Evaluation of Hippocampal Injury and Cognitive Function Induced by Embolization in the Rat Brain

HENG-AI ZHANG,[1,2,3] MEI GAO,[1,2] BAINIAN CHEN,[1,2] LILI SHI,[1,2] QIAOYUN WANG,[4] XIAOYAN YU,[1,2] ZHAOHONG XUAN,[1,2] LI GAO,[1,2] AND GUANHUA DU[1,2]*

[1]Beijing Key Laboratory of Drug Target Identification and Drug Screening, Beijing 100050, China

[2]National Center for Pharmaceutical Screening, Institute of Materia Medica, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing 100050, China

[3]Division of Cardiovascular Research, National Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China

[4]Binzhou Medical University, Huanghe San Road, Binzhou 256603, China

ABSTRACT

Embolism is responsible for at least 20% of all stroke and half of cerebral infarctions. A number of animal models have been developed to mimic thromboembolic stroke. However, little aimed directly at hippocampal damage and cognitive function. In the present study, three sizes of emboli (150–178 μm, 74–124 μm, and 48–74 μm) were employed to induce thromboembolic stroke model in rats. Results showed that the diameter of the particle was critical for animal behavioral and histopathological consequences. Hematoxylin–eosin (HE) staining revealed that CA1 and CA2–3, which are two of the main hippocampal subdivisions were injured seriously, especially induced by emboli$_{(48–74\ \mu m)}$. At 24 hr, the neurological deficit scores showed that emboli injection could cause significant neurological deficit, and the increase of neurological deficit scores correlated well to the diameter of emboli. At 60 days, emboli$_{(150–178\ \mu m)}$ and emboli$_{(48–74\ \mu m)}$ lead to obvious cognitive impairment, which correlated well to the hippocampal CA1 injury. Our research might be helpful to choose suitable size of emboli to induce animal model to research subcortical ischemia and vascular dementia. However, cognitive alterations and cerebral injury following different sizes of emboli injection in rats remains a topic for future investigation. Anat Rec, 296:1207–1214, 2013.

Key words: hippocampal injury; cognitive function; different sizes of emboli; cerebral thromboembolism

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*Correspondence to: Dr. Guanhua Du, Prof., Beijing Key Laboratory of Drug Target Identification and Drug Screening; National Center for Pharmaceutical Screening, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Xiannongtan Street, Beijing 100050, China. Fax: 86-010-63165184. E-mail: dugh@imm.ac.cn

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Stroke represents a major medical problem in the world and the leading cause of permanent disability, cognitive failure, and altered behavior (Ferri et al., 2011; Kim and Johnston, 2011; Wang et al., 2011). The gravity of this condition encourages the pursuit of novel treatments. Therefore, various animal models of ischemic stroke have been designed for the development of new drugs. Embolism is responsible for at least 20% of all stroke and half of cerebral infarctions (Schneider et al., 2004). Thus, the thromboembolic model induced by emboli injection is of great interest to stroke researchers because of its close resemblance to human ischemic stroke and its utility in evaluating thrombolytic therapies (Krakovsky et al., 2011; Tsai et al., 2011; Walvick et al., 2011).

Previous data showed that in the thromboembolic stroke model infarctions most frequently take place in cerebral cortex, hippocampus, and thalamostriate (Kudo et al., 1982). It is well known that the hippocampus is one of the brain regions most sensitive to ischemic damage and plays important roles in learning, memory, and epilepsy and is known to have high susceptibility to ischemic damage compared with other brain structures in both animals (Sugawara et al., 2002) and humans (Fujioka et al., 2000). Therefore, investigating the pattern of ischemic injury in the hippocampus may provide insights in pathogenetic mechanisms and may help develop new therapeutic strategies.

The severity of cerebral ischemia depends on the extent of cerebral blood flow (CBF) reduction. The major blood supply of the brain comes from two sources—the internal carotid (IC) arteries and the vertebral arteries. While one of the major blood supplies of the hippocampal formation is provided by branches of the posterior cerebral artery (PCA), originated from the anterior portion of the superior cerebellar artery very close to the midline, which comes from the vertebral arteries. However, the anterior choroid artery of the IC also supplies the portions of the hippocampus (Dorr et al., 2007; Barth and Mody, 2011). It is well known that IC is one of the most important blood vessels which are used to induce cerebral ischemia. Therefore, occlusion, ligation, or emboli from the IC and PCA can reach to the hippocampus to induce injury.

