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Comparison of Surgical Methods of Transient Middle Cerebral Artery Occlusion between Rats and Mice

Seunghoon LEE[1–4], Yunkyung HONG[1–4], Sookyoung PARK[3], Sang-Rae LEE[5], Kyu-Tae CHANG[5]# and Yonggeun HONG[1–4]#*

[1]*Department of Rehabilitation Science, Graduate School of Inje University, Gimhae, Korea*
[2]*Department of Physical Therapy, College of Biomedical Science & Engineering, Inje University, Gimhae, Korea*
[3]*Ubiquitous Healthcare & Anti-aging Research Center (u-HARC), Inje University, Gimhae, Korea*
[4]*Biohealth Products Research Center (BPRC), Inje University, Gimhae, Korea*
[5]*National Primate Research Center (NPRC), Korea Research Institute of Bioscience and Biotechnology (KRIBB), Ochang, Korea*

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ABSTRACT. Rodent models of focal cerebral ischemia that do not require craniotomy have been developed by intraluminal suture middle cerebral artery occlusion (MCAo). Mouse MCAo models have been widely used and extended to genetic studies of cell death or recovery mechanisms. Therefore, we compared surgery-related parameters and techniques between such rats and mice. In rodent MCAo models, has to be considered body temperature during the operative period, as well as the need for the use of a standardized tip in terms of the outer diameter of probes. Induction of focal cerebral ischemia was measured by neurological dysfunction parameters. Our methods could induce stable moderate-severity ischemic brain injury models and histological alteration at 24 hr after MCAo surgery. Moreover approximately 80% (rats) and 85% (mice) survival ratios were shown indicating with model engineering success. Finally, we described and compared major parameters between rats and mice, including probe size, thread insert length, operation and occlusion periods, and differences in the procedures.

KEY WORDS: focal cerebral ischemia, microsurgical procedure, rodent MCAo model

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Cerebral ischemia-related research has been extensively used rodents with various methods, including vessel occlusion, hypotension and hypovolemia [4, 14]. In order to investigate the mechanisms underlying the injury after ischemic brain injury as well as to exploit effective therapeutic approaches for the disease, several ischemic stroke models have been developed in a variety of species, including rodents, canines, rabbits, cats and primates [1]. Experimental stroke research commonly employs focal cerebral ischemic rat models. Most investigators utilize permanent or transient occlusion of the middle cerebral artery (MCA) in mice or rats [8]. In general, there are two major types of animal model of ischemic brain injury: (1) global cerebral ischemia [2, 24] and (2) focal cerebral ischemia [18]. Ischemic stroke in humans typically results from thrombotic or embolic occlusion in a major cerebral artery, most often the MCA, so experimental focal cerebral ischemia models have been exploited to mimic human stroke [7]. Rodent models of focal cerebral ischemia that do not require craniotomy have been developed by intraluminal suture MCAo [22]. Furthermore, mouse MCAo models have been widely used and extended to genetic studies of cell death or recovery mechanisms [13]. Genetically engineered mouse stroke models are particularly useful for ischemic pathophysiology and in designing potential new prophylactic, neuroprotective and therapeutic agents and interventions [2]. In the past 2 decades, MCAo surgery techniques have been developed in rodent models that are not easy to extend to mouse MCAo models. Previous studies have suggested detailed methods for rodent MCAo model engineering, which involved a variety of standards, such as in terms of filament size, tip outer diameter, insert length of probe and occlusion period [19, 25, 27]. Moreover, several studies suggested that rodent strains should be considered for transient MCAo model engineering [15, 16, 28]. Thus, ischemic stroke-related researchers have required a standard method for rodent MCAo model engineering.

Therefore, we established a procedure of mouse MCAo model engineering compared with a rat MCAo model using histological staining and behavioral measurement for neurological severity analysis.

