สัญญาเลขที่ MRG4880034

รายงานวิจัยฉบับสมบูรณ์

โครงการ

ผลของภาวะเลือดไปเลี้ยงสมองลดลงเรื้อรัง
ต่อการเปลี่ยนแปลงของแอสโตรไซต์ในสมองส่วนฮิปโปแคมปัส

The effect of chronic cerebral hypoperfusion
on astrocytes in the CA1 of hippocampus

โดย สมพล เทพชุม และคณะ

มีนาคม 2552

สัญญาเลขที่ MRG4880034

รายงานวิจัยฉบับสมบูรณ์

โครงการ

ผลของภาวะเลือดไปเลี้ยงสมองลดลงเรื้อรัง ต่อการเปลี่ยนแปลงของแอสโตรไซต์ในสมองส่วนฮิปโปแคมปัส

The effect of chronic cerebral hypoperfusion on astrocytes in the CA1 of hippocampus

คณะผู้วิจัย	**สังกัด**
1. สมพล เทพชุม พ.บ. Ph.D.	มหาวิทยาลัยมหิดล
2. รศ.กนกวรรณ ติลกสกุลชัย Ph.D.	มหาวิทยาลัยมหิดล
3. นางสาวอัญรัตน์ ทอนมาตร	มหาวิทยาลัยมหิดล
4. นางสาวธิดาพร วงศ์สุทิน	มหาวิทยาลัยมหิดล
5. นางฉัตรสุมาลย์ ผลแสง	มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา
และสำนักงานกองทุนสนับสนุนการวิจัย

สัญญาเลขที่ MRG4880034

กิตติกรรมประกาศ

งานวิจัยนี้จะสำเร็จลงได้ ด้วยการสนับสนุนทุนวิจัยสำหรับนักวิทยาศาสตร์รุ่นใหม่ ของสำนักงานคณะกรรมการการอุดมศึกษา (สกอ.) และสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) ขอขอบคุณภาควิชาสรีรวิทยา คณะแพทยศาสตร์ศิริราชพยาบาลซึ่งเป็นสถานที่ปฏิบัติงานวิจัย ขอบคุณอาจารย์ บุคลากรและนักศึกษาในสาขาประสาทวิทยาศาสตร์ ภาควิชาสรีรวิทยา คณะแพทยศาสตร์ศิริราชพยาบาล ที่มีส่วนร่วมในงานวิจัยครั้งนี้ รวมทั้งขอขอบคุณอาจารย์ และบุคลากรท่านอื่นๆ ในภาควิชาสรีรวิทยา คณะแพทยศาสตร์ศิริราชพยาบาล ที่สนับสนุนและให้ความคิดเห็นที่เป็นประโยชน์ต่องานวิจัยมาอย่างต่อเนื่อง ขอขอบคุณนักวิจัยที่ปรึกษา รศ.ดร.กนกวรรณ ติลกสกุลชัย ที่ให้คำแนะนำที่เป็นประโยชน์อย่างยิ่งต่องานวิจัย

ดร.นพ.สมพล เทพชุม
หัวหน้าโครงการวิจัยผู้รับทุน

สารบัญ

บทคัดย่อ

รหัสโครงการ : MRG4880034
ชื่อโครงการ : ผลของภาวะสมองขาดเลือดเรื้อรังต่อสมองส่วนฮิปโปแคมปัสและการเรียนรู้และความจำ
ชื่อนักวิจัย : สมพล เทพชุม
ภาควิชาสรีรวิทยา คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล
E-mail Address : sisth@mahidol.ac.th
ระยะเวลาโครงการ: มิถุนายน 2548 – มีนาคม 2552

การลดลงของความจำเป็นปัญหาที่สำคัญของประชากรสูงวัยทั่วโลก โดยระดับการลดลงของความจำเป็นได้ตั้งแต่ความจำลดลงตามอายุ จนถึงระดับความจำเสื่อมน้อยและมากตามลำดับ ทำให้มีการศึกษาเพื่อหากลไกการเสื่อมของความจำและการเปลี่ยนแปลงความรุนแรงของโรคโดยมีเป้าหมายหลักเพื่อที่จะหากลไกเริ่มต้นของการดำเนินสู่ระยะต่างๆ ของการเสื่อมของความจำ เพื่อที่จะได้ให้การป้องกันหรือรักษาก่อนที่โรคจะรุนแรง ปัจจัยที่พบร่วมในการเสื่อมถอยของความจำระดับต่างๆ ในผู้สูงอายุคือ การลดลงของเลือดที่ไปเลี้ยงสมอง ดังนั้นการศึกษาการเปลี่ยนแปลงที่เกิดจากการลดลงของเลือดไปเลี้ยงสมองเล็กน้อยที่ระยะเวลานานพอสมควร จะทำให้ทราบถึงการเปลี่ยนแปลงในระยะเริ่มต้นได้ ดังนั้นงานวิจัยชิ้นนี้จึงมีเป้าหมายเพื่อศึกษาผลของภาวะเลือดไปเลี้ยงสมองลดลงเล็กน้อยแต่เรื้อรัง ต่อความจำ ภาวะออกซิเดชั่น และการเปลี่ยนแปลงจำนวนและรูปร่างของเซลล์ประสาทและแอสโตรไซต์ในสมองส่วนฮิปโปแคมปัส โดยการผูกหลอดเลือดแดงใหญ่ที่คอด้านขวาในหนูขาวเพศผู้อายุ 4 เดือน ทดสอบความจำใน radial arm water maze (RAWM) และวัดภาวะออกซิเดชั่นในสมองส่วนฮิปโปแคมปัส หลังผูกหลอดเลือด 2 และ 6 เดือน ขณะเดียวกันก็ทำการทดลองเพื่อศึกษาการเปลี่ยนแปลงจำนวนและรูปร่างของเซลล์ประสาทและแอสโตรไซต์ในสมองส่วนฮิปโปแคมปัสที่ระยะเวลา 1, 3, 7 วัน, 2 สัปดาห์, 1 เดือน, 2 เดือน และ 4 เดือน หลังผูกหลอดเลือด ผลการทดลองแสดงให้เห็นว่า การผูกหลอดเลือดแดงใหญ่ที่คอด้านขวาเป็นระยะเวลา 2 และ 6 เดือน ทำให้เกิดการลดลงของความจำทดสอบโดย RAWM มีการเพิ่มระดับของภาวะออกซิเดชั่นในสมองส่วนฮิปโปแคมปัส แต่ไม่มีผลต่อการเปลี่ยนแปลงจำนวนและรูปร่างของเซลล์ประสาทและแอสโตรไซต์ในสมองส่วนฮิปโปแคมปัส ดังนั้นการผูกหลอดเลือดแดงใหญ่ที่คอด้านขวาในหนูขาวสามารถทำให้มีการเสื่อมของความจำลงเล็กน้อย อันน่าจะมีสาเหตุมากจากการเปลี่ยนแปลงของภาวะออกซิเดชั่นในสมองที่ไปกระทบต่อการทำหน้าที่ของเซลล์ประสาทและแอสโตรไซต์

คำหลัก : ภาวะสมองขาดเลือด, ฮิปโปแคมปัส, ความจำ, แอสโตรไซต์, ภาวะออกซิเดชั่น

Abstract

Project Code : MRG4880034

Project Title : Effect of mild chronic cerebral ischemia on hippocampus and spatial learning and memory

Investigator : Sompol Tapechum, Anyarath Tonmate and Kanokwan Tilokskulchai.
Department of Physiology, Faculty of Medicine Siriraj Hospital
Mahidol University, Bangkok, Thailand.

E-mail Address : sisth@mahidol.ac.th

Project Period : June, 2005 – March, 2009

Memory impairment ranging from aged-related memory loss, mild cognitive impairment (MCI) and dementia affects millions of elderly around the world. Though extensive researches have been invested, the pathogenesis of the transition from each stage of memory impairment is not well characterized. The focus of the research is on the characterization of the earliest stages of cognitive impairment. One factors consistently discovered in both normal and demented elderly is the reduction of cerebral blood flow. The mild cerebral hypoperfusion would then reveal the early change of the memory impairment process affected by elderly. To study the effect of mild chronic reduction of cerebral blood flow on memory, hippocampal oxidative stress, neurons and astroglia, 4-month old male Sprague-Dawley rats were subjected to permanent right common carotid artery occlusion (RCO). Behavioral assessment for spatial learning and memory in radial arm water maze (RAWM) was performed 2 or 6 months after occlusion. Following the behavioral test, the rats were sacrificed and brains were assayed for the oxidative stress parameters, malondialdehyde (MDA) and reduced glutathione. Parallel experiment was performed for histological study of number and morphology of CA1 hippocampal neurons and astroglia after arterial occlusion for 1 day, 3 days, 7 days, 2 weeks, 1 month, 2 months and 4 months. The results showed that unilateral common carotid artery occlusion for 2 and 6 months could cause spatial memory impairment in RAWM task, increase in level of oxidative stress parameters especially the reduced glutathione at 2 months after RCO but without the alteration in numbers and morphology of neurons or astroglia in CA1 hippocampus. The results indicated that unilateral common carotid artery occlusion could cause mild memory impairment. This could be resulted from brain oxidative stress affecting neurons and astroglial functions.

Keywords : cerebral ischemia, hippocampus, spatial memory, astrocyte, oxidative stress

1 Introduction

The progression of health care system in this era makes life expectancy of the population increase than ever occur before. Together with the success of the family planning system, the elderly becomes a large proportion of the population. The National Statistical Office Thailand reported in 2003 that 9.5% of 63 million population of Thailand were those of 60 years old or older. In the next 20 years, this number will reach 15% of population (70 million) or 11 million. Along with the advanced age, several illness and disabilities of ageing increase such as diabetes mellitus, hypertension, and stroke. Cognitive impairment is another condition frequently encountered by elderly. This condition disturbs activities of daily living and even causes disability and dependent on care providers.

Cognitive impairment presents with different degree of severity. The subclinical cognitive deficits are labeled benign senescent forgetfulness, age-associated cognitive decline (AACD), age-associated memory impairment (AAMI) and mild cognitive impairment (MCI). These conditions are capable of transition to more severe cognitive impairment. The most severe form of cognitive impairment is dementia which causes disability and incapacity to maintain normal activity of daily living. The worldwide prevalence of dementia are 5% in population of 65 years old or older and becomes 20% in population of 80 years or older. In 2002, the National Statistical Office Thailand revealed that 30% of elderly reported forgetful while 0.6% was in clinical stage of severe dementia. This numbers might be lower than expected because of poor access to medical care of those with dementia.

Because treatment of dementia is not very effective, the aim of health care system is to prevent the development of the disease. To effectively prevent the disease, the thorough understanding the pathophysiology and early detection of the condition must be achieved. It has been shown that the AACD, AAMI and MCI are capable of transition to overt dementia. More than 10% of MCI turns into dementia within 3 years and almost 50% turns into dementia within 5 years.

Despite extensive research, the early mechanism for the development of dementia is not well understood. This could be because of no appropriate animal model for an early stage of this condition has not been met. One factor consistently discovered in subclinical dementia; AADC, MCI or the overt dementia is the reduction of cerebral blood flow (Farkas and Luiten, 2001). Therefore, the animal models of cerebral blood flow reduction have been used extensively to understanding the mechanism of cognitive impairment. The two common models are bilateral common carotid arteries occlusion (2VO) and arteriovenous fistula (AVF) between common carotid arteries and external jugular veins. Both models cause reduction of cerebral blood flow which persists for several weeks together with impairment of learning and memory (Morgan et al., 1989;

Ouchi et al., 1998). In 2VO model, the cerebral blood flow initially reduces to the level below 50% in most parts of brain and gradually returns to almost normal within several months (Farkas et al., 2007). Motor deficit and death of animals were frequently encountered using these models which forced animals to be excluded from the experiment. Besides, it has been shown that bilateral common carotid artery ligation for 30 days caused significant functional and morphological damage to retina (Lavinsky et al., 2006). These eye problems could impair animals' performance during the visual-dependent behavioral tasks. Therefore, the mild model of cerebral blood flow reduction by ligation of unilateral common carotid artery has been used in this study. This model has been shown to cause maximal cerebral blood flow reduction to 65.5% of pre-ligation on the ipsilateral hemisphere at 2 hours after ligation in mice which then gradually improved but still at about 80% at 4 weeks after surgery. No animal died or showed impairment of motor function after unilateral common carotid artery ligation while cognitive impairment has been evidenced (Yoshizaki et al., 2008). In rats, which have a complete circle of Willis as human, the cerebral blood flow reduction after unilateral common carotid artery occlusion is mild and transient. However, the circulatory reserve was reduced as hypercabia significantly reduced cerebral blood flow (De Ley et al., 1985). This condition could mimic the situation of cerebrovascular compromise in elderly or dementia. There is no study of the effect of unilateral common carotid artery on the memory and mechanism in rats. In our research group, when compared between right and left common carotid arteries ligation, the spatial memory impairment was evidence in the animal with right common carotid artery ligation (Tilokskulchai., unpublished data). Therefore, the permanent right common carotid artery ligation was adopted in our experiment to study effect of chronic mild reduction of cerebral blood flow on cognitive function.

Several cellular mechanisms leading to cognitive impairment after cerebral ischemia have been proposed. Study of hippocampal pathology after 2VO showed delayed neuronal death concomitant with the impairment of learning and memory (Bennett et al., 1998; Weinstock and Shoham, 2004). The death of neurons increased with the ischemic duration and also enhanced with chronic stress (Ritchie et al., 2004). However, the early functional impairment can occur right before the death of neurons. Study in ageing rats showed the impairment of the maze learning test without reduction of CA1 hippocampal neurons (Rasmussen et al., 1996). Another experiment in 2VO models also showed the impairment of learning and memory prior reduction of hippocampal neurons (Ni et al., 1994). This impairment could be the result of functional alteration of neurons such as impairment of glucose metabolism (Tsuchiya et al., 1993). White matter lesion has also been evidenced in mice after unilateral common carotid artery occlusion (Yoshizaki et al., 2008). As we try to understand the earliest change in the brain during

the development of cognitive impairment, the milder animal models mimic AADC or MCI are preferential.

The mechanisms leading to neuronal dysfunction, death and cognitive impairment after cerebral ischemia have been studied extensively. Among these, oxidative stress is one of the major players in the process. It has been shown in several ischemic models including 2VO that ischemia increase brain oxidative stress causing cell dysfunction and apoptosis (Chavez et al., 1995; He et al., 2009). In our study, the role of oxidative stress in a chronic mild cerebral ischemia of unilateral common carotid artery occlusion was studied.

Beside neurons, astrocytes is the most abundant glia cells in brain and performs several major processes for neuronal survival and function. Astrocyte is part of a neurovascular unit important for normal function of nervous system. Astrocytes have been shown to involve in synaptic transmission, maintenance of extracellular glutamate and ions, blood-brain barrier function and glycogen storage. Impairment of astrocyte function would impair glutamate transportation and excitotoxicity. After ischemia, astrocyte water transport increased causing cerebral edema which worsens the ischemic condition (Manley et al., 2000). Astrocyte is also a major site for production of glutathione, a major brain antioxidant molecule (Dringen et al., 1999a; Dringen et al., 1999b; Shanker and Aschner, 2001; Wang and Cynader, 2000). Therefore, impairment of astrocytic function could impair glutathione production favoring oxidative condition in brain.

The effect of cerebral hypoperfusion on astrocyte has been equivocally reported in several studies. Ritchie et. al. showed that chronic cerebral hypoperfusion by bilateral common carotid artery occlusion did not alter the number of astrocyte but decreased the number of astrocytes when stress was present (Ritchie et al., 2004). While de la Torre showed that the number and size of astrocytes were increased in 2VO models (de la Torre et al., 1993). In most studies, the alteration of astrocyte including astrogliosis occurred late in the ischemic process (Farkas et al., 2004; Farkas et al., 2006; Pappas et al., 1996; Schmidt-Kastner et al., 2005). It is therefore very interesting to study the effect of mild chronic cerebral hypoperfusion by unilateral common carotid artery occlusion on the astrocyte.

