term of chronic in humans, is should be different than in rodents. A future study elaborates more in this definition warrants to be performed.

CONCLUSION

Rat's brain shows spontanenous improvement in chronic phase of stroke ischemia and angiogenesis and neurogenesis still happends until week 12th.

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Figure 7. Photomicrograph of doublecortin (DC) staining. Figure A, E DC staining 4th week post MCAO (macrophoto, 40x 0) Figure B, F, DC staining 8th week post MCAO (macrophoto, 400x) Figure C, G DC staining 12th week post MCAO (macrophoto, 400x). Figure D DC staining sham rat (400x magnification). The process of neurogenesis still took place until week 12th post MCAO.

COMPLIANCE WITH ETHICAL STANDARTS

(In case animals were involved) Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All protocols were approved by the Animal Care and Use Committee of Faculty of the Medicine Universitas Indonesia.

AUTHOR CONTRIBUTIONS

FM, TS, RHN, WS, reviewed the writing, and grammar, FM as main contributor and collected the microphotographs.

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