MATERIALS AND METHODS

Animals and pre-operative care: All animal procedures were approved by the Ethics Committee for Animal Care and Use at Inje University (Approval No. 2012–29). A total of 47 healthy adult male rats, weighing 240–260 g, and 37 mice, weighing 20–25 g, were subjected to transient MCAo surgery. All animals were individually housed in plastic cages at controlled temperature (22 ± 1°C), relative humid-

*CORRESPONDENCE TO: HONG, Y., Department of Physical Therapy, College of Biomedical Science & Engineering, Inje University, 197 Inje-ro, Gimhae, Gyeong-nam 621-749, Republic of Korea. e-mail: yonghong@inje.ac.kr

#These authors contributed equally in this work.

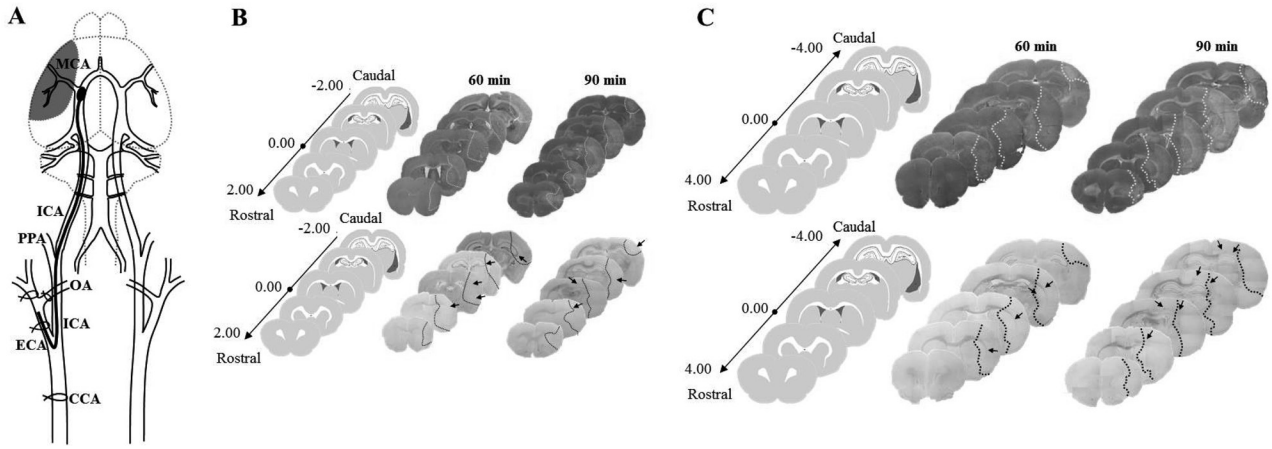

Fig. 1. Structural diagram of the MCAo surgery region and histological results followed by an occlusion period in rats and mice. (A) Monofilament was inserted into MCA via ECA stump. H&E- and Nissl-stained brains of (B) MCAo mice and (C) rats. Brain slices were cut at 1-mm (mice) and 2-mm (rats) intervals rostral and caudal to the bregma (0.00 mm). The yellow (H&E staining) and black (Nissl staining) dotted lines indicate the marginal penumbra lesion. The black arrow indicates the pale violet color of the ipsilateral lesion of the corpus callosum. The 90 min MCAo models not only showed severe infarct lesion but also failure to confirmed corpus callosum. CCA: common carotid artery; ECA: external carotid artery; ICA: internal carotid artery; OA: occipital artery; PPA: pterygopalatine artery; MCA: middle cerebral artery.

ity (55 ± 10%) and photoperiod (light/dark conditions 12/12 hr lights on 7:00 a.m.). Food and water were available *ad libitum*.

Preparations of middle cerebral artery occlusion microsurgery: MCAo surgery required 4–0 and 6–0 nylon threads (AILEE Co., Busan, Korea) in rats and mice, respectively. The nylon thread was cut into 4 cm (for rats) or 2 cm (for mice) pieces, and its tips were blunted to form a bulb shape by heating (outer diameter of bulb shape or corn dog form: 0.4–0.45 mm for rats and 0.15–0.18 mm for mice) [10].

