LABORATORY INVESTIGATION

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Photochemically Induced Cerebral Ischemia in a Mouse Model

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Objective : Middle cerebral artery occlusion(MCAO) has widely been used to produce ischemic brain lesions. The lesions induced by MCAO tend to be variable in size because of the variance in the collateral blood supply found in the mouse brain. To establish a less invasive and reproducible focal ischemia model in mice, we modified the technique used for rat photothrombosis model.

Methods : Male C57BL/6 mice were subjected to focal cerebral ischemia by photothrombosis of cortical microvessels. Cerebral infarction was produced by intraperitoneal injection of Rose Bengal, a photosensitive dye and by focal illumination through the skull. Motor impairment was assessed by the accelerating rotarod and staircase tests. The brain was perfusion-fixed for histological determination of infarct volume four weeks after stroke.

Results : The lesion was located in the frontal and parietal cortex and the underlying white matter was partly affected. A relatively constant infarct volume was achieved one month after photothrombosis. The presence of the photothrombotic lesion was associated with severe impairment of the motor performance measured by the rotarod and staircase tests.

Conclusion : Photothrombotic infarction in mice is highly reproducible in size and location. This procedure can provide a simple method to produce cerebral infarction in a unilateral motor cortex lesion. In addition, it can provide a suitable model for study of potential neuroprotective and therapeutic agents in human stroke.

KEY WORDS : Rose Bengal · Cerebral ischemia · Photothrombosis · Mouse.

Introduction

Stroke is one of the leading causes of death and the most common cause of adult disability. Stroke often causes devastating and irreversible loss of function. There is a wide range of sensory, motor, and cognitive deficits including tremor, lack of coordination and partial paralysis[6]. There is no specific treatment for improving functional recovery after stroke. A large number of neuroprotective drugs have failed to demonstrate efficacy in clinical trials[27].

Development of effective stroke therapy requires utilization of animal models that can simulate human pathology in a reproducible and physiologically relevant manner. Currently, the intraluminal suture model of the middle cerebral artery occlusion(MCAO) has been widely used to induce ischemic brain lesions. As the MCA is occluded via a cervical carotid approach, this obviates the requirement for a craniectomy and the associated problems with opening the skull[4,7,21]. However, the lesions caused by the MCAO tend to be variable in size; in addition, animals have been excluded from analysis by arbitrary criteria such as unexpected behavior in most series[1,23]. The collateral blood supply in the mouse brain is variable depending on the mouse strain; this results in variability of clinical outcome and stroke size[11,23]. The C57BL/6 strain, frequently used for genetic studies, is particularly unsuitable for MCAO[3]. The development of reliable and reproducible animal models for cerebral ischemia is needed for the systematic study of the pathophysiology and treatment of stroke.

Watson et al.[26] introduced photothrombosis as a technique that could be used to induce cerebral infarction; this technique is highly reproducible in size and location. In this study, we adapted the rat photothrombosis model by modifying the

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application route of the dye, illumination and stereotactic parameters to establish a less invasive and reproducible focal ischemia model in mice.

Materials and Methods

Photothrombotic cortical lesion

All surgical procedures and postoperative care was performed in accordance with guidelines of Animal Care and Use Committee. A total of 25 male C57BL/6 mice (8~10 weeks old) weighing 20~25g were maintained on a 12-h light/dark cycle. The mice were fasted overnight before the day of the experiment but were allowed free access to tap water.

Focal cortical ischemia was induced by photothrombosis of the cortical microvessels. Each mouse was anesthetized with 2% isoflurane and maintained with 1% isoflurane in an oxygen/air mixture using a gas anesthesia mask in a stereotaxic frame (Stoelting, Wood Dale, IL, USA). The rectal temperature was maintained during surgery at 37±0.5°C with a homeothermic blanket (Harvard Apparatus). Rose Bengal (Sigma Chemical Co., St. Louis, MO, USA), 0.1ml of a 10mg/ml solution in normal saline, was infused intraperitoneally via a 1mm peritoneal incision 5 min before illumination. The skull was exposed via a midline incision of the skin. For illumination, a fiber optic bundle of a cold light source (Zeiss FL1500 LCD, Berlin, Germany) with a 4.5mm aperture was centered 2.4 mm to the left of bregma. According to the atlas by Franklin and Paxonos[9] at this stereotactic position, the mouse sensorimotor cortex is located. The periosteum over the skull was removed completely and the cold light source was placed as close as possible to the skull. The brain was illuminated for 15 min through the exposed intact skull. The scalp was sutured and mice were allowed to awake.

