An Optimized Mouse Model for Transient Ischemic Attack

Eric Pedrono, MSc, Aysan Durukan, MD, Daniel Strbian, MD, PhD, Ivan Marinkovic, MD, Shashank Shekhar, MD, Miia Pitkonen, MSc, Usama Abo-Ramadan, PhD, and Turgut Tatlisumak, MD, PhD

Abstract

Transient ischemic attacks (TIAs) are brief neurological deficits of cerebrovascular origin that are followed by complete clinical recovery. Although a plethora of animal models exist for ischemic stroke, a verified TIA model is lacking. We aimed to optimize such a model in mice, investigating the impact of varying durations (from 2.5 to 20 minutes) of intraluminal middle cerebral artery occlusion (MCAo). Three conditions were required to mimic clinical TIA reliably: 1) an objective demonstration of occlusion and reperfusion (assessed by laser Doppler flowmetry); 2) no permanent neurological deficit (assessed by sensorimotor neurological evaluation); and 3) no lesion at 24 hours after reperfusion (assessed by magnetic resonance imaging [MRI]). We observed high incidences of MRI lesions with MCAo durations of 15 minutes or longer. In contrast, no permanent neurological deficits or MRI lesions were observed in animals with MCAo below or equal to 10 minutes. Middle cerebral artery occlusion of 12.5 minutes rarely induced MRI lesions, but histopathologic evaluation using routine and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling staining revealed minute ischemic changes even after 2.5-minute MCAo. Abundance of necrotic and apoptotic changes gradually increased with the duration of ischemia. These results indicate that 10 minutes or shorter focal cerebral ischemia proves a suitable mouse TIA model; in addition, they indicate that MRI-negative microscopic ischemic damage may occur with even a few minutes of arterial occlusion.

Key Words: Animal model, Cerebral ischemia, Magnetic resonance imaging, Middle cerebral artery, Mouse, Stroke, Transient ischemic attack.

INTRODUCTION

Transient ischemic attack (TIA) is classically defined as a sudden brief neurological deficit of cerebrovascular origin lasting less than 24 hours and resolving without any residual symptoms or signs (1). Advances in neuroimaging have provided new insight into TIA (2–4). According to a revised definition, a TIA with magnetic resonance imaging (MRI) evidence (“MRI-positive” TIA) is to be diagnosed as “brain infarction” (5).

Transient ischemic attack remains largely understudied. Until recently, it was considered to be a benign condition; however, we now know that TIA heralds forthcoming ischemic stroke (6, 7). Evidence suggests that TIA-induced tissue changes may vary from an ischemia-tolerant state (8) to clinically silent infarction. Furthermore, repeated TIAs may be associated with cognitive decline (9) or brain atrophy (10), even in “MRI-negative” TIA patients; this suggests that TIA likely leads to some degree of permanent brain injury that is beyond the detection limits of MRI. Because histopathologic studies of patients with TIA are not feasible, an appropriate animal model of TIA might facilitate analyses of changes within the neurovascular unit and the molecular determinants of tissue fate after TIA, and it would assist in identifying cellular and molecular substrates of mild ischemic injury in a spatial pattern and with a dose-response gradient corresponding to the increasing severity of ischemic insult. Furthermore, such a model may aid the discovery of therapies targeting the adverse consequences of TIA, such as necrotic and apoptotic injury cascades, and promoting neuroprotection.

Among existing models for ischemic stroke (11), most fail to accomplish well-controlled reperfusion, except for surgical and intraluminal suture middle cerebral artery occlusion (MCAo). The major disadvantage of the surgical approach is its invasiveness. The intraluminal suture MCAo method is less invasive and easier to perform and thus is preferable for the study of TIA in mice.

In this study, we aimed to develop a mouse TIA model that 1) ensures cerebral arterial occlusion and reperfusion assessed by laser Doppler flowmetry (LDF), 2) results in no permanent neurological deficit at 24 hours, and 3) shows no lesion on MRI (diffusion-weighted images [DWI], T2-weighted images) at 24 hours. Routine hematoxylin and eosin (H&E) and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) staining were used to identify microscopic evidence of ischemic injury.

MATERIALS AND METHODS

Animals

Adult male NMRI mice (22–44 g body weight) were purchased from Harlan Laboratories (Horst, The Netherlands).
Before surgery, the animals were housed in groups of 5 in a temperature- and humidity-controlled environment in a 12/12-hour light/dark cycle with free access to standard rodent food and tap water. All experiments were performed in conformity with institutional guidelines and in compliance with national and international laws and policies governing the use of animals in neuroscience research. The local Animal Research Committee approved the study protocol (STH 283 A). All efforts were made to minimize animal distress and the number of animals used.