Microsurgical procedures of middle cerebral artery occlusion: All animals were anesthetized with 40 mg/kg tiletamine/zolazepam cocktail (Zoletil) and 10 mg/kg xylazine (Rompun) via intraperitoneal injection. After anesthesia, the animals' body temperature was maintained at 36.5°C to 37.0°C using a heating pad on the surgical table. The animals were placed in the supine position and fixed to the surgical table using adhesive tape. The incision region was disinfected with povidone-iodine solution. The midline neck skin was incised, and the common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were carefully separated from the vagus nerve. The ICA bifurcates into the middle cerebral artery (MCA) and the pterygopalatine artery (PPA) from the CCA. The occipital artery (OA) originates from the bifurcation point of the ECA and the ICA. A transient knot was placed on the distal portion of the CCA, near the manubrium of the sternum. A microvascular clamp was transiently placed on the ICA proximal to the CCA junction. Two closely spaced permanent knots were then placed on the distal portion of the ECA, below the suprathyroid artery, to prevent the backflow of blood. The tied section of the ECA was dissected using microscissors to insert the probe, which reached the ICA through the CCA junction. The microvascular clamp that had been placed on the ICA was then removed to allow probe insertion. The probe was carefully inserted into the MCA from the CCA junction (up to 18–20 mm in rats and 9–11 mm in mice). After confirmation of MCA blockage (mild bending of the ICA), the rat model exhibited blood supply from the CCA, but the mouse model only exhibited blood supply after the occlusion period (dual occlusion by CCA ligation). After 60 to 90 min, the probe was carefully withdrawn until the tip was near the arteriotomy for reperfusion. After confirmation of blood flow reperfusion, topical lidocaine gel was applied onto the surgical region to relieve pain and discomfort in the postoperative period. Each animal also received 1.0 m*l* of normal saline subcutaneously for volume replenishment after the surgery. All surgical procedures were finished within 15 min, excluding the occlusion and reperfusion periods (Fig. 1A).

Measurements of neurological dysfunction in rats and mice followed by MCAo: The battery of rat MCAo model tests consisted of evaluation of the modified neurological severity score (mNSS) [12]. Table 1 shows a set of the mNSS. Neurological function was graded on a scale of 0 to 18 (normal score: 0, maximal deficit score: 18). The mNSS evaluation involves a composite of motor, sensory, reflex and balance tests. In the severity scores of injury, 1 point is awarded for the inability to perform the test or for the lack of a tested reflex; thus, the higher the score, the more severe the injury. Likewise, mouse MCAo models have been suggested to have an association with a neurological disability status score (NDSS) (Table 2) [21].

Evaluation of infarct lesion between rats and mice followed by MCAo: The subjects were anesthetized with an intraperitoneal injection of Zoletil (40 mg/kg) and Rompun (10 mg/kg). Subsequently, they underwent transcardial perfusion with 0.1 M PBS (phosphate-buffered saline) (pH

Table 1. Modified neurological severity score points (mNSS)

Motor functions	Score
Raising rat by tail	3
Flexion of forelimb	1
Flexion of hindlimb	1
Head moved > 10° to vertical axis within 30 sec	1
Placing rat on floor (normal=0; maximum=3)	3
Normal walk	0
Inability to walk straight	1
Circles toward paretic side	2
Falls down to paretic side	3
Sensory tests	2
Placing test (visual and tactile test)	1
Proprioceptive test (deep sensation, pushing paw against table edge to stimulate limb muscles)	1
Beam balance tests (normal=0; maximum=6)	6
Balances with steady posture	0
Grasps side of beam	1
Hugs beam and 1 limb falls down from beam	2
Hugs beam and 2 limbs fall down from beam, or spins on beam (>60 sec)	3
Attempts to balance on beam but falls off (>40 sec)	4
Attempts to balance on beam but falls off (>20 sec)	5
Falls off; no attempt to balance or hang on to beam (<20 sec)	6
Reflex absence and abnormal movements	4
Pinna reflex (head shake when auditory meatus is touched)	1
Corneal reflex (eye blink when cornea is lightly touched with cotton)	1
Startle reflex (motor responses to a brief noise from snapping a clipboard paper)	1
Seizures, myoclonus, myodystony	1
Maximum points	18

One point is awarded for inability to perform the tasks or for lack of a tested reflex: 13–18, severe injury; 7–12, moderate injury; 1–6, mild injury.