Although emboli-related hippocampal injury and cognitive impairment were previously described (Kudo et al., 1982; Rapp et al., 2003; Rasmussen et al., 2006), there is no current data about the effects of the size of emboli on the injury pattern and severity of hippocampus available, and also no data about the relationship between the size of the emboli and cognitive impairment. Therefore, in this study, three sizes of emboli were used to induce thromboembolic rat model to examine its behavioral and hippocampal histopathological consequences.

MATERIALS AND METHODS

Male SD rats (250–300 g, Vitalriver Company, China) were housed in a temperature-controlled room on a 12-hr light/12-hr dark cycle with food and water ad libitum. All animal experiments were carried out in accordance with institutional guidelines and ethics, and those experiments were approved by the local government authorities. Every effort was made to minimize the number of animals used and their suffering.

Emboli were produced as described by Kudo et al. (1982). Briefly, 10 mL of blood was obtained from two rats and then centrifuged at 4,000 rpm for 10 min. The serum was discarded and the rest was stored at 37°C for 72 hr for clot formation. The clots were separated and fragmented to emboli in a mortar box. The emboli were divided into three diameters: 150–178 μm, 74–124 μm, and 48–74 μm by five nets with 48 μm, 74 μm, 124 μm, 150 μm, and 178 μm meshes. Accordingly, the thromboembolic model was divided into three groups: group of 150–178 μm emboli (L group), group of 74–124 μm emboli (M group), and group of 48–74 μm emboli (S group). Furthermore, rats in the normal saline group (sham group) were injected with 0.5 normal saline and rats in the normal group (normal group) with no operation and injection.

Animals were anesthetized with 10% chloral hydrate (0.4 mL/100 g). The right common, internal and external carotid arteries were surgically exposed. Then a length of the external carotid artery was dissected free at proximal part. The free external carotid artery was stretched to align with IC artery. The vagus nerve and sympathetic plexus were left intact. A small opening cut was made around the external carotid artery just above its origin. Employing a 26-gauge needle, the suspension of emboli was slowly injected from the external carotid artery to the IC artery. The common carotid artery was temporarily occluded by a gentle lifting of a thread to avoid the emboli entering the common carotid artery during the injection which lasted about 4 min. Then the needle was removed and the external carotid artery was ligated. The thread around the common carotid artery was removed and the blood flow was re-established. For group sham, the same operation was performed with injection of 0.5 mL normal sodium (NS) with no emboli, and in the normal group, no operation and emboli. The rat body temperature was kept within the physiological range (37°C–38°C).

A neurological examination was performed as previously described (Bederson et al., 1986) with modifications at 24 hr after injection of emboli, by a single experimenter who was blinded to the experimental groups. Briefly, the scores were: 0, no apparent deficits; 1, contralateral forelimb flexion when suspended by tail; 2, consistently reduced resistance to lateral push toward the paretic side; 3, spontaneous contralateral circling; 4, the injury was very severe and rats hadn't spontaneous activities after recovery from the anesthesia.

After emboli injection for 30 days and 60 days the rat was placed in a 2-m diameter water tank (DMS-2, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, China). The temperature of the water was 22 ± 2°C. The tank was visually separated into four quadrants. In the center of first quadrant, the goal quadrant, a platform was hidden 2 cm below the waterline. The rat was placed in the tank at one of two start locations (equidistant from the platform location) and swam for up to 120 sec until it found the platform. Once the animal located the platform, it remained on it for 30 sec. If an animal did not locate the platform, the observer positioned the animal on the platform at the end of the 120-sec swimming period. At the end of the session, the rat was dried with a towel before being returned to its home cage. Rats were tested twice a day for five consecutive days. The time to

find the platform (escape latency) of the rat was measured by a video tracking system (TOTA-450III, TOTA.-Co.Ltd., Tokyo, Japan) connected to a computer.

The sixth day a probe trial was given in which the platform was removed from the tank. One of two starting positions in the hidden platform trial was used; this position was consistent for all rats. For the probe trial, the number of crossing over the former platform, the total distance, and the distance in the goal quadrant were recorded by the video tracking system.

After neurological examination and Morris water maze test, animals were perfusion-fixed and their brains were removed for histopathological analysis, respectively at 24 hr and 60 days. Briefly, rats were anesthetized with 10% chloral hydrate (0.4 mL/100 g) and perfused transcardially with 4% paraformaldehyde for the fixation of the brain tissue. Brain tissue was removed, post-fixed in 4% paraformaldehyde for three days. Then it was washed by distilled water for 12 hr and embedded in paraffin for histological examination. A 4-μm thick hippocampus sections were cut, deparaffinized, hydrated, and stained with hematoxylin–eosin (HE). Semiserial sections were examined under microscope (COIC, Olympus-IX62) and camera (EOS 450D Computer Cameras, Cannon Inc.).