2 Objectives

To study the effect of mild cerebral ischemia and ischemic duration by unilateral common carotid artery occlusion on

2.1. spatial learning and memory

2.2. hippocampal pathology (alteration of neuron and astrocyte)

2.3. hippocampal oxidative stress

3 Methods

3.1. Animals

Four-month old male Spraque-Dawley rats weighing 350-550 g were used in this study. The rats were obtained from National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom. They were housed under 12-hour light/dark cycle at temperature of 25±2 °C. Food and water were accessed ad libitum. The rats were divided into control, sham and right common carotid artery occlusion (RCO) groups (n=12, each).

3.2. Vascular occlusion

For the RCO, the rats were fasted overnight and then anesthetized with 40 mg/kg body weight of ketamine and 0.5 mg/kg body weight of xylazine intra-muscularly. A ventral cervical incision was performed and the right common carotid artery was identified, separated from nearby vagus nerve and ligated with silk suture. After surgery, the rats were allowed to recover for 1 hour under heating lamp before returning to their cages.

The sham rats undergo the same procedures as the RCO except that the artery was not ligated. The control group received no anesthetic and surgery.

3.3. Assessment for locomotors activity

After surgery, the rats were observed for their locomotors activity including spontaneous activity in the cage, symmetry of four limp movement, forepaw outstretching, climbing activity, body proprioception and response to vibrissae touch.

3.4. Assessment of spatial learning and memory

The radial arm water maze (RAWM) protocol was used in the experiment. The maze was constructed from a circular pool of 200 cm in diameter and 50 cm of height. Polystyrene plates were used to divide the pool into eight radial arms. Each arm was about 15 cm width, 75 cm in length. The pool was filled with water to the height of 30 cm. An escape platform made from transparent cylinder shape glass was located at the end of one arm, 2 cm below the surface of the water. Three extra maze cues of different shape and colors were hanged on three walls of the room. A video-camera fixed to the ceiling above the center of the pool was connected to a computer located in the adjacent room for recording. The room and water temperature were maintained at 20 °C during the experiment. The behavioral protocol was as follow.

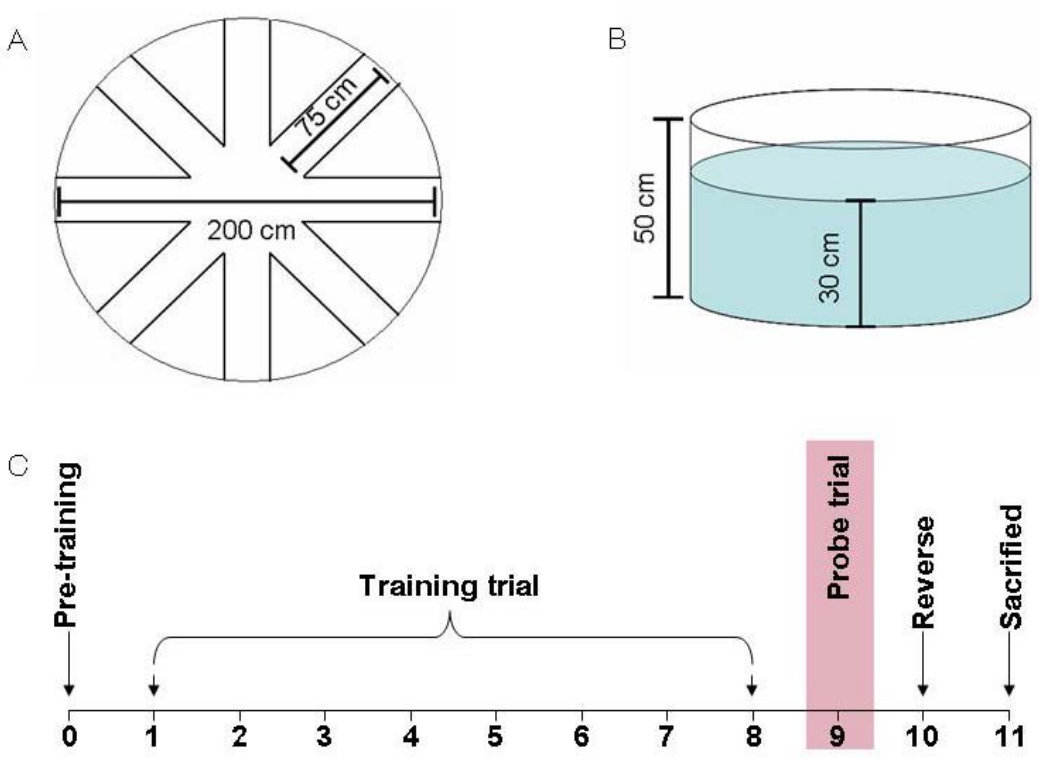


Figure 1 Experimental set up and protocol for spatial learning and memory assessment in RAWM. The 8 day training trial protocol was showed (c). For the 5 day training trial protocol, the sequence was similar to the 8-day protocol but with only 5 days of training trial.

Pretraining: The rats were allowed to swim for 60 s without hidden platform in the RAWM and extra-maze cues on the wall. The aims of this pretriainning trial were to acquaint the rats with the RAWM and also to observe their locomotor activity.

Training trial: The training trial consisted of 8 consecutive day protocol. On each day, each rat was given 4 one-minute trials with the interval of 30 minutes between trials. For each trial, the rats were place in the center of the pool facing different direction and allowed 60 s to find the escape platform. When the rats could not find the platform in 60 s, they were guide to the platform and allowed to stay on the platform for 10 s to get familiar. The mean escape latency was analyzed offline from the recorded.

Later, the 8-day training trial protocol was shorten to 5-day protocol and the rats were place in different arms of RAWM at the beginning instead of placing at the center.

Probe trial: Twenty four hours after the completion of the training trials, the rats were place in the RAWM without the hidden platform. The rats were allowed to swim for 60 s. The time spent in the target arm (previously occupied by the hidden platform) and the swimming paths were analyzed from the video record.

Reverse trial: The next day after the probe, the rats were given 4 one-minute trials with the interval of 30 minutes similar to the training trial except that the platform was placed on the opposite arm. The mean escape latency was analyzed offline from the recorded.

3.5. Brain biochemical study

After the completion of the behavioral tasks, the rats were deeply anesthetized with high dose of anesthetic drugs (48 mg/kg body weight of ketamine and 0.6 mg/kg body weight of xylazine intra-muscularly). The rats were then transcardially perfused with 200 ml of iced-cold phosphate buffer saline (PBS) to remove residual blood. Brains were quickly removed and hippocampi were dissected from both left and right hemispheres of the brains. Hippocampi were then homogenized with tissue homogenizer in ice-cold homogenize buffer (PBS with 0.1M EDTA, pH 7.4). The homogenized suspension was centrifuged at 14,000 g for 30 minutes at 4 °C. The supernatant was transferred to a new vessel and stored in -70 °C freezer until assayed (Gupta et al., 2003; Zhu et al., 2006).

3.5.1. Protein assay

Total protein concentration in each sample was assay using standard Bradford technique. Briefly, 20 μl of reaction was added into 1 ml of 20% Bradford reagent. The absorbance was read at 595 nm wavelength. Standard curve of protein concentration was prepared from dilution of albumin.

3.5.2. Lipid peroxidation assay

The level of malondialdehyde (MDA) was used as an indicator for lipid peroxidation. The reaction mixture consisted of 200 μl of sample, 750 μl of 20% acetic acid (pH3.5), 750 μl of 0.8% thiobarbituric acid, 100 μl of 8.1% sodium dodecyl sulfate (SDS). The reaction mixture was boiled in 100 °C water bath for 60 minutes before cooling down with tap water. Then, 2.5 ml of n-butanol was added. The mixture was vortexed vigorously and centrifuged at 2000 g for 10 minutes. The organic layer was removed to a cuvette. The absorbance was

measured at 532 nm wavelength using a spectrophotometer. The concentration of MDA was expressed as nmol/mg protein. A standard curve was prepared from 0-20 μM of malondialdehyde bis (dimethy acetal, 99%) in water.

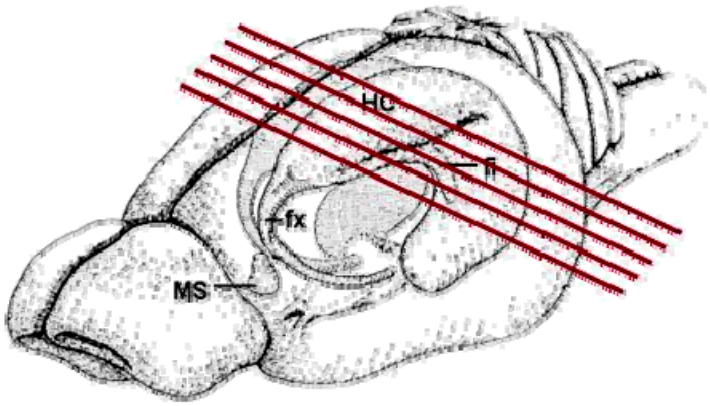
3.5.3. Reduced glutathione (GSH) assay

Glutathione was measured according to the method of Gupta et al. (Gupta et al., 2003). At first, protein in the sample was precipitated by adding 50 μl of 10% trichloroacetic acid (TCA) to an equal volume of sample. The reaction was then centrifuged at 1500 rpm for 15 minutes. Then, 20 μl of supernatant was transferred to a well of flat bottom microplate (96 wells) containing 200 μl of PBS (pH 8.4). 20 μl of 5'5-dithiobis (2-nitrobenzoic acid) was added into the reaction. The absorbance was read at 412 nm within 15 minutes. The concentration of reduced glutathione was expressed as nmol/mg protein. Standard curve of reduced glutathione ranging from 0-400 μM was prepared from commercially available glutathione.

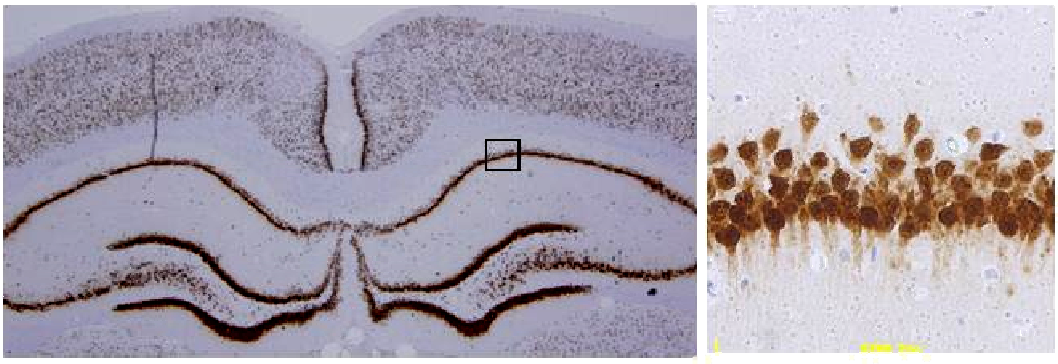
3.6. Histopathological study

After vascular occlusion for 1 day, 3 days, 7 days, 2 weeks, 1 month, 2 months and 4 months, the rats were deeply anesthetized with high dose of anesthetic drugs (48 mg/kg body weight of ketamine and 0.6 mg/kg body weight of xylazine intra-muscularly). The rats were then transcardially perfused with 0.9% saline for 10 minutes followed by 4% paraformaldehyde in 0.2 M PBS (pH7.4) for 10 minutes. Brain was quickly removed and post-fixed in 4% paraformaldehyde for 24 hours. The brain was then embedded in paraffin. Coronal sections of brain at 5 μm thickness were prepared using microtome. Sections from bregma-3.14 to -3.60 mm according to Paxinos and Watson which contained hippocampus were subjected for immunostaining with antibody to neuron specific nuclear protein (NeuN) and glial fibrillary acidic protein (GFAP). Briefly, the paraffin sections were deparaffinized in 45 °C water bath and mounted on glass slide coating with Silane. The slides were dried overnight at room temperature and 6 hours in 60°C incubator. The sections were incubated with primary antibodies against NeuN or GFAP (1:5,000 and 1:8,000 dilution, respectively) overnight. Then, the sections were incubated with secondary antibody conjugated with horse radish peroxidase (HRP) for 30 minutes and with diaminobenzidine (DAB) for 5 minutes. The immunohistochemical stained slides were examined under light microscope. Pictures were captured at magnification of 200x and 100x for anti-NeuN and anti-GFAP stained sections.

A. Section of brain



B. NeuN Immunoreactivity



C. GFAP-N Immunoreactivity

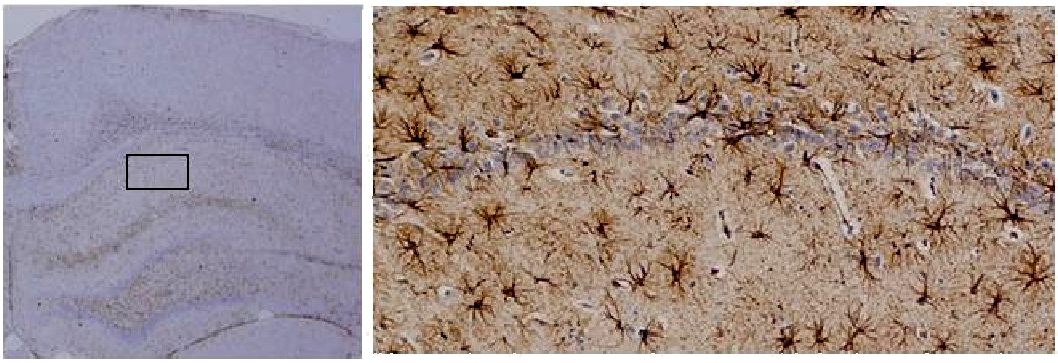


Figure 2 Immunohistochemical study of hippocampus. Coronal sections of hippocampus were prepared from brain at bregma-3.14 to -3.60 mm (A). After immunostaining with ant-NeuN or anti-GFAP, photomicrographs were taken from sections (B and C respectively). The

magnified pictures on the right of B and C were taken from the CA1 as indicated by square and rectangle for neurons and astroglia studies, respectively.

The number and morphology of neuron and astroglia were studied. Five sections with 45 μm spacing between each section were taken for the studies. The area of 500x500 and 750x1500 μm^2 at CA1 hippocampus as indicated in figure 2 were taken for quantification of the number of neurons and astroglia respectively. The number of neurons and astroglia in CA1 hippocampus were counted by the free UTHSCSA ImageTool program (University of Texas Health Science Center, San Antonio, Texas, USA.) available from internet by anonymous FTP (ftp://www.maxrad6.uthscsa.edu). Sections were analyzed by an investigator who was blinded for the experimental design. The neuronal number was corrected with a multiplication factor from the Abercombie method calculating from thickness and the average cell diameter. The mean cell size was also obtained by measuring the cross-sectional area of every cell counted.

$$P = A (M/L+M)$$

P = average number of cells per section.

A = crude count of number of cells seen in the section.

M = thickness (μm) of the section

L = average cell diameter (μm) in the section.

3.7. Statistic analysis

Data are presented as mean ± standard error of the mean (SEM). For behavioral studies, between groups and within groups comparison were analyzed using Kruskall-Wallis ANOVA followed by Mann-Whitney U-test and Firedman's ANOVA followed by Wilcoxon signed-rank test, respectively (StatView software, SAS Institute, NC, USA). Mann-Whitney U-test was used for analysis of brain lipid peroxidation, reduced glutathione and quantity of neurons and astroglia. $P \leq 0.05$ was considered statistically significant difference.

4 Results

4.1. Effects of right common carotid artery occlusion on spatial learning and memory

Rats subjected to right common carotid artery occlusion (RCO) did not show any sign of locomotors deficit when compared with sham and control. The body weight and tail blood pressure was also not different from those of sham and control groups. After two and six months of RCO, the spatial learning and memory were assessed using five or eight days radial arm water maze protocols as explained in the methods

As shown in figure 3 and 4, the mean escape latencies measured at two months after arterial occlusion was slightly longer in the RCO group. This difference in mean escape latency between sham (27.2 ± 3.2 s, n=12) and RCO (37.7 ± 4.1 s, n=11) was significant on the 5th training day of the five days protocol (p=0.017).