Table 2. Neurological disability status scale

Degree of deficit	Neurobehavioral alterations
0	None
2	Hypomobility (slight)
	Passivity
4	Hypomobility (moderate)
	Flattened posture
	Hunched back
	Ataxic gait
	Piloerection
	Decreased body tone
	Decreased muscular strength
	Motor incoordination (slight)
6	Circling
	Tremor/twitches/convulsions
	Forelimb flexion
	Motor incoordination (moderate)
8	Hypomobility (severe)
	Motor incoordination (severe)
	Respiratory distress
10	Death

7.4), followed by fix with 4% NBP (neutrally buffered paraformaldehyde). The brain tissues were rapidly removed and stored in the same solution (4% NBP) for 24 hr at 4°C and then passed through a gradient (10, 20, and 30%) of sucrose solutions for cryoprotection. Next, tissue samples were embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA, U.S.A.). Brain tissues were dissected at 10 and 20 μm using a cryostat (HM525; MICROM International GmbH, Walldorf, Germany). They were then stained with either H&E (10-μm thick sections) or cresyl violet acetate (Nissl; 20-μm thick sections), serially dehydrated in EtOH solutions, cleared with xylene and mounted with Permount (SP15-500; Fisher Scientific, Fair Lawn, NJ, U.S.A.).

Statistical analysis: Data were collected from repeated experiments and are presented as means ± SD. Student`s *t*-test was used for statistical analysis. Differences were deemed to be statistically significant when $P<0.05$. All analyses were performed using the SPSS software (SPSS ver. 19.0, IBM, Chicago, IL, U.S.A.).

RESULTS

Alteration of body weight in rodent MCAo animal models: MCAo surgery was applied to 8-week-old rats and mice, as well as equalization of the body weight from 240–260

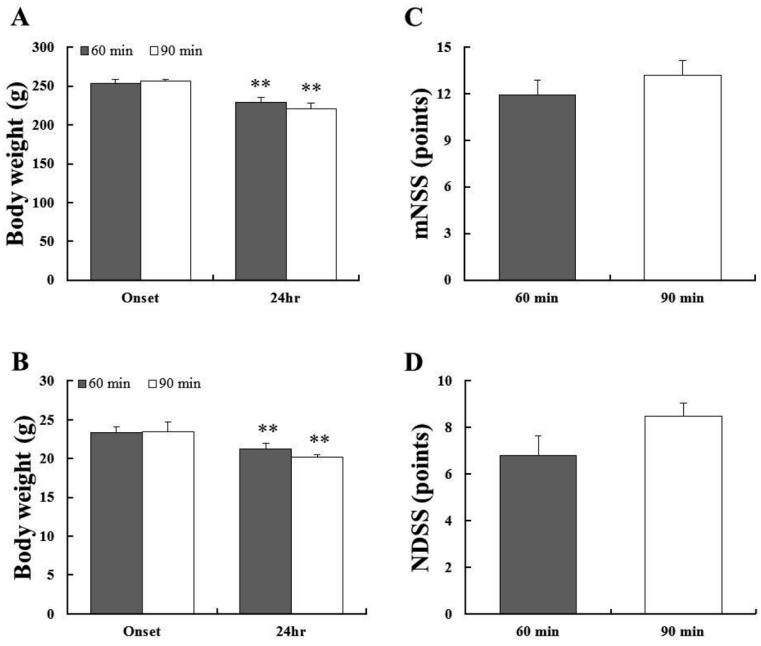

Fig. 2. Results of body weight alteration and neurological dysfunction symptoms. (A) Alteration of body weight of rat MCAo models followed by an occlusion period. (B) Alteration of body weight of mouse MCAo models followed by an occlusion period. (C) Neurological severity score of rat MCAo models at 24 hr after transient MCAo surgery by occlusion period. (D) Neurological disability status of mouse MCAo models at 24 hr after transient MCAo surgery by occlusion period. Occlusion period did not significantly affect neurological score. The 90 min occlusion period was clearly associated with a severe score, which affected survival ratio and model identity. mNSS: modified neurological severity score; NDSS: neurological disability status score. **P*<0.05, ***P*<0.01 compared with that at onset, Student's *t*-test.