The CA1 and CA2–3 regions of hippocampus were selected as the target areas to be analyzed for hippocampus injury. The numbers of normal integrate CA1 and CA2–3 pyramidal neuronal cells were counted within each microscale area of 1 mm sections from every brain slice. This was achieved under the light microscope (×400). The neuronal density (ND) at 24 h after ischemia was determined as the average number of surviving hippocampal CA1 and CA2–3 pyramidal neurons (complete whole cells) per microscale area of 1 mm sections from each rat that were counted, with three sections in the CA1 and CA2–3 area of right hippocampal slices counted for each rat.

The histological changes of the CA1 and CA2–3 infarction areas were evaluated by Image J (NIH image J) on every HE slice of ×400. The infarction areas at 60 days after ischemia was determined as the average of infracted CA1 and CA2–3 area per integrated hippocampus of 1 mm sections from each rat, with three sections in the CA1 and CA2–3 area of right hippocampal slices for each rat.

Statistical Analysis

Data analyses were performed with SPSS10.0. ND and data obtained from Morris water maze tests were analyzed by one way analysis of variance (ANOVA). When appropriate, *post hoc* tests were assessed using the least significant digit (LSD) test. The hippocampal infarction areas were analyzed by frequencies. The results were shown as mean ± S.E.M. In all statistical comparison, $P < 0.05$ was used as the criterion for statistical significance.

RESULTS

Rats injected with emboli showed apparent neurological deficits (Fig. 1). Furthermore, the neurological deficit scores of rats in the L group were higher than those of M and S group ($P < 0.05$). There was no difference between group M and S. Compared to sham group, the neurological deficit scores of the three thromboembolic model groups was significantly higher ($P < 0.05$).

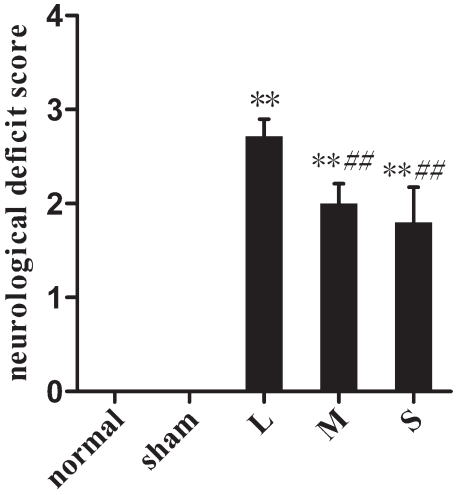

Fig. 1. Neurological deficit scores. Three sizes of emboli were injected to rats' brain from the IC arteries to induce the rats' cerebral thromembolism. A neurological examination was performed at 24 hr after emboli injection. (L: L group; M: M group; S: S group, Data were shown as mean ± S.E.M., $^{\#\#}P < 0.05$ vs. L, $^{**}P < 0.1$ vs. sham).

There were no statistical differences in the spatial learning and memory capabilities among the five groups at 30 days after emboli injection. At 60 days in the hidden platform trial, escape latency of rats in the normal and sham group decreased in a day-dependent manner. In the 1 and 2 training days, rats in the L group took significantly long time to find the platform ($P < 0.05$), while rats in the S group showed slight impairment in spatial learning and memory only in the first training day ($P < 0.1$) and no statistical difference was observed between group normal and sham (Fig. 2A). Rats in the M group also presented longer escape latency, but with no statistical difference compared to the sham group.

The data obtained from probe trial performance at 60 days were shown in Figure 2B–D. Analysis revealed that rats in the L and S group crossed over the former platform location significantly less frequently than the sham group ($P < 0.05$); rats in the M group also crossed over the former platform location less frequently than rats in the sham group, but with no statistical difference (Fig. 2B).

Furthermore, the total distance of rats swimming in the probe trial in 120 sec had no statistical difference (Fig. 2C), which meant that the motor function was undamaged at 60 days after emboli injection. While compared with the sham group, the percentage of swimming distance in the goal quadrant was decreased at 60 days after emboli injection (Fig. 2D).

In the present study, rats in the normal group showed no pathological changes, while rats in the sham group showed slightly reversible ischemic change at 24 hr (Fig. 3), and recovered at 60 days (Fig. 4). However, obvious morphological changes were found in the three model groups.