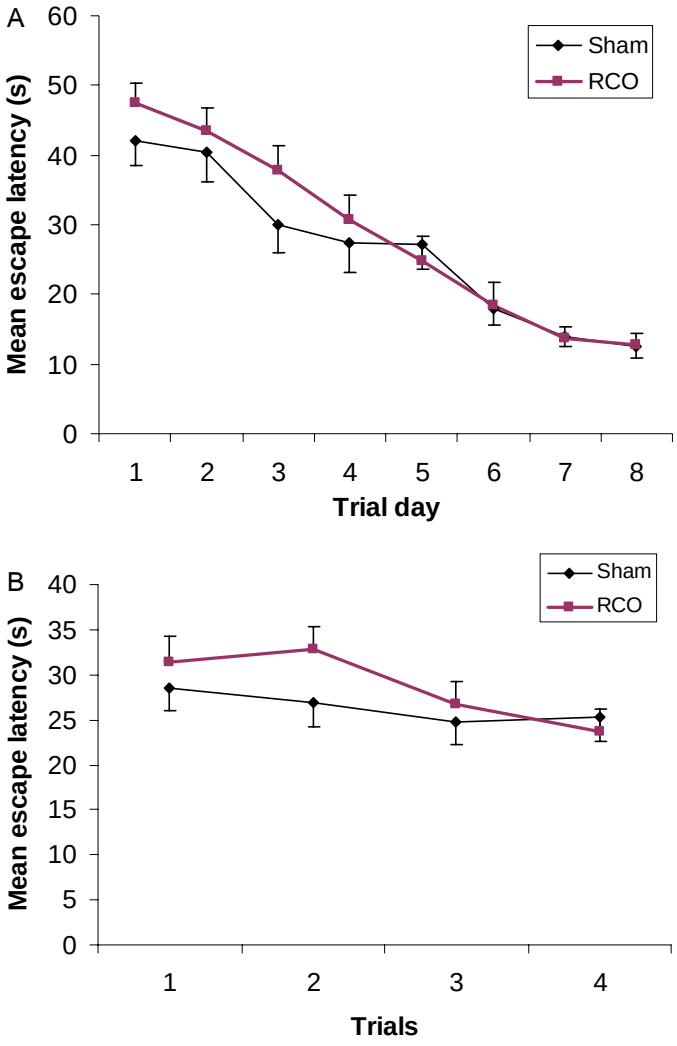


Figure 3 The mean escape latency during the 8-day radial arm water maze protocol in sham (n=7) and RCO (n=8) groups at 2 months after surgery. The data were displayed by days (A) and by trials (B).

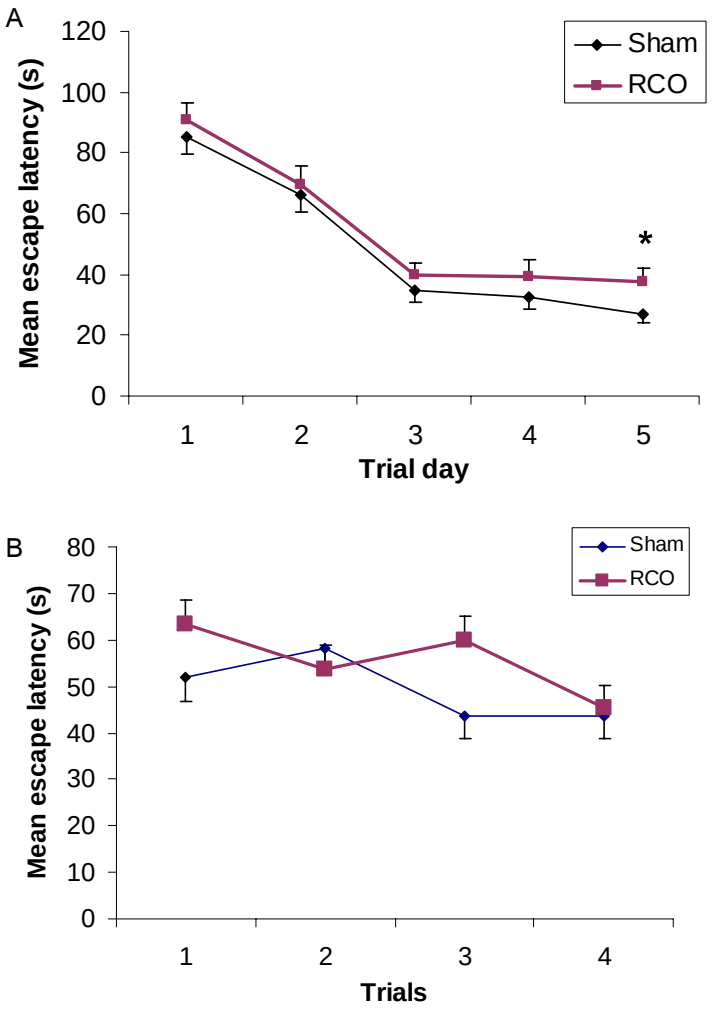


Figure 4 The mean escape latency during the 5-day radial arm water maze protocol in other series of sham (n=12) and RCO (n=11) groups at 2 months after surgery. The data were displayed by days (A) and by trials (B).

After the completion of five or eight days radial arm water maze protocols, the probe test was implemented. The mean time spent in the target arm (previously occupied by the platform) of the RCO group were shorter than those of sham in both the eight-day (time spent in target quadrant for sham and RCO groups were 15.75 $\pm$ 3.79 and 9.25 $\pm$ 2.93 s) and five-day (time spent in target quadrant for sham and RCO groups were 18.08 $\pm$ 2.50 and 14.91 $\pm$ 2.38 s) protocols as shown in figure 5. This result was consistent with the swimming pattern in the radial arm water maze where RCO rats showed diffused pattern of entranced into all arms of the maze while the sham rats entered mainly the target arm or the apposed arms (figure 6).

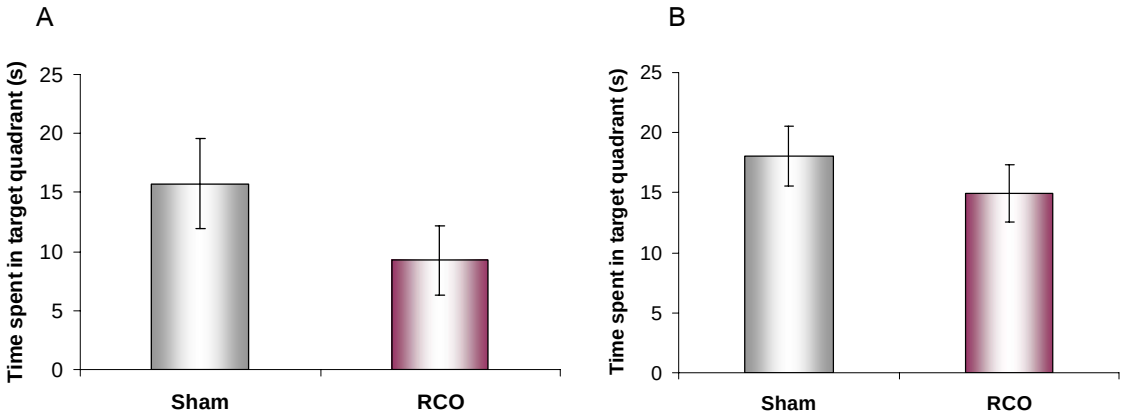


Figure 5 The mean time spent in target quadrant during the probe trial of sham and RCO groups after the 8-day (A) and 5-day (B) radial arm water maze protocol.

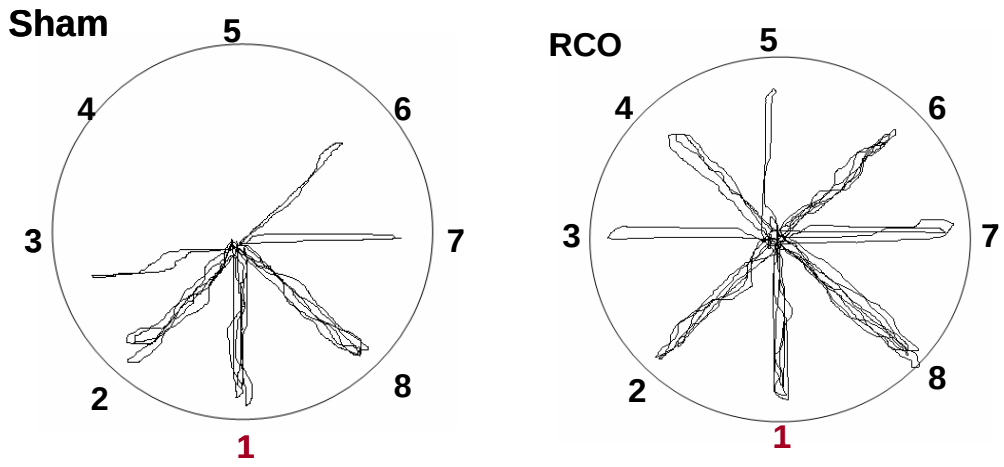


Figure 6 The swimming pattern of the sham and RCO rats during one-minute probe trial. The numbers indicated the arms of the radial arm water maze. Number 1 was the target arm. The patterns were the average of 4 rats in each group.

At the reversal trial, the RCO group took longer time to find the platform (figure 7). As shown in figure 7B, the difference of mean escape latency during the reversal trial were 19.67 $\pm$ 2.20 s for sham (n=6) and 46.35 $\pm$ 11.46 s for RCO (n=5). This difference was significant statistically (p= 0.01).

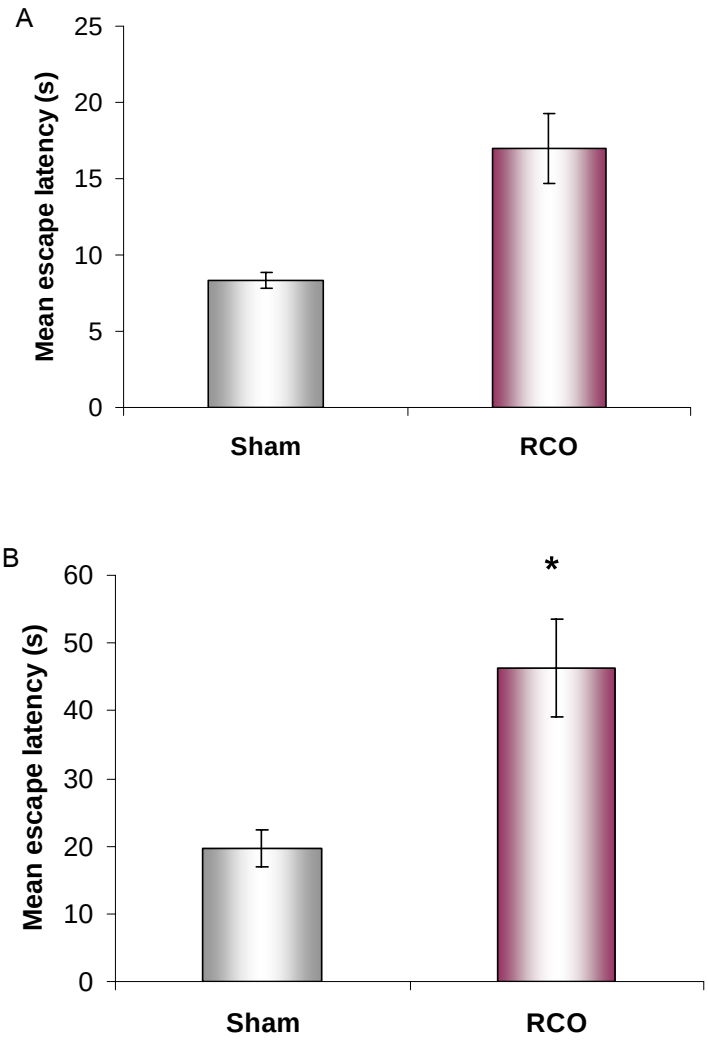


Figure 7 The mean escape latencies during reversal trial in sham and RCO after two months of arterial occlusion. * indicates p value of less than 0.05.

In another series of experiments, the rats were subjected for the same radial arm water maze protocol after arterial occlusion for 6 months. Similar results as those of two-month occlusion were observed. The mean escape latency during the 5-day radial arm water maze trials was longer for the RCO group especially on the 4th and 5th day of the protocol (figure 8A). The probe trial performed the next day after the completion of the 5-day trial protocol also showed that the RCO group spent less time in the target quadrant when compared to sham group (figure 8B). The mean escape latency during reversal trial was longer in the RCO group when compared to sham group (figure 8C). However, the difference in probe and reverse trial was not statistically significant.

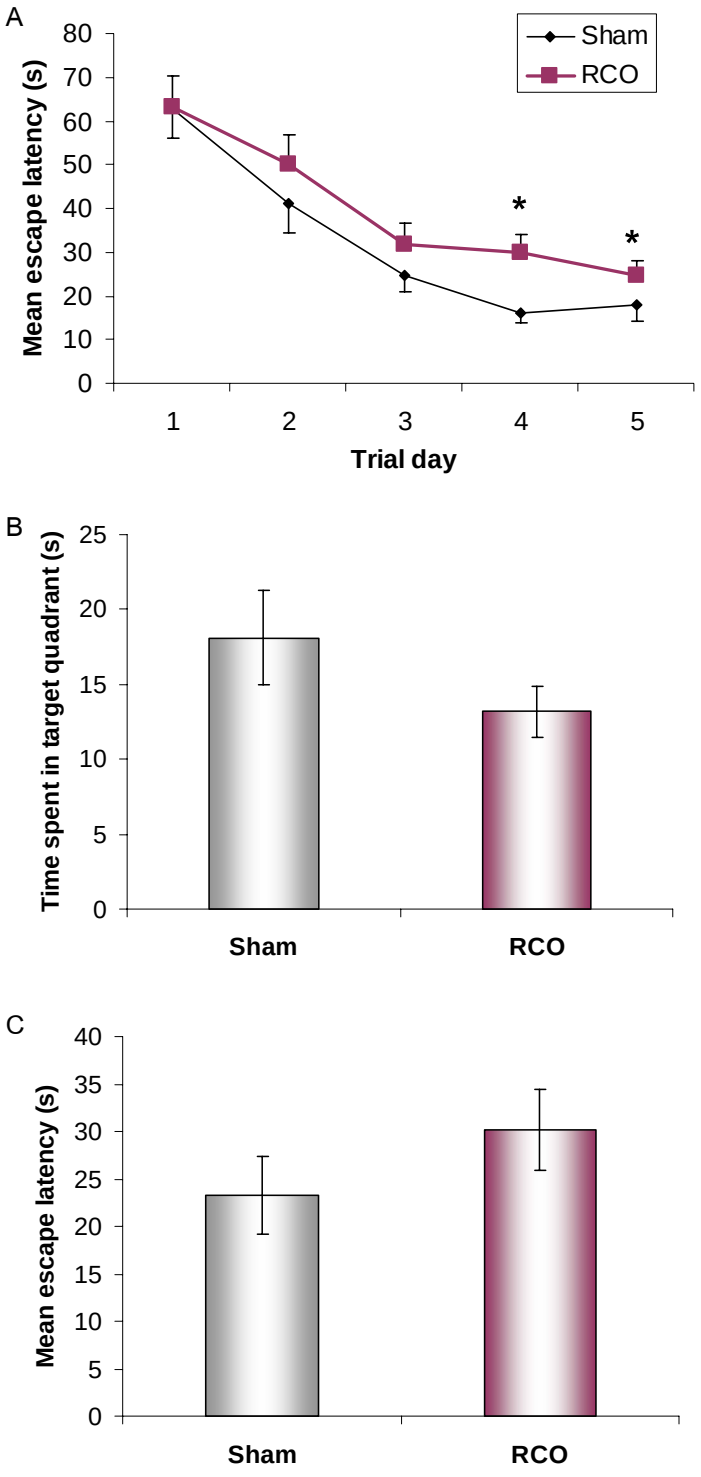


Figure 8 The mean escape latency during the 5-day trial protocol (A), Time spent in target quadrant during the probe trial (B) and mean escape latency during reversal trial (C) in sham and RCO groups at 6 months after surgery (n=8 and 10, respectively).