g (rats; 60 min: 253.08 ± 5.62 g and 90 min: 256.89 ± 2.26 g) and 20–25 g (mice; 60 min: 23.30 ± 0.79 g and 90 min: 23.50 ± 1.19 g). The subjects showed significant reductions of body weight compared with those before onset (rats; 60 min: 23.70 ± 7.43 g and 90 min: 36.29 ± 9.31 g) (mice; 60 min: 2.03 ± 0.54 g and 90 min: 3.17 ± 1.11 g) (P<0.01) (Fig. 2A, B). In 60 min occlusion models, there were reductions of approximately 10% compared with initial body weight in rats and mice. However, 90 min occlusion models showed approximately 15% reductions compared with initial body weight (P<0.01).

Survival ratio of MCAo rodent models: The 60 min occlusion rat model animals showed a survival ratio of 80.95%. A total of 6 rats were excluded in the moderate-severity MCAo model. Specifically, 3 rats were lost due to respiratory failure after reperfusion, 1 rat died from bleeding from the jugular vein, and 2 rats were excluded due to mild symptoms. Therefore, a total of 21 rats underwent MCAo surgery, and 15 of them showed moderate symptoms (model engineering success ratio: 72.43%). In the mice, 19 mice were subjected to MCAo surgery. A total of 16 mice also showed moderate-severity symptoms, as well as 3 mice that died due to respiratory failure. In terms of comparison of the 90 min occlusion MCAo models, 15 rats were excluded in the MCAo model. In detail, 1 rat was lost due to cardiac arrest at approximately 80 min during occlusion, 1 rat died from bleeding from the occipital artery, and 3 rats were lost due to cardiac arrest with respiratory failure. A total of 8 rats were also lost due to respiratory failure after reperfusion. Therefore, a total of 26 rats underwent MCAo surgery, and 11 rats showed moderate to severe symptoms. In the mouse MCAo model animals, 10 mice were also lost due to respiratory failure, as well as 2 mice that died from bleeding upon occipital artery rupture. Likewise, 18 mice were subjected to MCAo surgery, and 6 mice also showed high severity symptoms (Table 3).

Measurement of neurological dysfunction: All subjects were evaluated for neurological dysfunction following mNSS (for rats) and NDSS (for mice). The occlusion period is important for neurological severity. A period of occlusion of 60 min occlusion might be appropriate for moderate severity. A total of 19 rats showed moderate-severity symptoms (11.93 ± 0.96) (Fig. 2C). However, 2 rats showed mild symptoms, excluding circling gait, spasticity of paretic limbs or falling down on the beam. In the mice, 16 also exhibited moderate-severity symptoms (6.81 ± 0.83) (Fig. 2D). In contrast, 90 min occlusion more often showed higher severity than 60 min. A total of 11 rats showed high-severity symptoms (13.18 ± 0.98), including falling down on the paretic side in open field, falling down in 20 sec on the beam and the absence of a proprioceptive response (Fig. 2C). Likewise, 6 mice presented high-severity symptoms, including severe hypomobility, respiratory distress and motor in coordination (Fig. 2D).