Behavioral testing

For behavioral testing, all mice were housed in individual cages. Prior to induction of ischemia, all animals received training in the staircase and rotarod test. Animals that did not achieve criteria were excluded from the further study. All behavioral testing was carried out weekly.

The rotarod test was used to examine balance and coordination. All mice were trained on the rotarod on 3 consecutive days and a total 9 sessions before surgery. The rotating drum was accelerated from 4 to 40 rpm over 5 min and the latency in seconds for the animal to fall off the drum was recorded[13]. Each session included three consecutive trials, with a maximum time of 300 sec. The mean fall latency was calculated from the three trials. The rotarod performance at baseline is presented as the time spent on an accelerating rotarod at the final pre-stroke training session. Animals that did not stay on the rod for an average of at least 1 min at the end of training were excluded from the stroke surgery.

The staircase test was employed to test skilled forepaw use[2,19,25]. Food was restricted during the pre-stroke training period and 6 days following surgery in order to provide motivation for food rewards. All animals were food restricted to 85 to 90% of their free-feeding weight. Animals were returned to a free feeding schedule for 6 days following surgery to improve postoperative weight and recovery. All animals were fed 3~4g of standard laboratory chow at the end of each test. Food-restricted mice were trained to retrieve food pellets from a staircase that positioned the pellets at increasing distances from the body and allowed only the lesion-affected forepaw access to one set of pellets. Animals were placed in the staircase apparatus (Lafayette instrument). Each step of the stairs was baited with two food pellets of 14mg purified pellet (Bioserve). Eight steps were baited and each test session lasted 30 min. The number of pellets retrieved and eaten per side was used as a measure of forelimb reaching ability. Animals were trained for 2 weeks before being subjected to cortical ischemia to establish the baseline performance. Animals that did not learn to retrieve 4 pellets from each side, by the last training, were excluded from the stroke surgery.

Histology and analysis

Ten animals subjected to 24 hours of ischemia were used to confirm the ischemic injury. At 24 hours post-ischemia each animal was anesthetized with intraperitoneal ketamine (100mg/kg) and xylazine (15mg/kg), decapitated and the brain was removed. The brains were chilled at -80°C for 2 min to slightly harden the tissue and sectioned coronally into five 2-mm slices using a mouse brain matrix. Slices were immediately stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) in 0.9% phosphate-buffered saline and stained at 37°C for 20 min. After staining, the TTC was replaced with 10% formalin. The cerebral infarcts in the brain were unstained and clearly delineated from normal tissue that was stained red.

Four weeks after surgery, fifteen animals were perfused transcardially with phosphate-buffered saline(PBS), followed by 4% paraformaldehyde/PBS solution. The brain was dissected, postfixed for 48 hours and processed for histological quantification of ischemic damage. Coronal sections were cut serially in a cryostat at -20°C through the length of the lesion at a thickness of 0.5mm.

For quantification of stroke volume, eight coronal sections spaced at 0.5mm intervals, throughout the brain of each animal, were stained with cresyl violet acetate (Sigma) for determination of infarction size. Images of each section were digitized and the infarct area per section were traced and measured with image analysis software (Scion Image). Only the