Histological evaluation revealed that three sizes of emboli injection resulted in a severe infarction in the

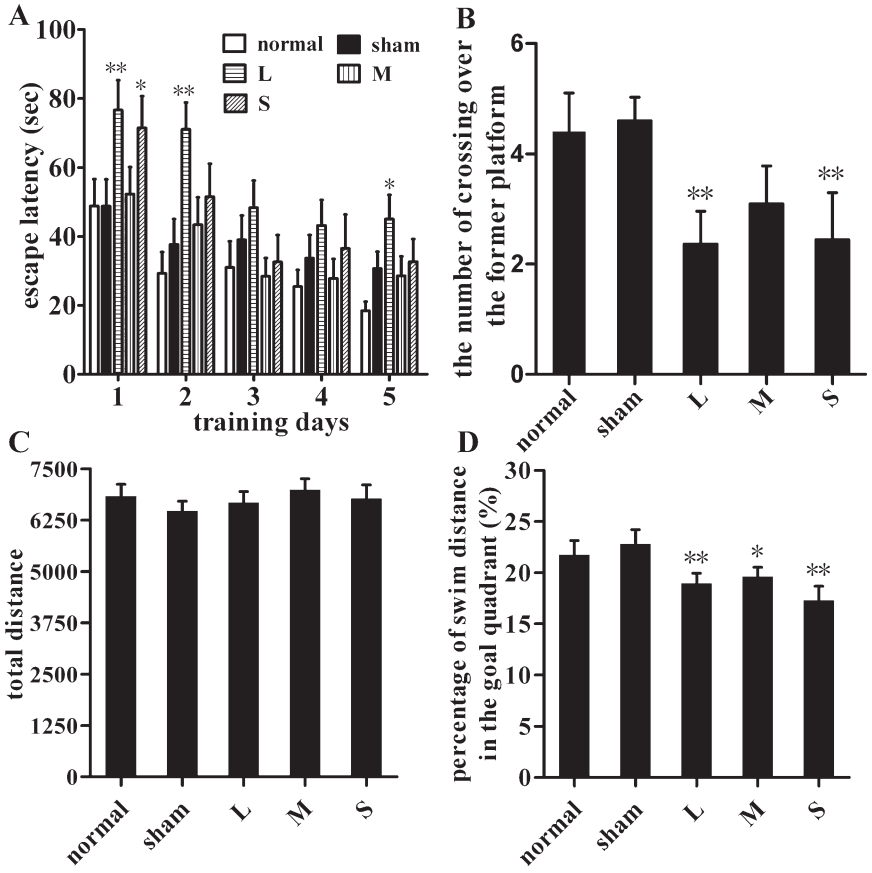

Fig. 2. Results in Morris water maze. Three sizes of emboli were injected to rats' brain from the IC arteries to induce the rats' cerebral thromboembolism. Animals were trained for five consecutive days at 55th day for the maze task and at 60th day for probe trial. **A**: Comparison of average escape latency time; (**B**) The number of crossings over the former platform location in probe trial performance; (**C**) The total distance in the probe trial performance. The total distance: rats' swimming distance in four quadrants of the Morris water maze in probe trial in 120 sec; (**D**) Percentage of swimming distance in goal quadrant. Percentage of swimming distance in the goal quadrant: ratio of swimming distance in goal quadrant and the total distance in the probe trial in 120 sec. (L: L group; M: M group; S: S group, Data were shown as mean ± S.E.M., $^{**}P < 0.05$ vs. sham, $^{*}P < 0.1$ vs. sham).

hippocampal CA1 and CA2–3 subfield of ischemic rats in comparison with the sham animal. As seen in Figure 3, 24 hr after ischemia the HE staining showed that most neurons appeared shrunken with eosinophilic cytoplasm and triangulated pycnotic nucleus, which was surrounded with necrotic neurons which exhibited condensed nuclear with no cytoplasm. By analysis, the ND in CA1 and CA2–3 region were different in the three groups. The ND in CA1 region (Fig. 5A) was M (45.78 ± 4.76) > L (35.96 ± 5.87) > S (15.73 ± 5.12), while the ND in CA2–3 region (Fig. 5B) was L (32.90 ± 4.25) > M (13.31 ± 3.26) > S (7.27 ± 3.07).