Comparison of infarct lesions of rodent MCAo animal

Table 3. Results of neurological behavior tests and alteration of body weight

Occlusion Period	Rats			Mice		
	mNSS	Body weight (g)		NDSS	Body weight (g)	
		Onset	24 hr		Onset	24 hr
60 min (n=19)	11.93 ± 0.96	253.08 ± 5.62	229.38 ± 6.51 (−23.70 ± 7.43)	6.81 ± 0.83 (n=16)	23.30 ± 0.79	21.28 ± 0.68 (−2.03 ± 0.54)
90 min (n=26)	13.18 ± 0.98	256.89 ± 2.26	220.60 ± 7.92 (−36.29 ± 9.31)	8.50 ± 0.55 (n=18)	23.50 ± 1.19	20.18 ± 0.33 (−3.17 ± 1.11)

Table 4. Results of infarct volume in rats and mice following occlusion period

Slice No.	60 min occlusion				
	#1	#2	#3	#4	#5
Rats	30.68 ± 4.50	41.01 ± 2.88	58.43 ± 0.38	55.09 ± 0.18	45.74 ± 3.00
Mice	20.94 ± 1.11	45.78 ± 1.53	46.70 ± 0.81	37.88 ± 1.54	31.86 ± 1.08
Slice No.	90 min occlusion				
	#1	#2	#3	#4	#5
Rats	36.75 ± 2.09	45.72 ± 1.18	60.63 ± 1.20	58.47 ± 0.68	58.79 ± 1.00
Mice	41.98 ± 2.42	50.84 ± 1.96	55.53 ± 1.53	52.49 ± 1.67	50.57 ± 2.36

*P<0.05, **P<0.01: 60 min vs. 90 min in rats and mice.

model: Transient MCAo models were affected by the occlusion period. Therefore, the occlusion period was divided into 60 and 90 min. All animals were sacrificed 24 hr after reperfusion. The animals were employed for the following histological studies: TTC staining (60 min occlusion: 3 rats and 3 mice; 90 min occlusion: 3 rats and 2 mice), H&E and Nissl staining (60 min occlusion: 12 rats and 13 mice; 90 min occlusion: 8 rats and 4 mice). We previously found that the infarct volume is affected by the occlusion period using the TTC staining method [10]. Here, we obtained quantitative results using general histological methods. Therefore, H&E and Nissl staining was performed according to the protocol described in the Methods section. In the comparison of the infarct volume using H&E staining, the bregma 0.00 mm (slice #3) region showed the largest infarct volume at 60 min of occlusion in both rat and mouse models (rats, 58.43 ± 0.38%; mice, 46.70 ± 0.81%). However, the infarct volume was slightly smaller in the caudal slices. Infarct lesion of MCAo animal models with occlusion for 90 min was significantly extended to retrograde at #3, #4 and #5 compared with those of MCAo animal models with occlusion for 60 min in rats and mice (*P<0.05, **P<0.01) (Table 4). The corpus callosum exhibited a dark violet contralateral lesion on Nissl staining. However, the ipsilateral corpus callosum showed a pale violet lesion (Fig. 1B and C, black arrow) without a clear division between superficial and profundus layer in cerebrum (Fig. 1B, C, lower panel).

DISCUSSION

The intraluminal MCAo model mimics one of the most common types of ischemic stroke in human. Nevertheless, MCAo models have typically involved rats for stroke-related research [23]. However, mouse stroke models have insteadoften involved genetically or molecularly manipulated mice in research using knockout and knockdown techniques [5, 6, 17]. Knockout mice have been informative in the discovery of unexpected biological functions of specific molecules [26]. In particular, targeted molecules were identified as having roles in pathophysiological mechanisms. In stroke-related studies, knockout mice have been used to identify the inhibitory mechanism of cell death signaling, as well as applied in the pharmacological developmental industry through elucidation of the effects of candidate molecules that exhibit therapeutic efficiency [9, 17]. Genetically mutated mouse models are essential for exploring genetic diseases or undisclosed disease mechanisms [26].

Numerous studies have described MCAo surgery procedures [3, 4, 10–12, 14, 19, 20]. However, there are different procedures, such as in terms of filament size and tip diameter, insert length, occlusion period and surgical techniques. The aim of the present study was to establish a standard mouse MCAo model surgery procedure, as well as to compare it with rat MCAo, which is extensively applied in stroke-related animal research.