4.2. Effects of right common carotid artery occlusion on hippocampal oxidative stress

The lipid peroxidation product MDA and the level of antioxidant glutathione were measured in hippocampus of sham and RCO groups at 2 months and 6 months after arterial occlusion.

The MDA level was not statistically different between left and right hippocampi. At 2 months after RCO, the MDA level in the RCO rats was slightly higher than those of sham but not significant statistically (figure 9A). While at 6 months after RCO, no different in the level of MDA was observed (figure 9B). .

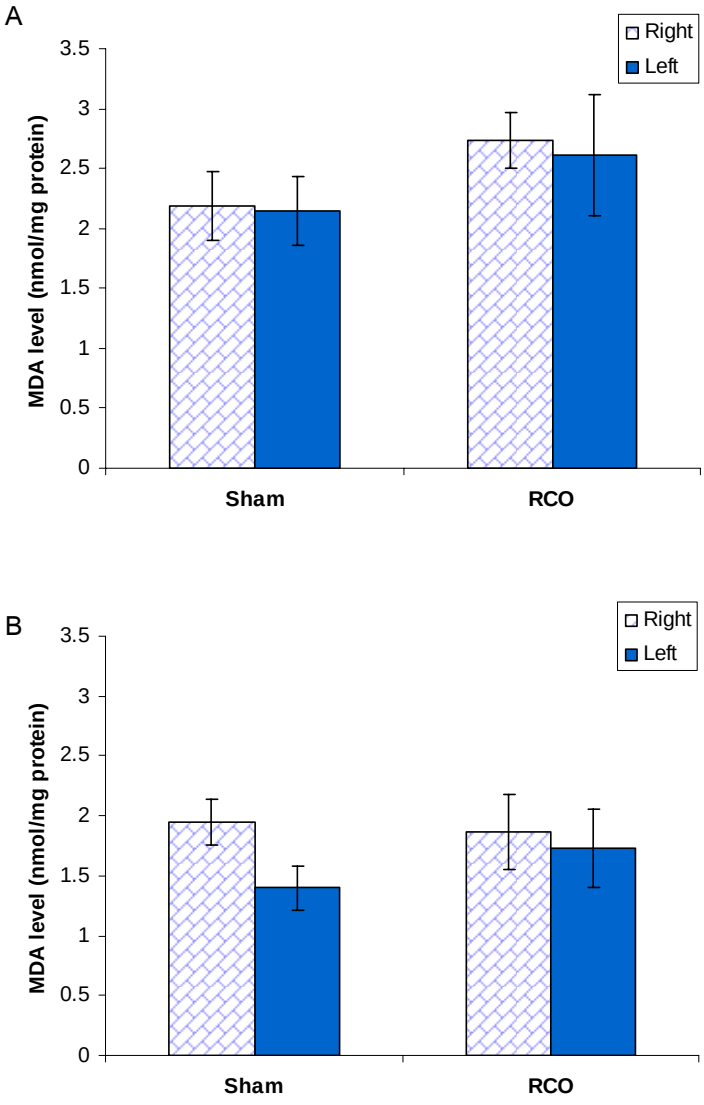


Figure 9 The MDA level in hippocampus from sham and RCO groups at 2 (A, n = 6 for each group) and 6 (B, n = 4 and 6 for sham and RCO, respectively) months after arterial occlusion.

The level of reduced glutathione (GSH) in the RCO group increased in hippocampus of the occluded side. This level was significantly higher than the GSH level in the non-occluded side of the RCO group (428.30 ± 59.01 and 196.66 ± 22.43 nmol/mg protein for right and left hippocampus, respectively). While, at 6 months after arterial occlusion, no alteration in the level of GSH was detected between sham and RCO.

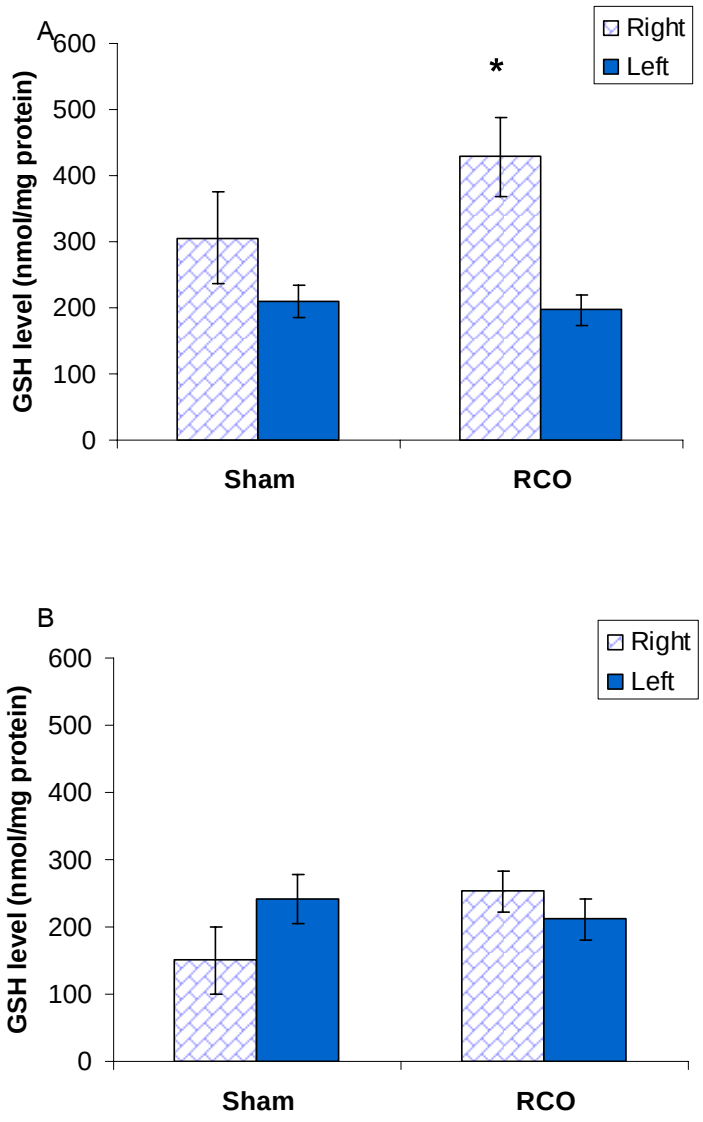


Figure 10 The GSH level in hippocampus from sham and RCO groups at 2 (A, n = 6 for each group) and 6 (B, n = 4 and 6 for sham and RCO, respectively) months after arterial occlusion. * indicated $p < 0.05$)

4.3. Histopathological alteration after various ischemic duration of right common carotid artery occlusion

The number and morphology of neurons and astroglia from sham and RCO at various ischemic durations were studied. No difference in the morphology of both neurons and astroglia were observed. When the numbers of neurons and astroglia were compared between different duration and between sham and RCO, no statistically difference was noted (figure 12 and 14).

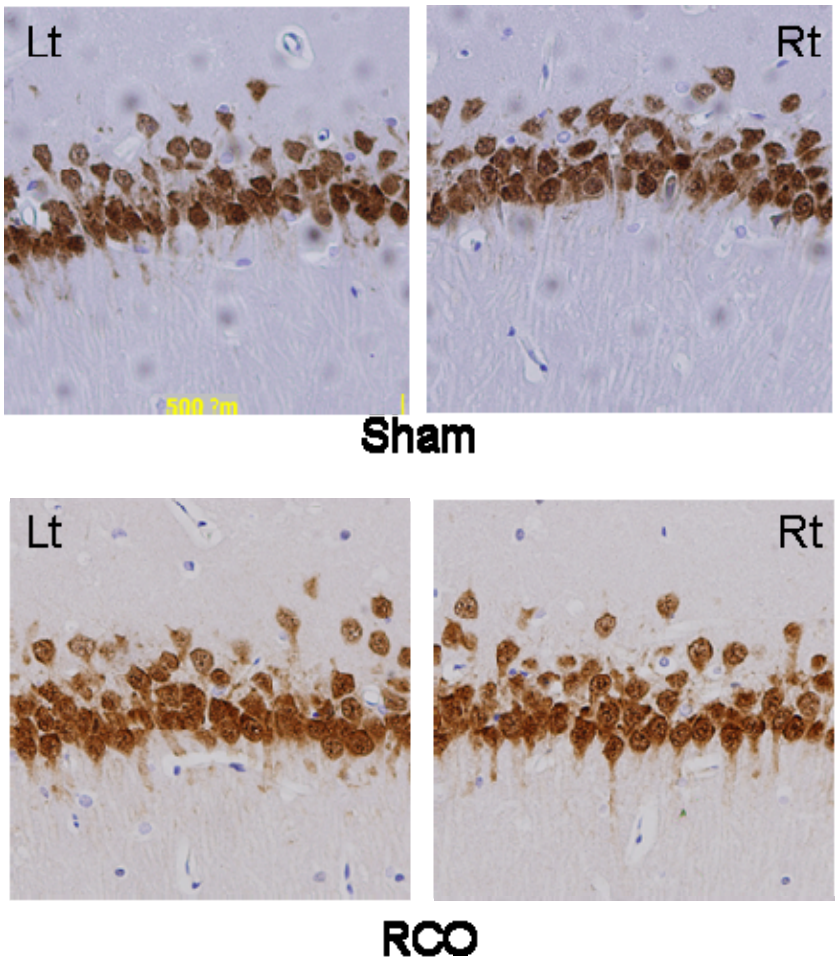


Figure 11 NeuN immunoreactivity in CA1 hippocampus. The representative pictures of left (Lt) and right (Rt) hippocampi of sham and RCO were shown.

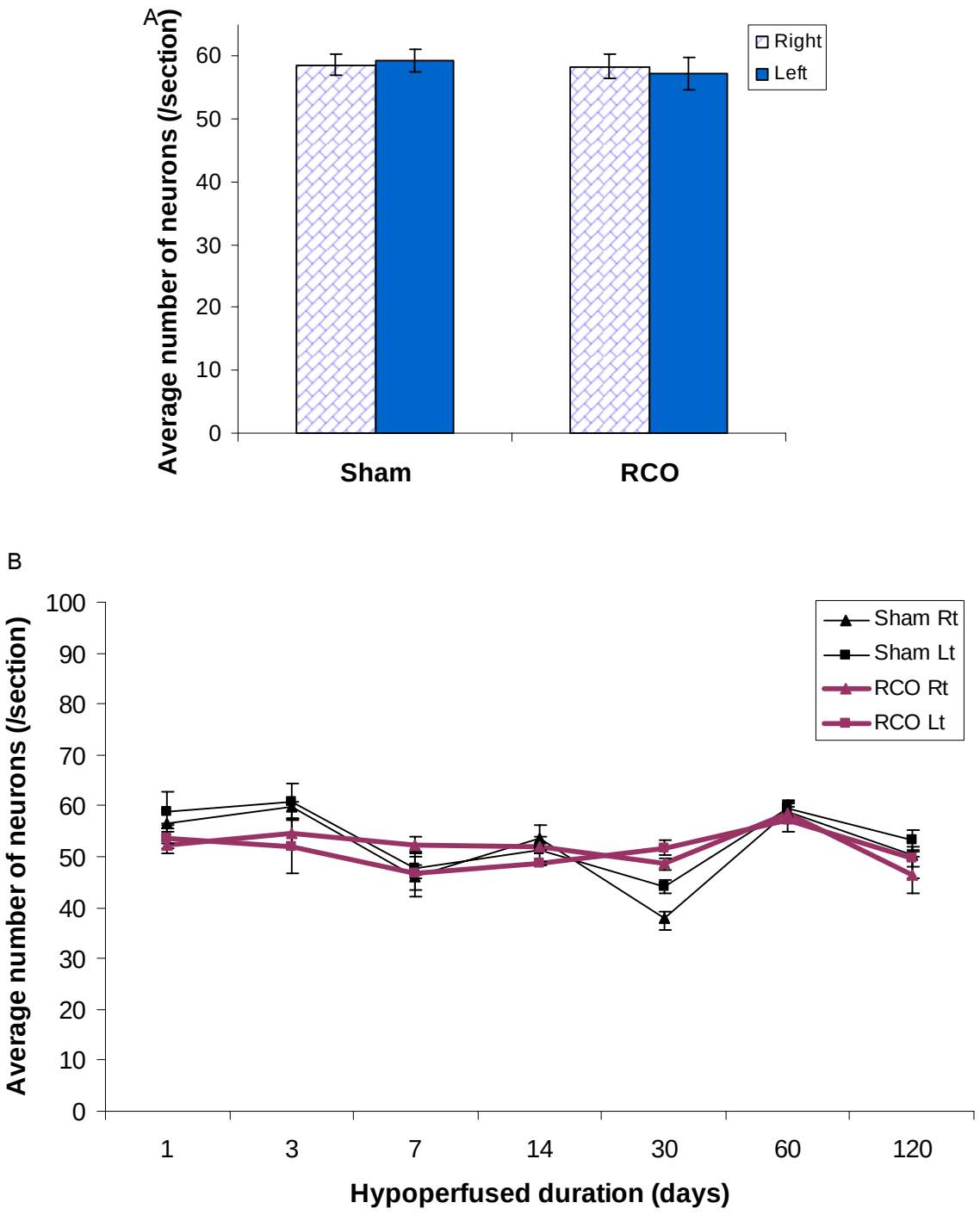


Figure 12 Average number of NeuN-positive neurons in CA1 hippocampus. A) After arterial occlusion for 2 months in sham and RCO (n=8 and 7, respectively). B) Average number of NeuN-positive neurons after various ischemic duration (n=4-8 for each duration).

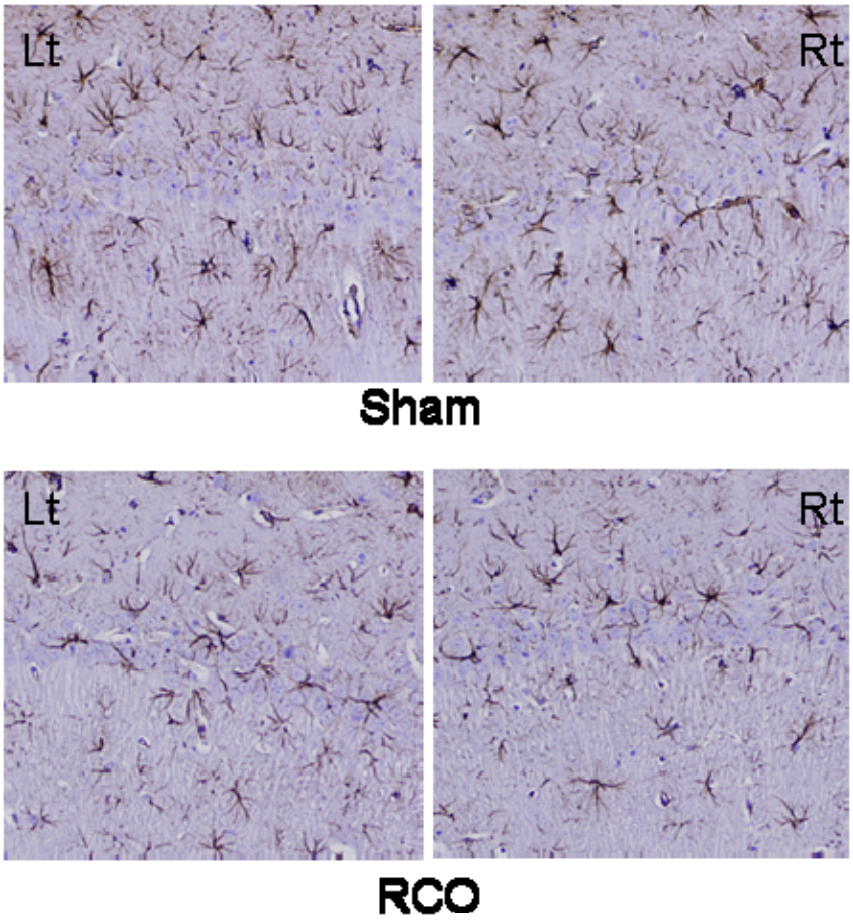


Figure 13 GFAP immunoreactivity in CA1 hippocampus. The representative pictures of left (Lt) and right (Rt) hippocampi of sham and RCO were shown.