Sixty days after ischemia most neurons in CA1 and CA2–3 displayed a different appearance. At the time, cell death occurs; a phenomenon termed neuronal death with pycnotic shape and condensed nuclear, which is surrounded with fibroplasia (Fig. 4). By analyzing the infarction areas with frequencies we found that the CA1 infarction areas (Fig. 5C) were S ($12{,}864.0 \pm 4{,}583.1$) > L ($12{,}334.1 \pm 3{,}874.3$) > M ($7{,}644.0 \pm 3{,}591.5$); the CA2–3 infarction areas (Fig. 5D) were M ($18{,}788.6 \pm 2{,}647.8$) > S ($17{,}363.0 \pm 5577.9$) > L ($15{,}838.6 \pm 5{,}119$).

DISCUSSION

Cerebrovascular disease is the second leading cause of cognitive impairment in the elderly, either alone or in combination with Alzheimer's disease. It may result from multiple cortical infarctions due to cerebral large vessel pathologies or to subcortical ischemic changes such as leukoaraiosis or lacunar infarction due to cerebral small artery disease. Clinical symptoms and signs vary depending on the location and size of the stroke lesion. It has been reported that the prevalence of stroke in China is nearly as high as in industrialized countries (Ferri et al., 2011). And, 30% of these individuals develop vascular dementia or vascular cognitive impairment (Savva and Stephan, 2010) and there is also a 9-fold increased risk of incident dementia in the first year after the first-ever cerebral infarct rising to a cumulative incidence of 23% within 10 years (Kokmen et al., 1996). Therefore, it is urgent to treat stroke and poststroke dementia. Valid experimental models and behavioral tests are indispensable for the development of therapies for stroke and poststroke dementia. The translational