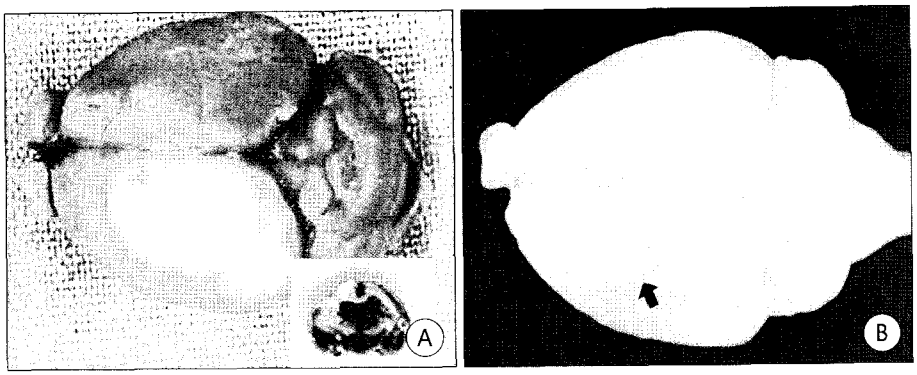

Fig. 1. Macroscopic appearance of the brain on day 1 and day 28 after injury. A : Macroscopic appearance of thrombotic cortical infarction shown in coronally sectioned mouse brain 24 hours after intraperitoneal injection of Rose Bengal and focal illumination. After incubation with triphenyltetrazolium chloride, infarcted area is visible due to absence of staining product in the irreversible damaged cells that lack dehydrogenase. The pale, non-stained area represents the lesion corresponding to ischemic injury, while viable tissue stained with deep red. B : Macroscopic appearance of cortical infarction on 28 days after injury. Arrow indicates the site of atrophy of the affected cortex.

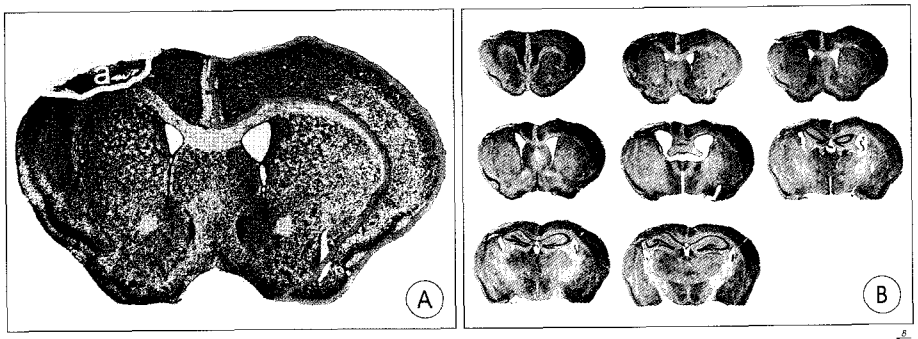

Fig. 2. A : Drawing of histological section showing how to measure the cortical infarct volume. $\sum_{n=1}^{8}(a_n/b_n)\times 1/8\times 100$. B : Coronal brain sections stained with cresyl violet showing a typical cortical infarct after photothrombosis on day 28 after injury.

Table 1. Cortical infarct volume on 28 days after injury

Animal No.	Contralateral cortical volume (pixels)	Infract volume (pixels)	Percentage (%)
1	2282421	279664	12.25
2	2535406	257172	10.14
3	2644856	365622	13.82
4	2698147	260048	9.64
5	2595433	345910	13.32
6	2309606	292195	12.65
7	2098633	242294	11.55
8	2306584	316261	13.71
9	2009010	195954	9.75
10	2116064	280658	13.26
11	2262342	308890	13.65
12	2256341	230701	10.22
13	2385358	293860	12.31
14	2217058	232668	10.49
15	2126703	265204	12.47
Average	2322931	277807	11.97

cerebral cortical area was included in the measurements, since all strokes were superficial. The cortical infarct volume was determined by the indirect method as area of the cortical infarct divided by area of the intact contralateral cortex and was then expressed as a percentage of the intact contralateral cortical volume[20]. Volume was interpolated across the eight sections (Fig. 2A).

Results

In this experiment, Rose Bengal dye was injected systemically and then the cortex was illuminated, at the left sensorimotor cortex, in a mouse to produce a discrete photothrombotic lesion based on light absorption by the dye. Histological examination of the brain at 24 hours after induction of ischemia showed a consistent pattern of ischemic brain damage, which was demarcated from the surrounding vital tissue after incubation with TTC. The lesion was located in the frontal and parietal cortex, as shown in Fig. 1A, B. The underlying white matter was partly affected by the ischemic insult. The total lesion volume at one month after injury was $11.97\pm0.99\%$ (Table 1). Cresyl violet staining revealed both gliotic scarring and atrophy of the affected cortex (Fig. 2B).