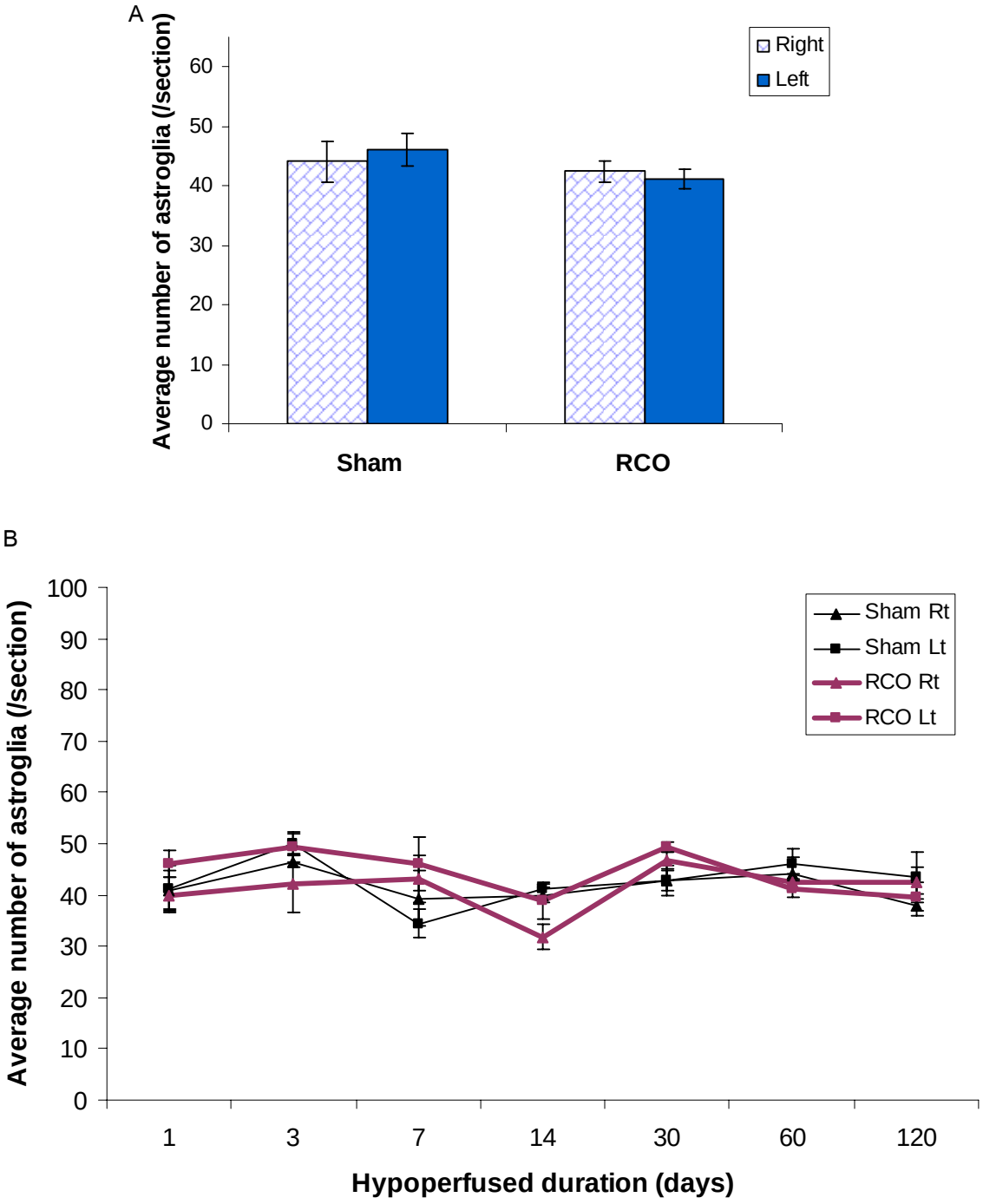


Figure 14 Average number of GFAP-positive cells in CA1 hippocampus. A) After arterial occlusion for 2 months in sham and RCO (n=8 and 7, respectively). B) Average number of GFAP-positive cells after various ischemic duration (n=4-8 for each duration).

5 Discussion

Cerebral ischemia is among the leading causes of disability and death. The severity of the condition depends on the degree and duration of blood flow reduction. Several mechanisms of cerebral ischemia leading to dysfunction of nervous system have been proposed (Taoufik and Probert, 2008). However, little is known about the sequence of occurring of those mechanisms from early to late changes. The basic knowledge of the early reaction of brain to cerebral ischemia starting from the very mild hypoperfusion is of very interesting because it could lead to prevention or early intervention before the condition becomes irreversible. Memory is one of the brain functions that alter early after cerebral hypoperfusion. This is because of the hippocampus, one of the brain area important for memory function, is very sensitive to cerebral hypoperfusion. Cerebral hypoperfusion is a condition encountered by individual during the ageing process (Farkas and Luiten, 2001). This leads to various degrees of memory impairments raging from benign senescent forgetfulness, age-associated cognitive decline (AACD), age-associated memory impairment (AAMI), mild cognitive impairment (MCI) and dementia. Although this process takes time to occur but it could lead eventually to the severe disability as the age progress. As we are in the era of longevity, the prevention and early intervention for this condition is important or loss due to disability and health care cost will happen.

Several models of mild cerebral hypoperfusion have been used for the research. Among this, extracranial vascular occlusion is often mentioned in the literatures. Bilateral common carotid artery occlusion provides a good model for memory impairment after cerebral hypoperfusion. But there are some draw backs as this model can be fatal to animals and motor or visual deficit can interfere with the interpretation of behavioral tasks commonly used for memory assessment. Unilateral common carotid artery occlusion in mice has been shown to cause more than 20% reduction of ipsilateral cerebral blood flow which persisted at least 4 weeks after surgery consistent with cognitive impairment (Yoshizaki et al., 2008). There are few studies about cerebral blood flow and memory impairment from unilateral common carotid artery occlusion in rats. It has been show that the reduction of cerebral blood flow by unilateral common carotid artery occlusion in was mild and transient (De Ley et al., 1985). However, we think that this condition would reduce the circulatory reserve of brain in the long run. We, therefore, studied the effect of permanent unilateral common carotid artery occlusion on spatial learning and memory, brain oxidative stress and histology of hippocampal neurons and astroglia. As shown in the result, mild chronic cerebral hypoperfusion by unilateral common carotid artery occlusion caused mild memory impairment at 2 and 6 months after surgery. This probably is the first report of memory impairment by unilateral common carotid artery occlusion alone. The reason for the memory impairment might not be the result of significant cerebral blood flow reduction but could be the

reduction of brain circulatory reserve that makes animals less flexible to stress encountered. Supporting this idea, the cerebral blood flow after unilateral common carotid artery occlusion was significantly reduced by a stress factor, hypercarbia (De Ley et al., 1985). We though that both the sham and RCO rats in our experiments went through the same stress from long duration housing condition. And stress has been shown to compromise brain function after bilateral common carotid artery occlusion through alteration of hippocampal neurons and astroglia (Ritchie et al., 2004).

The mechanism by which unilateral common carotid artery occlusion leads to cognitive impairment is not known. Oxidative stress has been proposed as an important factor involve with cognitive impairment after cerebral ischemia (Chavez et al., 1995; He et al., 2009). We studied the hippocampal oxidative stress after unilateral common carotid artery occlusion by assaying the lipid peroxidation and reduced glutathione level. Brain is rich with lipid component and oxidative stress could cause oxidation of lipid. Reduced glutathione is a major brain antioxidant mainly produced by astroglia. MDA, an indicator for lipid peroxidation, in hippocampus at 2 and 6 months after artery ligation were not statistically different between sham and RCO eventhough the level was slightly higher in RCO at two month after artery ligation. The only different observed was the level of reduced glutathione in the ipsilateral hemisphere at 2 months after unilateral common carotid artery occlusion. This condition could be the result of compensatory mechanism of antioxidative system as shown as a consequence of mild cerebral hypoperfusion (Choi et al., 2007). Oxidative stress depends on the balance between the oxidant and antioxidant. Even with the proposed compensatory mechanism, the MDA level was slightly higher in the RCO at 2 months after artery ligation and cognitive impairment was still observed. This could indicate the condition favoring oxidative stress in brain. However, others factor could possibly be the cause of this impairment such as glutamate excitotoxicity. Beside, we might have to look for other brain areas involving memory process assessed in our experiment such as the prefrontal cortex.

We further searched if the alteration of hippocampal neurons and astroglia lead to the memory impairment. As shown in the results, both numbers and morphology of neurons and astroglia in hippocampus were not altered by unilateral common carotid artery occlusion. However, it has been reported that functional impairment of neuron can occur before morphological change (Hayashi et al., 2006; Sugawara et al., 2002). This neuronal functional impairment could be the result of several intracellular mechanisms including the reduced expression of cytoskeletal protein MAP-2, a vesicular protein synaptophysin and growth factor BDNF (Hayashi et al., 2006; Liu et al., 2005). Besides, white matter lesion and inflammation in brain had been reported to be correlated with cognitive impairment after chronic cerebral ischemia

by unilateral common carotid artery occlusion in mice (Yoshizaki et al., 2008). These factors could lead to impairment of neural function including long-term potentiation (LTP). Also alteration of number and morphology of astroglia has been accepted to be a late response in mild chronic cerebral ischemia (Farkas et al., 2007). Impairment of astrocyte function has been associated with several neurological conditions (Araque, 2006; Chen and Swanson, 2003). Therefore, the function assessment of neurons and astroglia need further study.

6 Summary

Chronic mild reduction of cerebral blood flow and brain circulatory reserve has been associated with cognitive impairment in elderly and dementia. We have created a rat model of this condition and shown in this study that:

1. Unilateral common carotid artery occlusion for 2 and 6 months could cause spatial memory impairment in RAWM task. The memory impairment could be the result of chronic mild reduction of cerebral blood flow and the reduction of brain circulatory reserve.
2. The increase in level of reduced glutathione at 2 months after RCO could indicate the compensatory mechanism for the oxidative stress from reduction of cerebral blood flow and circulatory reserve. Hence, the lipid peroxidation product, MDA, was only slightly increase without statistical significant different from that of sham.
3. No alteration in numbers and morphology of neurons and astroglia in CA1 hippocampal after arterial occlusion from 1 day to 4 months. This result possibly indicated functional impairment of neurovascular unit, especially neurons and astroglia. However, the memory impairment in this study could be the result of white matter lesion as has been reported.
4. Further study is needed to clarify the functional impairment of neurons and astroglia. And longer duration of hypoperfusion might be applied to accentuate the memory impairment and the correlate change of neurons, astroglia and brain biochemistry.

7 References

1. Araque, A. (2006). Astrocyte-neuron signaling in the brain--implications for disease. Curr Opin Investig Drugs 7, 619-624.
2. Bennett, S.A., Tenniswood, M., Chen, J.H., Davidson, C.M., Keyes, M.T., Fortin, T., and Pappas, B.A. (1998). Chronic cerebral hypoperfusion elicits neuronal apoptosis and behavioral impairment. Neuroreport 9, 161-166.
3. Chavez, J.C., Pichiule, P., Boero, J., and Arregui, A. (1995). Reduced mitochondrial respiration in mouse cerebral cortex during chronic hypoxia. Neurosci Lett 193, 169-172.
4. Chen, Y., and Swanson, R.A. (2003). Astrocytes and brain injury. J Cereb Blood Flow Metab 23, 137-149.
5. Choi, Y.S., Cho, K.O., Kim, E.J., Sung, K.W., and Kim, S.Y. (2007). Ischemic preconditioning in the rat hippocampus increases antioxidant activities but does not affect the level of hydroxyl radicals during subsequent severe ischemia. Exp Mol Med 39, 556-563.
6. de la Torre, J.C., Fortin, T., Park, G.A., Pappas, B.A., and Richard, M.T. (1993). Brain blood flow restoration 'rescues' chronically damaged rat CA1 neurons. Brain Res 623, 6-15.
7. De Ley, G., Nshimyumuremyi, J.B., and Leusen, I. (1985). Hemispheric blood flow in the rat after unilateral common carotid occlusion: evolution with time. Stroke 16, 69-73.
8. Dringen, R., Kussmaul, L., Gutterer, J.M., Hirrlinger, J., and Hamprecht, B. (1999a). The glutathione system of peroxide detoxification is less efficient in neurons than in astroglial cells. J Neurochem 72, 2523-2530.
9. Dringen, R., Pfeiffer, B., and Hamprecht, B. (1999b). Synthesis of the antioxidant glutathione in neurons: supply by astrocytes of CysGly as precursor for neuronal glutathione. J Neurosci 19, 562-569.
10. Farkas, E., Donka, G., de Vos, R.A., Mihaly, A., Bari, F., and Luiten, P.G. (2004). Experimental cerebral hypoperfusion induces white matter injury and microglial activation in the rat brain. Acta Neuropathol 108, 57-64.
11. Farkas, E., Institoris, A., Domoki, F., Mihaly, A., and Bari, F. (2006). The effect of pre- and posttreatment with diazoxide on the early phase of chronic cerebral hypoperfusion in the rat. Brain Res 1087, 168-174.
12. Farkas, E., and Luiten, P.G. (2001). Cerebral microvascular pathology in aging and Alzheimer's disease. Prog Neurobiol 64, 575-611.
13. Farkas, E., Luiten, P.G., and Bari, F. (2007). Permanent, bilateral common carotid artery occlusion in the rat: a model for chronic cerebral hypoperfusion-related neurodegenerative diseases. Brain Res Rev 54, 162-180.

14. Gupta, Y.K., Veerendra Kumar, M.H., and Srivastava, A.K. (2003). Effect of Centella asiatica on pentylenetetrazole-induced kindling, cognition and oxidative stress in rats. Pharmacol Biochem Behav 74, 579-585.
15. Hayashi, Y., Tomimatsu, Y., Suzuki, H., Yamada, J., Wu, Z., Yao, H., Kagamiishi, Y., Tateishi, N., Sawada, M., and Nakanishi, H. (2006). The intra-arterial injection of microglia protects hippocampal CA1 neurons against global ischemia-induced functional deficits in rats. Neuroscience 142, 87-96.
16. He, X.L., Wang, Y.H., Gao, M., Li, X.X., Zhang, T.T., and Du, G.H. (2009). Baicalein protects rat brain mitochondria against chronic cerebral hypoperfusion-induced oxidative damage. Brain Res 1249, 212-221.
17. Lavinsky, D., Arterni, N.S., Achaval, M., and Netto, C.A. (2006). Chronic bilateral common carotid artery occlusion: a model for ocular ischemic syndrome in the rat. Graefes Arch Clin Exp Ophthalmol 244, 199-204.
18. Liu, H.X., Zhang, J.J., Zheng, P., and Zhang, Y. (2005). Altered expression of MAP-2, GAP-43, and synaptophysin in the hippocampus of rats with chronic cerebral hypoperfusion correlates with cognitive impairment. Brain Res Mol Brain Res 139, 169-177.
19. Manley, G.T., Fujimura, M., Ma, T., Noshita, N., Filiz, F., Bollen, A.W., Chan, P., and Verkman, A.S. (2000). Aquaporin-4 deletion in mice reduces brain edema after acute water intoxication and ischemic stroke. Nat Med 6, 159-163.
20. Morgan, M.K., Anderson, R.E., and Sundt, T.M., Jr. (1989). A model of the pathophysiology of cerebral arteriovenous malformations by a carotid-jugular fistula in the rat. Brain Res 496, 241-250.
21. Ni, J., Ohta, H., Matsumoto, K., and Watanabe, H. (1994). Progressive cognitive impairment following chronic cerebral hypoperfusion induced by permanent occlusion of bilateral carotid arteries in rats. Brain Res 653, 231-236.
22. Ouchi, Y., Tsukada, H., Kakiuchi, T., Nishiyama, S., and Futatsubashi, M. (1998). Changes in cerebral blood flow and postsynaptic muscarinic cholinergic activity in rats with bilateral carotid artery ligation. J Nucl Med 39, 198-202.
23. Pappas, B.A., de la Torre, J.C., Davidson, C.M., Keyes, M.T., and Fortin, T. (1996). Chronic reduction of cerebral blood flow in the adult rat: late-emerging CA1 cell loss and memory dysfunction. Brain Res 708, 50-58.
24. Rasmussen, T., Schliemann, T., Sorensen, J.C., Zimmer, J., and West, M.J. (1996). Memory impaired aged rats: no loss of principal hippocampal and subicular neurons. Neurobiol Aging 17, 143-147.
25. Ritchie, L.J., De Butte, M., and Pappas, B.A. (2004). Chronic mild stress exacerbates the effects of permanent bilateral common carotid artery occlusion on CA1 neurons. Brain Res 1014, 228-235.