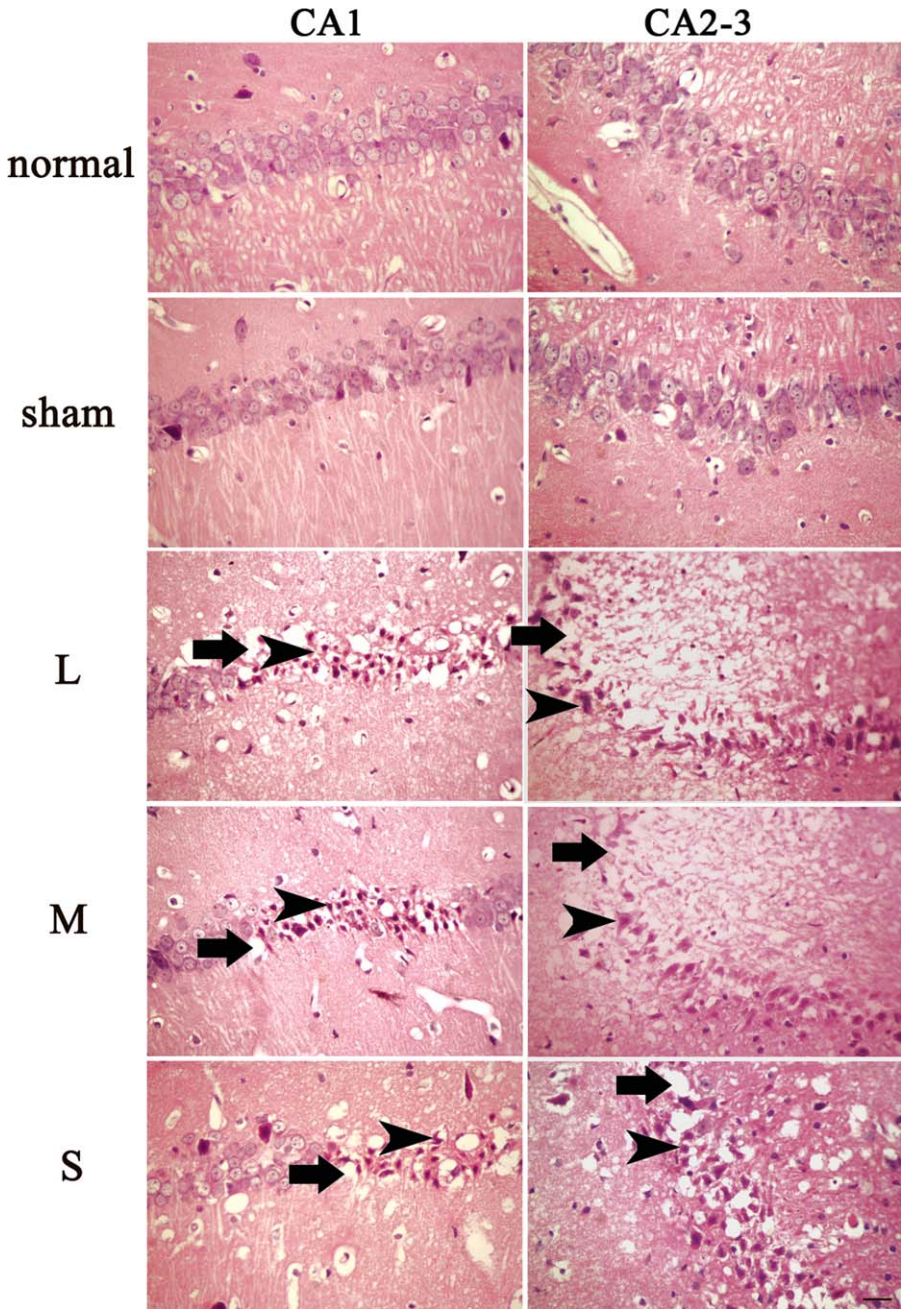

Fig. 3. Representative coronal sections of hippocampal CA1 and CA2–3 in rats at 24 hr after ischemia. Three sizes of emboli were injected to rats' brain from the IC arteries to induce the rats' cerebral thromboembolism. After neurological examination at 24 hr, the brain tissues obtained and stained with HE to observe CA1 and CA2–3 under a 400× light microscope (scale bar = 25 μm). The pycnosis (arrow heads) and liquefaction necrosis (arrows) in the three model groups were marked, respectively.

failure with neuroprotective drugs has forced us to work hard to look for alternative approaches.

A number of animal models have been developed to mimic thromboembolic stroke. These involve injection of microspheres, macrospheres, a thrombotic clot, or purified thrombin into the IC artery. Emboli of various types of particles are characteristics of brain ischemic injury. Autologous or heterologous blood clots have the best face validity to assess safety and efficacy of antithrombotic therapies (Zhang et al., 1997). Both spontaneously developing clots and thrombin-induced clots of variable number and size have been used. The generation of thrombus in the middle cerebral artery (MCA) is also possible by local injection of purified thrombin (Orset et al., 2007). As reported recently, the size of the particle was critical in occluding the cerebral vessels which produce different injury pattern of cerebral infarction (Tsai et al., 2011). It was reported that particle$_{(212–250\ \mu m)}$