Behavioral recovery was assessed by the forepaw pellet retrieval task and the rotarod test. The unilateral lesion in the sensorimotor cortex produced a significant contralateral deficit implicating an impairment of contralateral motor control. Limb movement and open field locomotion were improved quickly after insult with these cortical lesions. However, persistent deficits in motor performance were detectable using two additional complicated tasks in mice up to 4 weeks after the ischemic insult. The lesion significantly disrupted the number of pellets collected with the contralateral paw in the staircase test. The number of food pellets retrieved with the affected right forepaw in this 30 minute test dropped from 7 per session in pre-stroke training to 2 per session at one week post-stroke and recovered slowly to 3-3.5 pellets per session over a month (Fig. 3A). The average time spent on the rotarod dramatically dropped from 219.2 ± 14.6 seconds in pre-stroke training to 139.3 ± 13.4 seconds at one week post-stroke and slightly improved to 151.9 ± 12.7 seconds over one month (Fig. 3B); these findings suggest that ische-

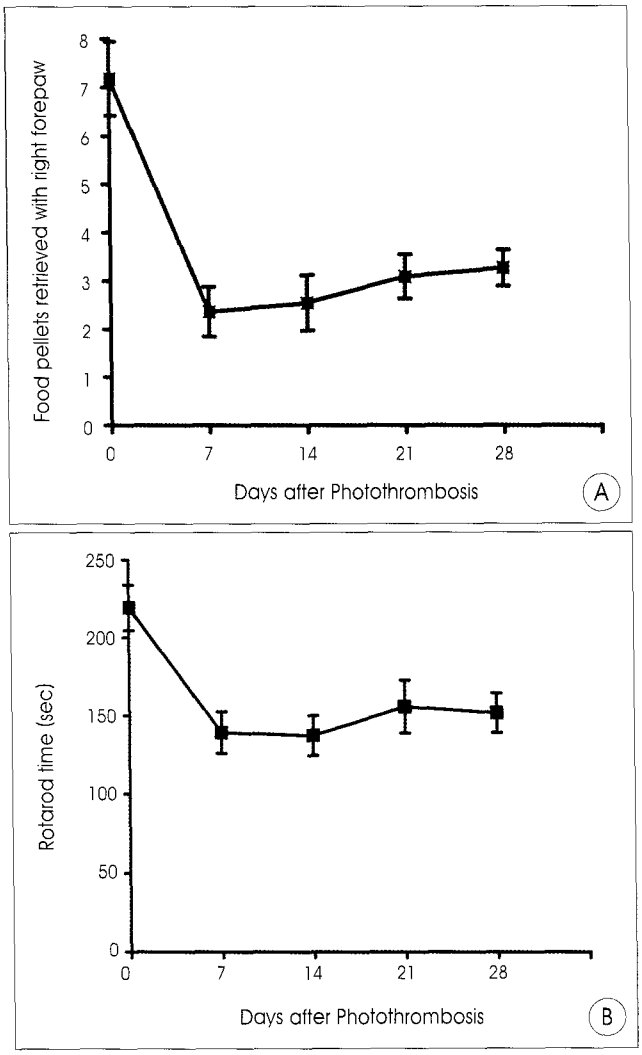

Fig. 3. Behavioral performance following cortical photothrombosis. A : Staircase test of skilled forelimb reaching ability. The photothrombotic lesion reduces the number of pellets retrieved with the contralateral forepaw. The number of food pellets retrieved with the affected right forepaw dropped from 7/session in pre-stroke training to 2/session at one week post-stroke and recovered slowly to 3-3.5 pellets per session over a month. B : Effect of ischemia on rotarod performance. The average time spent on the rotarod was significantly decreased at one week post-injury and performance across the postoperative period was slightly improved. Data are expressed as mean (SEM of time spent on the accelerating rotarod from different animals. n=15 mice, SEM = standard error of the mean.

mia had induced a marked deficit in motor co-ordination and balance.