In MCAo surgery, when all procedures have been completed, recovery of the body temperature must be achieved, since numerous studies have proposed a body temperature range of 37.0 ± 0.5°C, as well as indicating that the operation period can also affect hypothermia. Body temperature is one of the essential factors affecting the extent of infarction, hypothermia decreases and hyperthermia increases the infarct lesion size [6]. Thus, completion of operation within 15 min has been recommended [8]. Consequently, we exclude animals that have undergone operation for longer than 15 min. The rat MCAo model used a 4–0 monofilament nylon, which is made of a 0.4–0.45-mm outer-diameter thread by heating. Numerous studies have suggested that the optimal thread re-

quires 6–0 monofilament nylon for occlusion, which is made with a 0.17–0.2-mm outer-diameter by heating [6, 13]. We utilized 0.15–0.18-mm outer-diameter 6–0 nylon by heating. Mouse MCAo models showed similar infarct volume to the rat MCAo models upon 60 min occlusion. In particular, the bregma 0.00 mm region showed the largest infarct volume in both rat and mouse brains, as well as presenting a pattern of infarct lesion from rostral to caudal in 60 min occlusion models. The 90 min occlusion models also showed a similar pattern of infarct lesion. Conversely, infarct volume was shown to be slightly reduced from bregma 0.00 to caudal (Fig. 2).

We previously suggested that the occlusion period by MCAo surgery affects the infarct volume in rat and mouse models of focal cerebral ischemia; rat and mouse 90 min MCAo models exhibited significantly augmented infarct volume from bregma 0.00 to −4.00 mm and from 2.00 to −2.00 mm, respectively [10]. Brain infarct volume might be associated with the survival rate.

In terms of the survival ratio, the 60 min occlusion rat model showed an approximately 80% survival ratio, as well as a 72.43% ischemic model engineering success ratio. Likewise, mouse model animals showed approximately 85% survival and 85% ischemic model engineering success ratio. However, 90 min occlusion models showed a poor model engineering success ratio followed by cardiac arrest, respiratory failure and severe ischemic damage. As a result, 90 min was associated with 42.03% and 33.34% model engineering success ratios in rats and mice, respectively.

We reported that transient MCAo models exhibited a moderate severity score by the mNSS test [12, 19, 20]. In terms of a comparison of neurological dysfunction, 60 min occlusion rat model animals revealed optimal moderate-severity symptoms, including flexion pattern of forelimb, circling gait toward the paretic side and poor balance or falling down on the beam. However, 90 min occlusion rat model animals exhibited severe neurological symptoms, such as falling down on the paretic side during gait, not attempting to balance on the beam, and myodystony. Moreover, there reduced survival ratio during 24 hr after MCAo. Mouse MCAo model animals also showed moderate neurological disability status, as well as presenting with moderate-severity symptoms, including circling gait, moderate hypomobility, excessive urination, forelimb flexion and body rotation by tail suspension. Likewise, 90 min mouse MCAo model animals showed a severe neurological disability status, such as severe motor in coordination namely no attempt to climb to the top of the edge on the inclination board, severe hypomobility namely no movement during 30 sec and respiratory distress.

In conclusion, we have elucidated that the mouse MCAo model procedure could induce a moderate-severity MCAo model, as well as that our method might obtain a higher survival ratio and stable model engineering using mice.

To produce results relevant for a stroke study, standardized and high-quality rodent models are very important in experimental stroke research. In addition, rodent MCAo model engineering has to consider monofilament standard for a hand-made probe, tip outer diameter (unrelated to silicone coating or rounding by heating), probe insert length (is needed regarding hemorrhage or ACA infarction) and maintenance of body conditions during operation (body temperature control) and occlusion time (moderate versus severe score models and identity of model).

The following is a summary of rodent MCAo models:

(1) 0.40–0.45 mm probe of 4–0 nylon (for rats) and 0.15–0.18 mm probe of 6–0 nylon (for mice) by heating
(2) Inserted probe length from bifurcated ICA and ECA: 18–20 mm (for rats) and 9–11 mm (for mice)
(3) Operation time: a maximum of 15 min
(4) Occlusion time: 60 min
(5) MCA occlusion allows CCA reperfusion (for rats) or CCA binary occlusion (for mice)

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