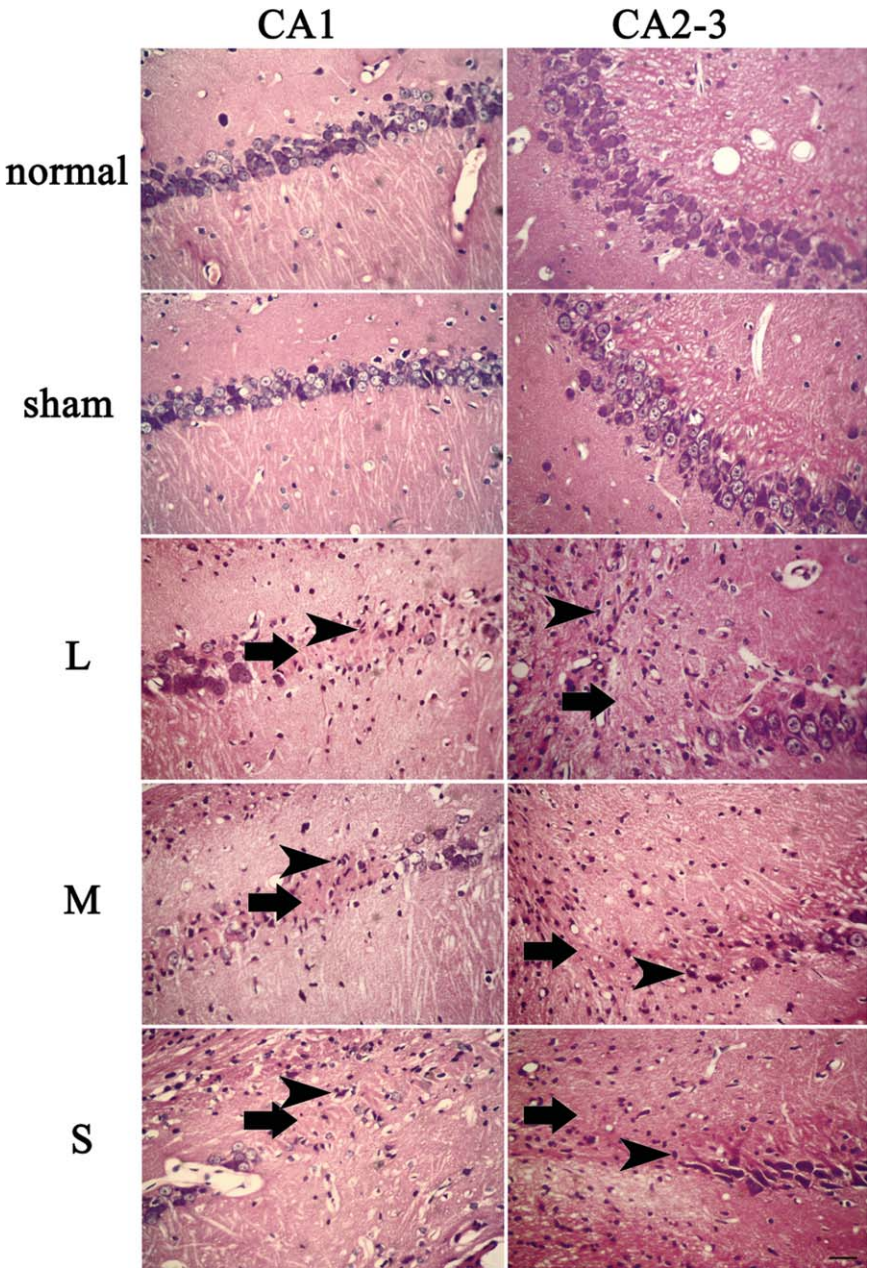

Fig. 4. Representative coronal sections of hippocampal CA1 and CA2–3 in rats at 60 days after ischemia. Three sizes of emboli were injected to rats' brain from the IC arteries to induce the rats' cerebral thromboembolism. After Morris water maze at 60 day, the brain tissues obtained and stained with HE to observe CA1 and CA2–3 under a 400× light microscope (scale bar = 25 μm). The condensed nuclea (arrow heads) and fibroplasia (arrows) in MCAO group were marked, respectively.

produced the greatest diffuse infarction in the ipsilateral hemisphere, including the cortex, hippocampus, basal ganglion, thalamus, midbrain, and cerebellum. Particle$_{(75–90\ \mu m)}$ induced single or sparse isolated infarcts mainly located in the subcortical region, resembling lacunar stroke observed in humans. Particle$_{(38–45\ \mu m)}$ frequently crossed to the contralateral hemisphere and induced diffuse infarctions in both hemispheres. However, as an important part of brain, there is no research about the hippocampal injury pattern. Furthermore, there is no research about the relationship between post-thromboembolic stroke dementia and the size of particles.

According to references (Zang et al., 2006; Tsai et al., 2011) and previous research in our lab, we chose three sizes of emboli (48–74 μm, 74–124 μm, and 150–178 μm) to evaluate the alterations of cognitive function and hippocampal injury following cerebral thromboembolism. At 24 hr, the neurological deficit scores of L group were higher than M group, and the S group was the least,