26. Schmidt-Kastner, R., Aguirre-Chen, C., Saul, I., Yick, L., Hamasaki, D., Busto, R., and Ginsberg, M.D. (2005). Astrocytes react to oligemia in the forebrain induced by chronic bilateral common carotid artery occlusion in rats. Brain Res 1052, 28-39.
27. Shanker, G., and Aschner, M. (2001). Identification and characterization of uptake systems for cystine and cysteine in cultured astrocytes and neurons: evidence for methylmercury-targeted disruption of astrocyte transport. J Neurosci Res 66, 998-1002.
28. Sugawara, T., Lewen, A., Noshita, N., Gasche, Y., and Chan, P.H. (2002). Effects of global ischemia duration on neuronal, astroglial, oligodendroglial, and microglial reactions in the vulnerable hippocampal CA1 subregion in rats. J Neurotrauma 19, 85-98.
29. Taoufik, E., and Probert, L. (2008). Ischemic neuronal damage. Curr Pharm Des 14, 3565-3573.
30. Tsuchiya, M., Sako, K., Yura, S., and Yonemasu, Y. (1993). Local cerebral glucose utilisation following acute and chronic bilateral carotid artery ligation in Wistar rats: relation to changes in local cerebral blood flow. Exp Brain Res 95, 1-7.
31. Wang, X.F., and Cynader, M.S. (2000). Astrocytes provide cysteine to neurons by releasing glutathione. J Neurochem 74, 1434-1442.
32. Weinstock, M., and Shoham, S. (2004). Rat models of dementia based on reductions in regional glucose metabolism, cerebral blood flow and cytochrome oxidase activity. J Neural Transm 111, 347-366.
33. Yoshizaki, K., Adachi, K., Kataoka, S., Watanabe, A., Tabira, T., Takahashi, K., and Wakita, H. (2008). Chronic cerebral hypoperfusion induced by right unilateral common carotid artery occlusion causes delayed white matter lesions and cognitive impairment in adult mice. Exp Neurol 210, 585-591.
34. Zhu, Y., Carvey, P.M., and Ling, Z. (2006). Age-related changes in glutathione and glutathione-related enzymes in rat brain. Brain Res 1090, 35-44.

Output ที่ได้จากโครงการ

Presentations and Publications

1. Tidaporn Wongsuthin, Chatsumal Pholseang, Ratana Ninthuk, Kanokwan Tilokskulchai, Uraiwan Panich, Suwattanee Kooptiwut and Sompol Tapechum

   Effect of mild chronic cerebral ischemia on learning, memory and lipid peroxidation in adult male rats

   FAON symposium 2008, Bangkok, Thailand. May 13-17, 2009. (Proceeding)

2. Tidaporn Wongsuthin, Chatsumal Pholseang, Ratana Ninthuk, Kanokwan Tilokskulchai, Uraiwan Panich, Suwattanee Kooptiwut and Sompol Tapechum

   Effect of mild chronic cerebral ischemia on learning, memory and brain oxidative stress in adult male rats.

   Physiological society of Thailand meeting 2009, Petchboon, Thailand. April 1-3, 2009. (Oral presentation)

3. Anyarath Tonmat, Sompol Tapechum, Chatsumal Srimongkol, Ratana Ninthuk, Kanokwan Tilokskulchai

   Effect of mild chronic cerebral ischemia on hippocampal CA1 astroglial reaction and role in spatial memory.

   Physiological society of Thailand meeting 2007, Ayuthya, Thailand.

4. Sompol Tapechum, Anyarath Tonmat, Kanokwan Tilokskulchai.
   Effect of chronic mild global cerebral hypoperfusion on spatial memory in rats
   Thailand research fund 2007, The regent hotel, Cha-um, Petchburi, Thailand.

Expected Further Publications

1. Effect of mild chronic cerebral hypoperfusion on brain oxidative stress, hippocampus and spatial learning and memory

Effect of mild chronic cerebral ischemia on learning, memory and lipid peroxidation in adult male rats

Tidaporn Wongsuthin[1], Chatsumal Pholseang[1], Ratana Ninthuk[1], Kanokwan Tilokskulchai[1], Uraiwan Panich[2], Suwattanee Kooptiwut[1] and Sompol Tapechum[1]

[1]Department of Physiology, [2]Department of Pharmacology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

Background and Objective

Memory impairment in elderly presents in various degrees ranging from age-related memory loss, mild cognitive impairment (MCI) and dementia. MCI is particularly interesting because it is the pre-state of dementia, 50% of MCI turns to dementia in 3-5 years(1). Though extensive researches have been invested, the pathogenesis of the transition from each stage of memory impairment is not well characterized. One factor consistently discovered in both normal and demented elderly is the reduction of cerebral blood flow. Neurons are very sensitive to cerebral blood flow reduction especially in the sensitive area such as hippocampus and cerebral cortex. Only 5 minutes of global ischemia can cause delayed death in almost all CA1 pyramidal neurons. The reduction of blood supply during cerebral ischemia produces oxidative stress, alterations in calcium homeostasis and endothelial damage, which coincide with reduced efficacy of amyloid clearance and increasing cerebrovascular disease. These events cause synaptic damage, transmitter and receptor loss, and inflammation in brain which leads eventually to neuronal dysfunction and death (2). Neurons and glial cells may adapt to the problem of reduction of cerebral blood flow by increasing their ability to cope with stress, compensating for lost or damaged cells by producing new neurons and glial, and remodeling neuronal circuits (3).

Hippocampal lesions following cerebral blood flow reduction produce a functional impairment of the brain, which present as behavioral changes including loss of spatial memory (4). The degree of cerebral blood flow reduction is correlated with the severity of memory impairment. The long-lasting cerebral blood flow reduction by permanent bilateral common carotid arteries occlusion combined with aging can result in the enhanced cognitive impairment of aged individuals(5). The

minor degree of cerebral blood flow reduction would then reveal the early change of the memory impairment process in elderly (1, 6). The aims of this study were to establish that mild reduction of cerebral blood flow for a considerable period would lead to memory dysfunction and to reveal the underlying biochemical alteration particularly the oxidative stress that followed the cerebral blood flow reduction.

Materials and Methods

Adult male Sprague-Dawley rats (4-month old) were randomly allocated in to sham operated (sham) or permanent right common carotid artery occlusion (RCO) groups. For surgery, the rats were anesthetized with ketamine (40mg/kg) and xylazine (0.5mg/kg) injected intra-muscularly. Right common carotid artery was permanently occluded with single silk suture. After surgery, the rats were observed for their level of consciousness and allowed to recover for 1 hour under heating lamp before returning to their cages.

Spatial learning and memory was assessed at 6 month after occlusion by using radial arm water maze (RAWM). RAWM is a circular pool (200 cm diameter x 50 cm high) filled to a depth of 30 cm with water located in a room with cues for spatial orientation. The maze consists of eight radial arms (30 cm width x 60 cm long x 50 cm high) projected from the center. An invisible platform was hidden 2 cm below the surface of the water in a fixed location in one of the eight arms. The rats were given 4 training trials with a 30-min interval, on each day for 5 consecutive days. The duration the rats used to reach the platform(escape latency) was recorded in second. The animal's performances were recorded using an overhead video camera. The probe trial was performed 24 hours after the last trial of the final day. The rats were allowed to swim with no platform for 60 seconds. The spatial memory was determined by the time that the rats spent in the arm previously occupied by the platform. The escape latency was analyzed by repeated measures ANOVA followed by Fisher's post hoc test and the time spent in the target arm was analyzed by student t-test.

Following the behavioral test, the rats were sacrificed and brains were dissected for measurement of malondialdehyde (MDA), a marker of lipid peroxidation, as described by Gupta et al.(7),Lei et al.(8) and Ohkawa et al. (9). Briefly, the brain tissues from sham or RCO were divided into two subgroups consisting of whole brain (sham, n=2 and RCO, n=3), and hippocampus (sham, n=2 and RCO, n=3). The brain tissues were homogenized in ice-cold buffer (0.1 M PBS,

pH 7.4 and 0.1 mM EDTA) and centrifuged at 14,000 x *g* for 30 min at 4°C. The supernatant was employed for analysis. Each reaction contained 0.2 ml of processed tissue sample, 0.75 ml of 20% acetic acid (pH 3.5), 0.75 ml of 0.8% thiobarbituric acid and 0.1 ml of 8.1% sodium dodecyl sulphate. The reactions were then heated at 100°C for 60 min and cooled with tap water. The mixture were added with 2.5 ml of *n* – butanol and vortexed vigorously. After centrifugation at 2000 x *g* for 10 min, the organic layer was separated and the absorbance at 532 nm was measured using a spectrophotometer. The protein content in the sample was measured using Bradford method. The MDA concentration (nmol/µg protein) were calculated from standard curve and analyzed by student t-test.

Results and discussion

The behavioral performance of the rats subjected to sham or RCO operation were examined 6 months after surgery. The performance in the RAWM during five day training showed improvement in both sham and RCO groups as demonstrated by reduction of escape latency. When compared between sham and RCO groups, the mean escape latencies from the 2^{nd} -5^{th} day were longer in the RCO group, especially on the 4^{th} day (mean ± S.E.M.: 29.925 ± 3.980 vs. 15.969 ± 2.138, $P < 0.01$). In the probe test, the RCO group spent shorter duration in the target arm (time spent; mean ± S.E.M.: 13.200 ± 1.711 vs. 18.125 ± 3.193) compared with sham group. However, there was no significant difference in spatial memory between groups ($P = 0.17$).

MDA level, a maker of oxidative stress, in RCO groups were higher than that of sham group in whole brain (mean ± S.E.M.(nmol/µg protein): 418.912± 27.382 for RCO, 342.873± 48.598 for sham). Similarly, the MDA level in hippocampus was higher in RCO group (317.670± 95.988 for RCO, 230.057± 19.847 for sham). There was no significant difference in all MDA levels between groups.

The present study suggested that mild chronic cerebral ischemia induced by permanent right common carotid artery occlusion caused spatial learning and memory impairment. The rise in MDA level in RCO group indicates increased free radical, which could responsible for producing for the neuronal changes mediating the behavior deficit. Eventhough this study showed potential spatial learning and memory impairment and oxidative stress by cerebral blood flow reduction, further studies need to be performed because the small sample size was used in this study.

In conclusion, the mild chronic cerebral ischemia induced by RCO resulted in impairment of spatial learning. The corresponding increase of lipid peroxidation could possibly suggest the role of oxidative stress in the pathogenesis of memory impairment in this rat models.

Summary

Adult male Sprague-Dawley rats (4-month old) were subjected to permanent right common carotid artery occlusion for 6 months (RCO). Radial arms water maze test was assessed 6 months after occlusion. Following the behavioral test, the rats were sacrificed and the hippocampus was dissected for estimation of malondialdehyde (MDA), a marker of lipid peroxidation. These brains were divided into two subgroups of whole brain (sham, n=2 and RCO, n=3), and hippocampus (sham, n=2 and RCO, n=3). After mild chronic cerebral hypoperfusion insult, the rats subjected to RCO resulted in a significant deficit in spatial learning assessed by RAWM (indicated by escape latency on day 4^{th} $P< 0.05$). the MDA level in RCO groups were higher than that of sham group in whole brain and hippocampus, but not significant statistically because the small sample size was used in this study. The study revealed that mild chronic cerebral ischemia induced by RCO resulted in impairment of spatial learning. The corresponding increase of lipid peroxidation could possibly suggest the role of oxidative stress in the pathogenesis of memory impairment in this rat models.

Acknowledgement

This work was supported by Siriraj Graduate Thesis Schorlarship and Thailand research fund.

References

1. Meyer JS, Quach M, Thornby J, Chowdhury M, Huang J. MRI identifies MCI subtypes: vascular versus neurodegenerative. Journal of the Neurological Sciences. 2005;229-230:121-9.

2. Lipton P. Ischemic cell death in brain neurons. Physiological Reviews. 1999;79:1431-568.

3. Mattson MP, Chan SL, Duan W. Modification of Brain Aging and Neurodegenerative Disorders by Genes, Diet, and Behavior. Physiol Rev. 2002;82:637-72.

4. Broadbent NJ, Squire LR, Clark RE. Spatial memory, recognition memory, and the hippocampus. PNAS. 2004 October 5, 2004;101:14515–20.

5. Sopala M, Danysz W. Chronic cerebral hypoperfusion in the rat enhances age-related deficits in spatial memory. J Neural Transm. 2001;108(12):1445-56.

6. Petersen; RC, Doody; R, Kuez; A, Mohs; RC, C.Morris; J, Rabins; PV, et al. Current Concepts in Mild Cognitive Impairment. Arch Neurol. 2001;58:1985-92.

7. Gupta YK, Veerendra Kumar MH, Srivastava AK. Effect of Centella asiatica on pentylenetetrazole-induced kindling, cognition and oxidative stress in rats. Pharmacology Biochemistry and Behavior. 2003;74(3):579-85.

8. Lei M, Hua X, Xiao M, Ding J, Han Q, Hu G. Impairments of astrocytes are involved in the d-galactose-induced brain aging. Biochemical and Biophysical Research Communications. 2008;369(4):1082-7.

9. Hiroshi Ohkawa, Nobuko Ohishi, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry. 1978;95:351-8.

Effect of mild chronic cerebral ischemia on learning, memory and lipid peroxidation in adult male rats

Tidaporn Wongsuthin[1], Chatsumal Pholseang[1], Ratana Ninthuk[1], Kanokwan Tilokskulchai[1], Uraiwan Panich[2], Suwattanee Kooptiwut[1] and Sompol Tapechum[1]

[1]Department of Physiology, [2]Department of Pharmacology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

Background and Objective

Memory impairment in elderly presents in various degrees ranging from age-related memory loss, mild cognitive impairment (MCI) and dementia. MCI is particularly interesting because it is the pre-state of dementia, 50% of MCI turns to dementia in 3-5 years. Though extensive researches have been invested, the pathogenesis of the transition from each stage of memory impairment is not well characterized. One factor consistently discovered in both normal and demented elderly is the reduction of cerebral blood flow. Neurons are very sensitive to cerebral blood flow reduction especially in the sensitive area such as hippocampus and cerebral cortex.

Hippocampal lesions following cerebral blood flow reduction produce a functional impairment of the brain, which present as behavioral changes including loss of spatial memory. The degree of cerebral blood flow reduction is correlated with the severity of memory impairment. The aims of this study were to establish that mild reduction of cerebral blood flow for a considerable period would lead to memory dysfunction and to reveal

Materials and Methods

reduction.

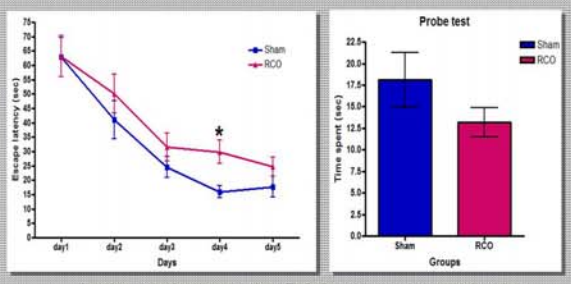
Adult male Sprague-Dawley rats (4-month old) were randomly allocated in to sham operated (sham) or permanent right common carotid artery occlusion (RCO) groups. For surgery, the rats were anesthetized with ketamine (40mg/kg) and xylazine (5mg/kg) injected intra-muscularly. Right common carotid artery was permanently occluded with single silk suture. Spatial learning and memory was assessed at 6 month after occlusion by using radial arm water maze (RAWM).