Discussion

Cerebral ischemia in mice has been introduced by transient and permanent occlusion of the middle cerebral artery[7,14]. However, the intraluminal filament MCAO model, which was initially described by Koizumi et al.[15] and modified by Zea Longa et al.[17] in the rat, requires sophisticated microsurgical skills because of the proximity to the vagus nerve and the tiny anatomy of the carotid system in mice. The size of the cerebral infarct is highly dependent on the vascular anatomy of the MCA and collateral vessels which differs considerably among mouse strains[18] Furthermore, the size of the infarction corresponding to the MCA territory area is so large and the neurologic deficit is so severe that intraluminal suture occlusion for 60 minutes results in more than an 80% mortality in the C57BL/6 mice followed for 7 days[14]. Therefore, this model is not suitable for a study where survival over long periods with long-term follow-up is necessary (e.g. regeneration study)[14].

In this study, we introduce a simple model of focal cortical ischemia in mice using photothrombotic occlusion of cerebral microvessels. Photothrombosis is a simple method used to induce cerebral infarction; it is highly reproducible in size and location. This approach for inducing brain infarction is based on a photochemical reaction between photosensitive dyes and light in the generation of singlet molecular oxygen. The method was initially proposed by Rosenblum and El-Sabban[22] who injected sodium fluorescein in craniotomized mice and irradiated pial vessels using ultraviolet light from a mercury lamp. The technology was improved by Watson et al.[26] with use of another dye, Rose Bengal, a more efficient generator of oxygen radicals. Irradiation was made through the intact calvarium of the rat, thereby rendering craniotomy unnecessary and ensuring the noninvasive character of the method. In subsequent studies of the rat model, the use of a conventional light source considerably simplified the procedure[8,10]. The photosensitive dye, Rose Bengal, is absorbed into the blood flow and focal illumination of the skull activates the local dye. Free radical formation leads to peroxidative damage of the endothelial membrane. Consequently disturbance of the endothelium activates platelet aggregation and the coagulation cascade leading to thrombotic occlusion of small vessels[5,23]. The features associated with the Rose Bengal model include acute severe endothelial cell damage, blood-brain barrier damage and edema formation[29]. The events leading to blood vessel occlusion provide some advantage to the model because 85% of human strokes are caused by thrombotic arterial occlusion[28]. In addition, the size and location of infarction can be altered by changing the position of the light source and the duration/intensity of illumination, allowing focal ischemia in the different cortical regions[5]. Furthermore, the surgical procedure is noninvasive because it can be performed without craniectomy and brain manipulation. Therefore, this approach can reduce postsurgical stress on the animals and animals rapidly recover from surgery with improved long term survival. The focal infarction was large enough to demonstrate a behavioral deficit without causing a decrease in survival of the mice.

The illuminated area of the skull was chosen because it overlies the sensorimotor cortex. The lesion itself occupied an area overlapping the sensorimotor cortex. It was anticipated that a lesion in this area would largely destroy the forelimb cortex and hence produce a deficit in the animals' use of their forepaws. The lesions also encompassed parts of the parietal and hindlimb cortex, but there was mild reduction in spontaneous activity and open field locomotion was improved quickly; this suggests that the mice hindlimbs were not seriously compromised. After ischemic stroke, partial recovery of function frequently occurs and may depend on the plasticity of axonal connections. It is likely that some reorganization of brain function can take place after focal infarction by increased axonal plasticity. This natural plasticity of the cortical area in the nondamaged hemisphere and adjacent to denervated areas may underlie at least a portion of stroke recovery[16,20].