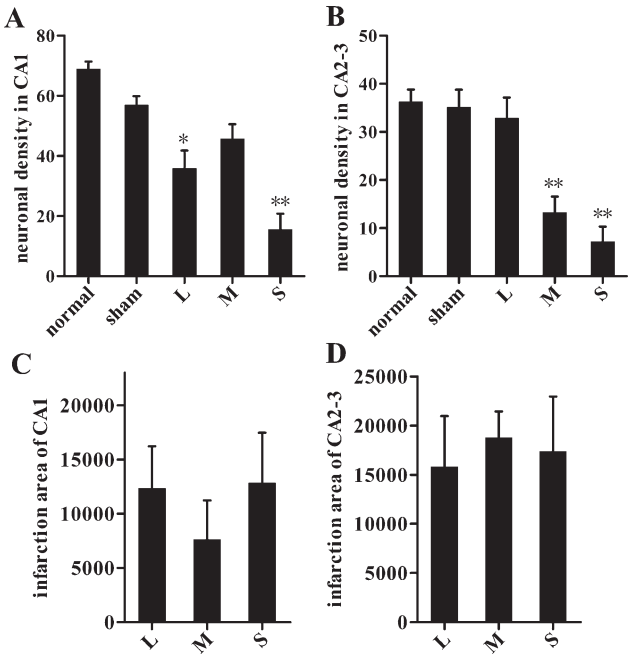

Fig. 5. Result of neuronal density (ND) and infarction areas (IA) of hippocampus. Three sizes of emboli were injected to rats' brain from the IC arteries to induce the rats' cerebral thromboembolism. After neurological examination at 24 hr, the brain tissues obtained and stained with HE. ND of CA1 (**A**) and CA2–3 (**B**) were counted within each microscale area of 1 mm sections from every brain slice under the light microscope (400×) which was determined as the average number of surviving hippocampal pyramidal neurons (complete whole cells) per microscale area of 1 mm sections from each rat that were counted. After Morris water maze tests at 60 days, the brain tissues obtained and stained with HE. IA of the CA1 (**C**) and CA2–3 (**D**) were evaluated by Image J (NIH image J) on every HE slice of 40×, which was determined as the average of infracted area per integrate hippocampus of 1 mm sections from each rat, with three sections of right hippocampal slices for each rat (L: group L; M: group M; S: group S, Data shown as mean ± S.E.M., $^{**}P < 0.05$ vs. sham, $^{*}P < 0.1$ vs. sham).

which not only showed that emboli injection could cause significant neurological deficit but also suggested that the increase of neurological deficit scores correlated well to the diameter of emboli. At 60 day, rats in the S and L groups showed obvious cognitive impairment in Morris water maze. Moreover, the total distance of the rats swimming in the probe trial in 120 sec had no difference which meant that rats' motor function completely recovered at 60 days after emboli injection. However, the percentage of swimming distance in goal quadrant was significantly decreased in the L and S groups.

HE staining demonstrated that the territories of the left middle cerebral artery and anterior choroidal artery were invariably involved. There were scattered, mostly microscopic sized lesions in the regions of anterior and/or posterior cerebral arteries including parietotemporal cerebral cortex, hippocampus, and thalamostriate areas. The neurons of the hippocampus are one of the most vulnerable to loss of blood supply to the brain in humans and rodents (Pulsinelli and Brierley, 1979). In the present study, HE staining at 60 days showed that hippocampus infarction of rats' rate reached 93.8% (15/16) and 90.9% (10/11) in the L and S groups, while those of M group reached 88.9% (16/18) and most infarctions were in the right cerebral hippocampus. It was higher than data reported by Kudo et al. (60%) (Kudo et al., 1982). Results at 24 hr suggested that the neuron loss of hippocampal CA1 and CA2–3 in the three model groups were quite prominent, especially induced by $\text{emboli}_{(48–74\ \mu m)}$ in the S group. The CA1 region of the hippocampus is the area most often associated with learning and memory. The Morris water maze is a hippocampus dependent memory task that has been commonly employed in the evaluation of cognitive status in the ischemic brain (Kuang et al., 2008). A direct correlation between the cerebral hypoperfusion-induced memory deficit and CA1 damage has been demonstrated by various studies (De Jong et al., 1999; Chao et al., 2010). HE staining analyses at 60 days suggested that the infarction area of CA1 in the three groups at 60 day was different: Group S > Group L > Group M. Taken together, both Morris water maze and HE staining suggested that injection of $\text{emboli}_{(150–178\ \mu m)}$ and $\text{emboli}_{(48–74\ \mu m)}$ (15 mg/0.5 mL NS) lead to obvious cognitive impairment and more obvious hippocampus CA1 injury. In addition, from the results we proved again that damaged hippocampus, especially injury of CA1 region, was probably the critical reason leading to obvious cognitive impairment.

In conclusion, first we characterized the patterns of hippocampus infarction induced by the three sizes of emboli in rats. Our results proved that the three sizes of emboli caused obvious hippocampus injury, especially CA1 and CA2–3. Second, we examined the behavioral consequences induced by emboli with three different sizes. Results showed that the increase of neurological deficit scores at 24 hr correlated well to the diameter of emboli. Previous study reported those neurological deficits were caused not only by hippocampal damage, other cerebral region like cortex might be involved in (Tsai et al., 2011). At 60 days after emboli injection, results showed that $\text{emboli}_{(150–178\ \mu m)}$ and $\text{emboli}_{(48–74\ \mu m)}$ lead to obvious cognitive impairment, which correlated well to the hippocampus CA1 injury. Our research might be helpful to choose suitable size of emboli to induce animal model for subcortical ischemia and vascular dementia.

However, our study has many limitations. Given that the present study does not include the histopathological consequences of cerebral cortex and other brain regions and it is very interesting to know the change of the structure of hippocampus at 30 days and the exact correlation between structural damage and cognitive impairment. Therefore, cognitive alterations and cerebral injury following different sizes of emboli injection in rats remains a topic for future investigation.

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