Following the behavioral test, the rats were sacrificed and brains were dissected for measurement of malondialdehyde (MDA), a marker of lipid peroxidation. The brain tissues from sham or RCO were divided into two subgroups consisting of whole brain (sham, n=2 and RCO, n=3), and hippocampus (sham, n=2 and RCO, n=3). The protein content in the sample was measured

Results and Discussion

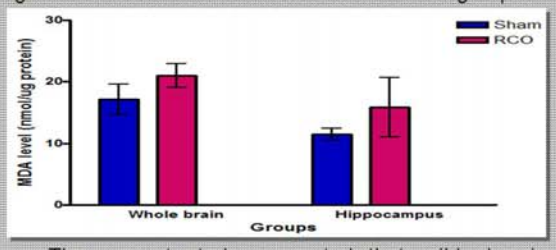
The behavioral performance of the rats subjected to sham or RCO operation were examined 6 months after surgery. The performance in the RAWM during five day training showed improvement in both sham and RCO groups as demonstrated by reduction of escape latency.

When compared between sham and RCO groups, the mean escape latencies from the 2nd -5th day were longer in the RCO group, especially on the 4th day (mean ± S.E.M.: 29.925 ± 3.980 vs. 15.969 ± 2.138, $P <$



In the probe test, the RCO group spent shorter duration in the target arm compared with sham group. However, there was no significant difference in spatial memory between groups ($P = 0.17$).

MDA level, a maker of oxidative stress, in RCO groups were higher than that of sham group in whole brain (mean ± S.E.M.(nmol/µg protein): 21.028 ± 1.933 for RCO, 17.144 ± 2.430 for sham). Similarly, the MDA level in hippocampus was higher in RCO group (15.884 ± 4.799 for RCO, 11.503 ± 0.992 for sham). There was no significant difference in all MDA levels between groups.



The present study suggested that mild chronic cerebral ischemia induced by permanent right common carotid artery occlusion caused spatial learning and memory impairment. The rise in MDA level in RCO group indicates increased free radical, which could responsible for producing for the neuronal changes mediating the behavior deficit.

Conclusion

The mild chronic cerebral ischemia induced by RCO resulted in impairment of spatial learning. The corresponding increase of lipid peroxidation could possibly suggest the role of oxidative stress in the pathogenesis of memory impairment in this rat model.

Acknowledgement

This work was supported by Siriraj Graduate Thesis Scholarship and Thailand Research Fund

References

1.Meyer JS, Quach M, Thornby J, Chowdhury M, Huang J. MRI identifies MCI subtypes: vascular versus neurodegenerative. Journal of the Neurological Sciences 2005;229-230:121-9.

2.Broadbent NJ, Squire LR, Clark RE. Spatial memory, recognition memory, and the hippocampus. PNAS 2004 October 5, 2004;101 14515-20.

3.Gupta YK, Veerendra Kumar MH, Srivastava AK. Effect of Centella asiatica on pentylenetetrazole-induced kindling, cognition and oxidative stress in rats. Pharmacology Biochemistry and Behavior. 2003;74(3):579-85.

4.Lei M, Hua X, Xiao M, Ding J, Han Q, Hu G. Impairments of astrocytes are involved in the d-galactose-induced brain aging. Biochemical and Biophysical Research Communications. 2008;369(4):1082-7.

5.Hiroshi Ohkawa, Nobuko Ohishi, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry. 1979;95:351-358.

Effect of mild chronic cerebral ischemia on hippocampal CA1 astroglial reaction and role in spatial memory

Anyarath Tonmat, Sompol Tapechum , Chatsumal Srimongkol, Ratana Ninthuk and Kanokwan Tilokskulchai

Objective

To study the effects of mild chronic cerebral ischemia on hippocampal CA1 neurons and astroglias and the role in spatial learning and memory.

Introduction

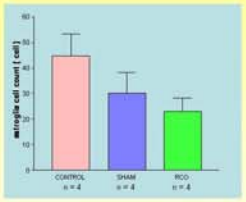
Chronic reduction of cerebral blood flow is one of the major cause of memory decline in ageing and neurodegenerative disease. Evidence showed that severe chronic cerebral ischemia in rats model causes memory deficite , hippocampal CA1 neuronal death and the alteration in astroglial number, function and morphology. However effect of mild chronic ischemia was not fully studied. Therefore, we studied the effect of mild chronic ischemia on the numbers of neuron and astroglia which may help to understand the progression of memory decline in both ageing and neurodegenerative disease.

Material and Method

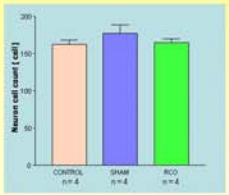
Four-month old male Spraque-Dawley rats were divided into three groups (n=8, each), control, sham and permanent RCO (right common carotid artery occlusion). Two months after surgery, 4 rats in each group were sacrificed and the other four in each group were subjected to behavioral test in radial arms water maze (RAWM) to assess spatial memory before sacrification. Staining with primary antibodies against neuron-specific nuclear protein (NeuN) and glial fibrilary acidic protein (GFAP) was performed on the serial 5-micron thickness coronal paraffin sections of both hemispheres for immunohistological study of neurons and astroglias, respectively.

Result

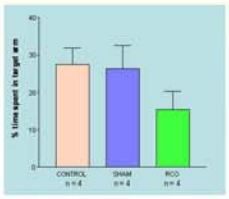
(1) The number of astroglia in CA1 hippocampus was decreased in RCO rats, especially in the right hemisphere (RCO = 23 ± 5.21 , sham = 30 ± 8.21 , control = 44.75 ± 8.51 cell , p = 0.446), while the number of neuron was not altered (RCO = 164.75 ± 5.17 , sham = 177.00 ± 11.57 , control = 162.75 ± 5.36 cell).



Astroglia in right hemisphere in group without testing in RAWM

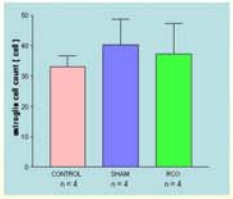


Neuron in right hemisphere in group without testing with RAWM



Time spent in target in probe trial

(2) The duration the RCO rats spent in target arm was shorter during probe trial when compared with those of sham and control (RCO = 15.42 ± 4.87, sham = 26.26 ± 6.33 , control = 27.50 ± 4.33 second).



Astroglia in right hemisphere in group testing with RAWM

(3) The activity in RAWM helped increase the number of astroglia in RCO compared with those of sham and control (RCO = 37.33 ± 9.96 , sham = 40.25 ± 8.49, control = 33 ± 3.70 cell).

Conclusion

(1) Mild chronic cerebral ischemia induced by RCO resulted in decrease number of astroglia in CA1 hippocampus. (2)The reduction of number of astroglias in CA1 hippocampus was correlated with the spatial memory impairment. (3) The activity could improve the number of astroglia in CA1 in RCO group to baseline.

This research was supported by Thailand Research Fund (TRF)

Effect of chronic mild global cerebral hypoperfusion on spatial memory in rats

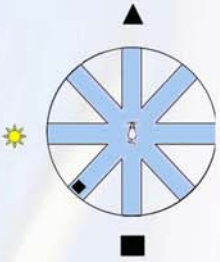
Sompol Tapechum, Anyarat Tonmart, Kanokwan Tilokskulchai

Department of Physiology, Faculty of Medicine Sirirraj Hospital, Bangkok, Thailand.

Cognitive impairment is one of the most leading health problems in elderly ranging from normal ageing process, mild cognitive impairment (MCI) and various degrees of dementia. Dementia is the extreme degree of cognitive impairment and is one of the leading causes of disability affecting 5% of individuals of age over 65 years old. Treatment and prevention of dementia are inefficient because the lack of the thorough knowledge of the disease process and the earliness of diagnosis. MCI is a syndrome defined as cognitive decline greater than that expected for an individual's age and education level but does not interfere with activities of daily living. It is demonstrated MCI is an early state of dementia *(Mayer, JS., 2002)*. Fifty percentages of MCI turns into dementia within 5 years. It is well accepted that vascular risk factor such as hypertension is a major contribution to all type of cognitive decline. Therefore an appropriate animal model of vascular occlusion which produces MCI would be necessary for the understanding of the early pathological process in cognitive impairment and to assess the efficiency of any possible interventions.

Methods

Four-month old male Sprague-Dawley rats (from Division of laboratory animal, Mahidol university) were allocated into three groups of control, sham (no arterial ligation) and right common carotid artery occlusion (UCO). All rats were kept in separated cages at 27 C with free access to food and water. Two months after surgery, the rats were subjected to radial arm water maze (RAWM) test. The time rats took to find the hidden platform (escape latency) was record. The protocol for RAWM consisted of 4 trials/day for 7 days of training. The probe trial was then performed 24 hours after the completion of training. The rats were then sacrified and the brains were subjected to immunohistochemical study using anti-NeuN and anti-GFAP antibody.



Results and Discussion

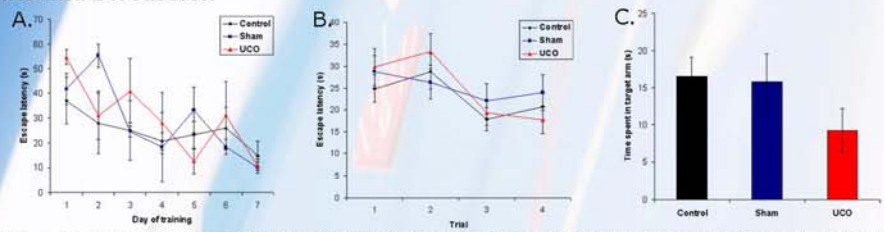


Figure 1 — The average escape latency of first trial of each training day (A) and the average escape latency of each trials of 7-day training showed that the UCO rats were able to learn to perform in the RAWM similar to control and sham animals (n=4 each). However, in the probe trial, the UCO rats performed worse than control and sham animals (C).

The RAWM performance demonstrated that the UCO rats were able to learn to find the platform similar to the control and sham rats. However, retention of the memory seemed impair in the UCO rats as showed by the probe trial. It has been evidenced that this type of spatial learning and memory is hippocampal dependent *(Block, F., 1997)* . Therefore, this chronic mild global cerebral ischemic model could possibly induce mild hippocampal damage that does not impair spatial learning but the retention of memory. As shown in the immunohitochemistry, there was reactive astrocytosis in CA1 of hippocampus in UCO rats without significant change in pyramidal neurons.

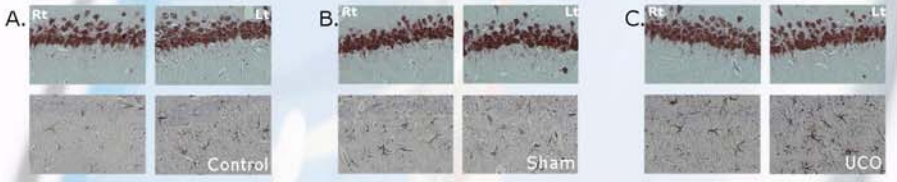


Figure 2 — Immunohitochemical studied showed pyramidal neurons (upper panel) and astroglial (lower panel) in right (Rt) and left (Lt) hemisphere CA1 of hippocampus staining with anti-NeuN and anti-GFAP antibody, respectively. The number of astroglial in UCO rats (C) was increased when compared to those of control (A) and sham (B) animals. The neurons were not significantly different among groups.

Conclusion

Chronic mild global cerebral hypoperfusion by UCO produced mild degree of cognitive impairment in rats. This animal model could represent MCI or mild degree of dementia which would be benefit for the study of pathogenesis of early stages of dementia and the possible intervention to prevent or treat dementia.

References

1. Mayer, JS. et al. (2002) Is mild cognitive impairment prodromal for vascular dementia like Alzheimer's disease? Stroke. 33, 1981-1985.

2. Block, F. and Schwarz, M. (1997) Correlation between hippocampal neuronal damage and spatial learning deficit due to global ischemia. Pharmacology Biochemistry and Behavior. 56, 755-761.

The Thailand Research Fund (TRF)

ปัญหาและอุปสรรค

การศึกษานี้ทำในสัตว์ทดลอง และต้องเลี้ยงสัตว์ทดลองให้ได้อายุตามที่ต้องการแล้วจึงจะเริ่มทำการทดลอง โดยการผูกหลอดเลือดแดง common carotid artery และหลังจากผูกหลอดเลือดแล้วยังต้องเลี้ยงสัตว์ทดลองไปจนได้ระยะเวลาที่ตั้งไว้เช่น 2, 6 เดือน จึงเริ่มทำการทดลอง ด้วยเหตุนี้จึงมีปัญหาและอุปสรรคหลายประการที่ทำให้งานวิจัยล่าช้ากว่ากำหนดและไม่เป็นไปตามแผนที่เสนอไปตอนต้น เช่น

1. จำนวนสัตว์ทดลองในแต่ละการทดลอง ในบางการทดลองอาจได้จำนวนน้อยเนื่องจากบางส่วนของสัตว์ทดลองที่เลี้ยงไว้เกิดป่วย ตายระหว่างเลี้ยงอันเกิดจากสาเหตุอื่นที่ไม่ได้เกี่ยวข้องกับการทดลองเช่น ติดเชื้อทางเดินหายใจ ทำให้บางการทดลองต้องเพิ่มจำนวนสัตว์ทดลองเข้าไปในตอนหลัง ทำให้ต้องใช้ระยะเวลานานขึ้น
2. สถานที่เลี้ยงสัตว์ทดลอง เนื่องจากอาคารสัตว์ทดลองใหม่กำลังอยู่ในระหว่างการก่อสร้าง ทำให้ต้องใช้สถานที่เลี้ยงและทดลองชั่วคราวซึ่งมีปัญหาแออัดในแง่การเลี้ยง ทำให้สัตว์เจ็บป่วยได้ง่าย และอาจมีผลต่อการทดลองบ้าง นอกจากนี้ยังมีความแออัดในการใช้สถานที่ทดลองในสัตว์ทำให้การทดลองล่าช้าออกไป
3. เนื่องจากในระยะเวลาที่ทำวิจัย ผลความแตกต่างระหว่างกลุ่ม sham และ RCO มีไม่มาก ดังนั้นการให้ intervention ด้วย FK 506 จึงทำไม่ได้ตามแผน คาดว่าถ้าให้ระยะเวลาผูกหลอดเลือดนานขึ้นอาจจะเห็นความแตกต่างมากขึ้นและจะทำให้การทดสอบ intervention ได้ผล

อย่างไรก็ตามผู้วิจัยได้พยายามวางแผนการทดลองอย่างรัดกุมที่สุด และทำการทดลองเพิ่มในบางกรณีที่มีจำนวนสัตว์ทดลองน้อย ทำให้ได้ผลเป็นที่น่าพอใจระดับหนึ่ง และมีองค์ความรู้ใหม่เกิดขึ้นซึ่งสามารถนำไปตีพิมพ์ได้

ภาคผนวก

Table 1 Escape latency in the radial arm water maze of the 8 days trial protocol of control rats at 2 months after the occlusion time

Group	Trial	Escape latency (s)							
		Day1	Day2	Day3	Day4	Day5	Day6	Day7	Day8
Control1	t1	10	60	60	13	16	35	10	22
	t2	28	26	60	60	26	14	20	19
	t3	21	55	18	60	9	11	13	16
	t4	3	13	5	10	60	10	10	16
Control2	t1	48	5	9	16	39	45	32	23
	t2	60	3	45	60	60	60	10	60
	t3	31	4	10	32	11	32	10	23
	t4	60	42	29	25	58	9	11	37
Control3	t1	52	13	22	29	22	13	7	7
	t2	40	21	28	9	6	3	8	7
	t3	3	28	17	11	9	10	6	6
	t4	15	30	16	9	5	17	13	6
Control4	t1	38	33	9	24	16	10	10	7
	t2	30	60	12	28	14	5	11	8
	t3	6	5	10	21	12	19	25	17
	t4	28	27	30	19	9	5	13	9