Studies of cortical and subcortical motor systems require sensitive tests of the functional motor performance in animals. An evaluation of potential therapeutic interventions for human stroke, in the animal model, requires appropriate behavioral testing. Most behavioral assessments have been developed in the rat model. These include limb placing, beam walking, grid walking, rotarod, the sticky label test and the staircase test. Spontaneous recovery can occur in some of easier behavioral tasks such as beam walking. However, deficits in more complex cognitive or skilled motor tasks such as the staircase test appears to be more stable and may persist for several months after stroke[12,13,24]. Mice are commonly used to study genetic factor in disease based on transgenic and knock-out strategies. However, assessment of sensorimotor function in the mouse after focal ischemia has been rarely described. Therefore, a sophisticated behavioral test that allows for the evaluation of more complex sensorimotor functions, in mouse model, is necessary. The rotarod and staircase test for motor performance provide quantitative, objective and reproducible measures of functional impairment of mice following stroke. These tests provide the sensitivity required to track behavioral recovery over time; therefore, they are useful for comparing outcome after infarcts of differing severity and for comparing the effects of different doses and/or drugs. The rotarod test is a well-established procedure for testing balance and coordination aspects of motor performance in rats and mice. A more complex objective assessment of skilled motor function, the staircase test has been developed by Montoya et al[19]. This test provides a measurement of skilled paw use, where independent forelimb reaching and grasping abilities can be quantitatively assessed in rats and mice. A lesion can significantly disrupt the number of pellets collected with the paw contralateral to the lesion. The only drawback of this test is a need for extensive training over a number of sessions before the stroke surgery. Two tests of the sensorimotor battery appear to be sensitive for detection of deficits after photothrombotic infarction in mice. These studies can provide information integral to future studies evaluating the effects of potential neuroprotective agents.

Conclusion

The results from this study demonstrate that photothrombotic occlusion of cerebral microvessels in mice is a minimally invasive and simple method for making focal cortical ischemia. The cortical lesion produced is consistent in location and size. Therefore, this model offers a reliable method for the study of cerebral ischemia especially for the evaluation of potential neuroprotective and therapeutic agents in human stroke.

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Commentary

Since there have been few reports about this traditional stroke model in Korea, I read this article with great interest and welcome. This article introduced photochemical thrombotic infarction model which is being used, as far as I know, only recently at a few domestic laboratories. The authors well described the technique for producing lesion in mouse and the mechanism of photochemical thrombosis and infarct.

Using this model the authors described mainly the results of behavioral tests. Considering that they did not show detailed informations about the pathobiologic differences or different fate of lesion from other models, the main contribution of this article may be about the performance of behavioral test of mouse with stroke. Actually it is known to be very troublesome to train mouse for behavioral test. Nevertheless, the authors performed nicely the tests and described impressively about them. With my reviewing these data, they showed acceptable ranges of variations, and these may be due to presurgery control with the exclusion criteria that the authors mentioned. I think that key factor for successful performances in behavioral evaluation of mouse is such sophisticated selection of candidates for the tests. It is impressive that the mice showed relatively consistent scores of staircase test in spite of weekly performing postoperative tests. Probably these results suggest that mouse can remember the related behaviors even after a few weeks and that mouse can also be trained successfully for animal behavioral tests. The authors made lesions at left hemispheres in all mice without predetermination of forepaw dominance. Although few are known about animal forelimb dominance, it seems more resonable to determine the dominant forepaw of mouse during pretest training period. The mice in this experiment showed continuing recovery pattern of staircase test even in 4 weeks after infarct, so it is somethat regretable that the point of time of final sequelae was not determined.

The authors reported that they used 4.5mm aperture of fiber-optic light source without mentioning about the intensity of light, and the size of aperture seems relatively large for mouse brain. Nevertheless, their results showed acceptable pattern of recovery of behavioral test. The extent, width and depth, of photochemical stroke lesion invariably change according to the intensity and the diameter of emitting light and the amount of delivered Rose Bengal dye. These factors will also determine the pattern of the motor recovery of mouse after stroke. The reason why the authors succeeded in obtaining their behavioral results with such large aperture may be relatively week intensity (more scientifically, low energy, expressed as Watt/cm^2) of light. When designing the animal behavioral study using this model, the factors for lesion size should be carefully controled, and appropriate and sensitive tests for behavioral evaluation should also be selected. This article shows that staircase test is a excellent tool for this aim in cortical infarction.

Although the volumes of the atrophied and gliotic lesion on 28 days postinjury were presented and such chronic lesions will attenuate the variation of the size of infarct, I agree to the opinion that this model have a advantage of reproducibility of size compared with other animal stroke models. And also, I totally agree with that this model provides reliable method for the functional or neuroprotective study of ischemia because photochemically induced ischemia model can offer higher control of location, more reproducible size of lesion, and longer survival than any other stroke models.

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