Table 1 (continue) Escape latency in the radial arm water maze of the 8 days trial protocol of control rats at 2 months after the occlusion time

Group	Trial	Escape latency (s)							
		Day1	Day2	Day3	Day4	Day5	Day6	Day7	Day8
Control5	t1	60	60	60	15	12	12	12	8
	t2	60	60	23	41	60	60	13	12
	t3	60	45	60	60	14	8	11	8
	t4	60	60	60	10	13	11	15	7
Control6	t1	25	31	47	27	27	24	8	13
	t2	58	59	60	43	7	8	23	7
	t3	18	57	60	5	13	24	14	21
	t4	59	60	14	23	14	5	12	6
Control7	t1	60	60	60	13	19	44	8	7
	t2	60	60	60	20	10	7	13	6
	t3	60	60	12	60	15	18	10	4
	t4	60	60	60	12	32	19	6	5
Control8	t1	20	59	33	43	36	17	36	9
	t2	60	36	60	60	59	32	13	14
	t3	60	60	48	41	60	51	9	17
	t4	60	45	60	60	22	9	12	16

Table 2 Escape latency in the radial arm water maze of the 8 days trial protocol of sham rats at 2 months after the occlusion time

Group	Trial	Escape latency (s)							
		Day1	Day2	Day3	Day4	Day5	Day6	Day7	Day8
Sham1	t1	60	60	19	4	28	17	15	9
	t2	54	52	43	16	12	11	11	14
	t3	35	4	60	3	11	10	10	9
	t4	58	11	60	3	12	11	10	8
Sham2	t1	38	60	28	5	19	14	5	3
	t2	60	60	21	54	8	13	5	6
	t3	10	53	28	3	3	9	6	5
	t4	20	60	7	9	11	15	5	4
Sham3	t1	38	60	28	60	60	27	10	11
	t2	30	15	13	60	14	24	27	24
	t3	60	15	4	60	60	10	9	29
	t4	30	60	4	60	28	7	16	34
Sham4	t1	31	41	23	4	26	15	10	13
	t2	3	60	40	4	10	9	9	9
	t3	43	8	41	5	19	31	9	7
	t4	41	43	4	60	10	6	9	8

Table 2 (continue) Escape latency in the radial arm water maze of the 8 days trial protocol of sham rats at 2 months after the occlusion time

Group	Trial	Escape latency (s)							
		Day1	Day2	Day3	Day4	Day5	Day6	Day7	Day8
Sham5	t1	32	60	8	60	60	26	23	20
	t2	17	60	45	52	60	17	17	11
	t3	60	29	60	23	52	30	19	15
	t4	60	46	60	32	34	21	15	12
Sham6	t1	60	60	30	22	16	11	31	15
	t2	53	60	13	55	39	10	17	8
	t3	60	60	15	25	11	13	6	8
	t4	60	5	60	20	9	15	14	7
Sham7	t1	60	11	44	17	23	8	35	27
	t2	9	11	5	12	60	60	11	14
	t3	35	55	15	22	42	39	19	9
	t4	60	13	60	16	26	21	19	9

Table 3 Escape latency in the radial arm water maze of the 8 days trial protocol of RCO rats at 2 months after the occlusion time

Group	Trial	Escape latency (s)							
		Day1	Day2	Day3	Day4	Day5	Day6	Day7	Day8
RCO1	t1	60	60	41	3	4	48	14	4
	t2	56	60	60	32	60	16	8	13
	t3	32	60	38	4	12	22	9	8
	t4	22	13	38	4	15	17	5	4
RCO2	t1	49	25	59	35	12	5	9	10
	t2	60	41	17	51	13	9	11	6
	t3	20	18	12	26	9	7	7	5
	t4	60	5	23	17	9	4	8	7
RCO3	t1	50	16	60	14	27	11	3	6
	t2	47	60	42	34	17	6	3	3
	t3	28	47	18	5	10	5	18	7
	t4	48	45	22	4	5	5	6	4
RCO4	t1	60	23	4	60	7	60	16	6
	t2	47	60	15	56	25	6	22	21
	t3	14	58	17	23	8	4	9	39
	t4	25	53	10	14	7	4	8	27

Table 3 (continue) Escape latency in the radial arm water maze of the 8 days trial protocol of RCO rats at 2 months after the occlusion time

Group	Trial	Escape latency (s)							
		Day1	Day2	Day3	Day4	Day5	Day6	Day7	Day8
RCO5	t1	60	60	60	60	35	10	13	9
	t2	60	60	60	34	38	40	12	10
	t3	60	60	60	59	54	5	6	6
	t4	60	60	60	53	12	22	22	14
RCO6	t1	36	60	42	60	60	25	5	6
	t2	60	23	60	42	60	9	16	5
	t3	60	51	60	59	31	30	8	5
	t4	60	60	60	7	14	4	6	27
RCO7	t1	60	60	60	60	29	55	26	21
	t2	60	47	27	38	21	37	42	23
	t3	60	60	27	9	42	56	28	22
	t4	55	38	27	27	60	40	35	25
RCO8	t1	51	8	40	37	5	5	14	20
	t2	60	54	27	20	30	6	23	16
	t3	15	33	60	28	6	14	20	21
	t4	28	14	5	4	60	5	5	6

Table 4 Escape latency in the radial arm water maze of the 5 days trial protocol of sham rats at 2 months after the occlusion time

Group	Trial	Escape latency (s)				
		Day1	Day2	Day3	Day4	Day5
Sham1	t1	55	92	10	104	7
	t2	119	90	58	64	30
	t3	120	120	10	7	75
	t4	120	29	35	17	33
Sham2	t1	20	26	59	8	22
	t2	51	108	11	120	22
	t3	9	120	11	8	40
	t4	10	120	49	10	8
Sham3	t1	11	14	10	18	9
	t2	120	120	81	18	31
	t3	120	29	10	11	42
	t4	110	120	20	36	50
Sham4	t1	120	120	31	73	120
	t2	87	60	96	25	55
	t3	27	91	10	38	52
	t4	42	39	97	45	24
Sham5	t1	64	120	55	120	17
	t2	120	93	34	62	53
	t3	27	120	28	37	14
	t4	116	52	113	36	13
Sham6	t1	120	18	43	21	39
	t2	120	120	42	7	8
	t3	120	50	58	25	14
	t4	120	29	8	17	8

Table 4 (continue) Escape latency in the radial arm water maze of the 5 days trial protocol of sham rats at 2 months after the occlusion time

Group	Trial	Escape latency (s)				
		Day1	Day2	Day3	Day4	Day5
Sham7	t1	120	110	20	27	25
	t2	103	42	6	7	18
	t3	27	16	17	7	19
	t4	120	82	17	14	14
Sham8	t1	120	120	61	15	62
	t2	40	120	120	40	24
	t3	118	49	21	41	31
	t4	106	120	69	23	33
Sham9	t1	74	29	58	28	37
	t2	120	39	30	110	21
	t3	120	78	40	42	59
	t4	8	14	9	66	11
Sham10	t1	120	51	35	35	8
	t2	78	44	17	22	26
	t3	120	55	29	10	24
	t4	46	29	9	18	7
Sham11	t1	120	46	35	10	8
	t2	120	62	8	20	10
	t3	22	17	11	7	34
	t4	73	32	8	38	8
Sham12	t1	120	20	34	14	8
	t2	120	62	9	14	10
	t3	86	37	21	6	13
	t4	49	15	14	27	9

Table 5 Escape latency in the radial arm water maze of the 5 days trial protocol of RCO rats at 2 months after the occlusion time

Group	Trial	Escape latency (s)				
		Day1	Day2	Day3	Day4	Day5
RCO1	t1	65	120	44	32	31
	t2	120	109	26	30	32
	t3	120	109	23	21	13
	t4	120	24	59	16	28
RCO2	t1	31	77	65	16	21
	t2	10	102	21	17	47
	t3	120	97	58	16	18
	t4	115	21	17	7	18
RCO3	t1	43	20	8	115	42
	t2	51	44	9	36	29
	t3	52	36	25	11	43
	t4	10	31	51	18	74
RCO4	t1	120	13	120	120	45
	t2	120	59	41	15	120
	t3	120	39	35	120	107
	t4	36	120	24	10	13
RCO5	t1	17	46	39	20	43
	t2	120	91	20	66	8
	t3	68	37	41	57	34
	t4	64	42	11	11	11
RCO6	t1	89	120	120	14	27
	t2	95	66	50	120	10
	t3	120	39	52	34	120
	t4	120	40	26	27	71

Table 5 (continue) Escape latency in the radial arm water maze of the 5 days trial protocol of RCO rats at 2 months after the occlusion time

Group	Trial	Escape latency (s)				
		Day1	Day2	Day3	Day4	Day5
RCO7	t1	120	120	56	87	39
	t2	120	120	11	43	9
	t3	120	120	79	9	16
	t4	120	53	28	9	16
RCO8	t1	120	37	38	56	37
	t2	91	45	11	103	43
	t3	96	52	31	23	60
	t4	37	11	40	10	24
RCO9	t1	120	120	42	33	69
	t2	120	74	12	32	20
	t3	120	64	65	10	29
	t4	120	120	23	90	8
RCO10	t1	120	120	54	10	46
	t2	81	49	10	76	36
	t3	120	59	70	25	57
	t4	120	51	44	22	24
RCO11	t1	120	120	47	43	25
	t2	54	13	33	36	33
	t3	81	120	15	24	37
	t4	58	101	59	46	25

Table 6 Escape latency in the radial arm water maze of the 5 days trial protocol of sham rats at 6 months after the occlusion time

Group	Trial	Escape latency (s)				
		Day1	Day2	Day3	Day4	Day5
Sham1	t1	9	22	8	15	21
	t2	120	60	20	7	6
	t3	42	23	7	7	7
	t4	9	27	26	31	6
Sham2	t1	67	25	12	6	25
	t2	7	5	18	26	22
	t3	44	25	25	27	8
	t4	15	12	7	5	16
Sham3	t1	22	38	23	10	8
	t2	25	17	18	5	14
	t3	98	15	11	8	7
	t4	111	21	8	8	6
Sham4	t1	35	120	12	11	30
	t2	52	23	10	24	17
	t3	32	24	43	61	10
	t4	23	23	26	11	19

Table 6 (continue) Escape latency in the radial arm water maze of the 5 days trial protocol of sham rats at 6 months after the occlusion time

Group	Trial	Escape latency (s)				
		Day1	Day2	Day3	Day4	Day5
Sham5	t1	120	107	10	9	8
	t2	100	34	14	9	8
	t3	120	41	18	9	8
	t4	64	18	23	9	8
Sham6	t1	100	120	38	27	12
	t2	51	16	17	15	13
	t3	54	36	7	7	6
	t4	85	8	29	27	20
Sham7	t1	45	120	99	35	120
	t2	105	120	48	28	15
	t3	120	36	66	21	40
	t4	106	20	54	17	21
Sham8	t1	19	96	7	7	16
	t2	106	37	16	14	10
	t3	68	16	50	7	7
	t4	39	9	16	8	35

Table 6 Escape latency in the radial arm water maze of the 5 days trial protocol of RCO rats at 6 months after the occlusion time

Group	Trial	Escape latency (s)				
		Day1	Day2	Day3	Day4	Day5
RCO1	t1	39	14	5	5	5
	t2	120	21	26	16	34
	t3	18	35	6	14	6
	t4	7	6	24	23	12
RCO2	t1	8	19	8	6	5
	t2	19	18	5	6	13
	t3	54	35	5	23	10
	t4	7	120	6	5	25
RCO3	t1	23	47	50	51	39
	t2	21	47	9	25	16
	t3	16	20	18	33	14
	t4	68	120	25	24	27
RCO4	t1	12	120	30	24	31
	t2	88	25	54	38	14
	t3	19	66	57	15	31
	t4	15	30	53	43	24
RCO5	t1	54	11	12	11	27
	t2	36	23	7	6	7
	t3	15	35	6	31	10
	t4	8	18	13	14	16

Table 6 (continue) Escape latency in the radial arm water maze of the 5 days trial protocol of RCO rats at 6 months after the occlusion time

Group	Trial	Escape latency (s)				
		Day1	Day2	Day3	Day4	Day5
RCO6	t1	60	11	6	45	27
	t2	52	18	34	7	9
	t3	17	28	7	49	21
	t4	62	15	15	12	23
RCO7	t1	120	31	33	47	120
	t2	120	21	22	46	10
	t3	87	65	24	6	20
	t4	67	120	12	17	68
RCO8	t1	120	120	120	41	23
	t2	120	120	120	9	8
	t3	120	120	79	61	33
	t4	120	120	54	16	21
RCO9	t1	120	11	113	99	62
	t2	78	38	47	120	42
	t3	120	120	33	67	31
	t4	120	53	46	46	36
RCO10	t1	120	17	19	20	20
	t2	120	111	18	36	14
	t3	16	30	25	8	8
	t4	120	9	21	32	32

Table 7 Time spent in target quadrant during the probe trial in control, sham and RCO at 2 and 6 months after RCO after the completion of 5 or 8 days radial arm water maze (RAWM) protocols

Groups	Control	Sham	RCO
	2-month RCO after 8-day RAWM protocol		
1	10	26	6
2	19	8	3
3	15	13	16
4	22	16	12
	2-month RCO after 5-day RAWM protocol		
1		16	7
2		14	20
3		11	17
4		15	0
5		24	22
6		0	9
7		24	12
8		18	29
9		17	18
10		19	17
11		23	13
12		36	
	6-month RCO after 5-day RAWM protocol		
1		23	13
2		5	12
3		26	12
4		26	14
5		28	10
6		18	7
7		8	25
8		11	13
9			19
10			7

Table 8 The mean escape latencies during reversed trials at 2 months of RCO after the 8 days radial arm water maze protocol.

Rat No.	Trial	Mean escape latency (s)		
		Control	Sham	RCO
1	t1	6	11	7
	t2	5	8	7
	t3	5	8	8
	t4	9	7	8
2	t1	6	8	43
	t2	10	8	11
	t3	8	10	19
	t4	4	7	12
3	t1	5	12	21
	t2	4	7	23
	t3	4	8	19
	t4	24	6	22
4	t1	6		14
	t2	7		24
	t3	8		14
	t4	6		20

Table 9 The mean escape latencies during reversed trials at 2 months of RCO after the 5 days radial arm water maze protocol.

Rat No.	Trial	Mean escape latency (s)	
		Sham	RCO
1	t1	24	67
	t2	17	120
	t3	38	92
	t4	14	55
2	t1	27	68
	t2	13	27
	t3	9	36
	t4	21	120
3	t1	16	43
	t2	43	25
	t3	25	7
	t4	10	36
4	t1	8	25
	t2	60	16
	t3	10	36
	t4	26	26
5	t1	9	41
	t2	6	53
	t3	8	14
	t4	26	20
6	t1	23	
	t2	25	
	t3	8	
	t4	6	

Table 9 The mean escape latencies during reversed trials at 2 months of RCO after the 5 days radial arm water maze protocol.

Rat No.	Trial	Mean escape latency (s)	
		Sham	RCO
1	t1	8	22
	t2	22	24
	t3	6	20
	t4	27	17
2	t1	5	73
	t2	17	49
	t3	120	18
	t4	9	72
3	t1	60	48
	t2	21	20
	t3	12	6
	t4	6	12
4	t1	58	35
	t2	22	21
	t3	9	30
	t4	9	9
5	t1	11	48
	t2	9	108
	t3	8	98
	t4	11	8

Table 9 (continue) The mean escape latencies during reversed trials at 2 months of RCO after the 5 days radial arm water maze protocol.

Rat No.	Trial	Mean escape latency (s)	
		Sham	RCO
6	t1	23	45
	t2	23	95
	t3	20	60
	t4	11	7
7	t1	39	13
	t2	58	11
	t3	39	20
	t4	16	15
8	t1	9	13
	t2	29	29
	t3	20	28
	t4	9	7
9	t1		19
	t2		19
	t3		29
	t4		17
10	t1		10
	t2		8
	t3		15
	t4		8