**Transient Focal Brain Ischemia**

The rats received anesthesia through a subcutaneous injection of ketamine hydrochloride (75 mg/kg; Ketalar, Parke-Davis, Detroit, MI) and medetomidine hydrochloride (0.5 mg/kg; Domitor, Orion). Rectal temperature was monitored (Therm Alert TH-8, Physitemp, Clifton, NJ) and maintained at 37°C using a heating blanket during surgery and until recovery from anesthesia. Transient focal cerebral ischemia was induced using the intraluminal suture MCAo model (12). Briefly, the right common carotid artery and the right external carotid artery were permanently ligated. The 6-0 nylon monofilament (Ethicon, Edinburgh, UK), with its tip rounded by heating, was inserted through an arteriotomy below the carotid bifurcation and advanced rostrally into the internal carotid artery (10–11 mm) until mild resistance was felt. This indicated that the monofilament had entered the anterior cerebral artery and occluded the blood flow to the MCA through the circle of Willis. Reperfusion was achieved by withdrawing the occluder after varying durations of MCAo. Regional cerebral blood flow (CBF) was measured in all animals with LDF (OxyFlo; Oxford Optronix Instruments, Oxford, UK) by applying a flexible fiber-optic probe onto the intact and bare cranial surface on the territory receiving blood supply from the MCA (1 mm posterior and 3 mm lateral to the bregma). Cerebral blood flow values at baseline, occlusion, and reperfusion (immediately, and at 10, 20, 30 minutes, and 24 hours after reperfusion) were recorded. All animals with inappropriate MCAo and inadequate reperfusion were excluded (Criterion 1).

**Neurological Evaluation**

At the end of the reperfusion period (i.e. at 24 hours, except in the group with 72-hour reperfusion), neurological status was assessed using a 6-point scale of sensorimotor skills as follows: 0, no deficit; 1, failure to fully extend the left forepaw; 2, circling to the left; 3, decreased resistance to lateral push; 4, no spontaneous walking with a depressed level of consciousness; and 5, dead (13). No deficit was required for fulfilling Criterion 2.

**Magnetic Resonance Imaging**

After neurological assessment, the animals were anesthetized for MRI, that was performed using a 4.7 T scanner (PharmaScan, Bruker BioSpin, Ettlingen, Germany) with a 90-mm shielded gradient capable of producing a maximum gradient amplitude of 300 mT/m with an 80-microsecond rise time. A linear birdcage radio frequency coil with an inner diameter of 19 mm was used. A triplane imaging sequence served for reproducible positioning of the animals in the scanner. Diffusion-weighted images were acquired using a spin-echo echo-planar imaging sequence with b values of 0 and 1,034 seconds/mm²; diffusion was measured in the read gradient direction (TR/TE, 4,000/69 milliseconds; matrix size, 128 × 128; field of view, 20 × 20 mm²; resolution, 156 × 156 μm²). Then, a T2-weighed spin-echo (rapid acquisition with relaxation enhancement) sequence (TR/TEeff, 3,000/36 milliseconds; matrix size, 256 × 192; field of view, 20 × 20 mm²; resolution, 78 × 104 μm²) was run. Both DWI and T2-weighted sequences had 1-mm thick, covering the entire brain. The first axial slice was selected posterior to the olfactory bulb (approximately 3.5 mm anterior to the bregma); the following slices were placed caudally. During MRI, body temperature was maintained at 37°C with an MRI-compatible heating blanket (TP472 T/Pump, Gaymar Industries, Inc, Orchard Park, NY). Images were evaluated visually to detect regions with increased signal (i.e. restricted diffusion in DWI and increased water content in T2-WI), which indicated an ischemic lesion.

**Tissue Handling and Histological Evaluations**

After a lethal dose of pentobarbital (60 mg/mL; 1 mL/kg, intraperitoneally; Mebunat, Orion, Espoo, Finland), the mice underwent cardiac perfusion-fixation with ice-cold 4% paraformaldehyde. The brains were quickly harvested, fixed in formaldehyde, and embedded in paraffin blocks. Four-micrometer-thick sections (0.30–1.30 mm posterior to bregma) were cut with a microtome (Leica SM2000 R, Leica Microsystems, Nussloch, Germany) and stained with H&E or TUNEL kit (In Situ Cell Death Detection Kit, Fluorescein; Roche Diagnostics Oy, Espoo, Finland), according to the manufacturer’s instructions. Stained sections were assessed under a light microscope (Axioplan2 Imaging, Carl Zeiss MicroImaging, Göttingen, Germany) by investigators who were blinded to the experimental groups.

In routinely stained specimens, ischemic changes and necrosis, that is, the presence of hypereosinophilic neurons, shrunken neurons, dark neurons, vacuolization, and loss of affinity for hematoxylin (pallor, ghost neurons) were evaluated in the hippocampus, caudoputamen, and frontoparietal cortex of both hemispheres. Pathological alterations in each area were scored as follows: 0, no change; 1, scattered neuronal changes; 2, selective neuronal necrosis (necrotic findings limited mainly to specific neuron populations); and 3, infarction (pan-necrosis characterized as the loss of affinity for hematoxylin in all cell types) with modifications from Garcia et al (14). Total scores were determined by summing scores from all 3 areas of both hemispheres.

In TUNEL-stained sections, TUNEL-positive cells in both hemispheres were counted and expressed as the TUNEL positivity index (i.e. the ratio of the ipsilateral hemisphere count to the contralateral hemisphere count). In addition, TUNEL positivity was analyzed in the same 3 regions as in the H&E evaluation. Regional TUNEL positivity indices for these areas were calculated as the ratio of the count in each region to that of the contralateral homologue.
Statistics

Parametric data are expressed as means (± SD) and non-parametric data as medians. Parametric data sets (rectal temperature and CBF values) in the 2 groups were compared with unpaired t-test and in multiple groups with 1-way analysis of variance followed by the Holm-Sidak post hoc test. Nonparametric H&E scores and non-normally distributed TUNEL positivity data were analyzed using Mann-Whitney U test for 2 group comparisons and Kruskal-Wallis test followed by Dunn method for multiple group comparisons. Correlations were investigated using the Spearman correlation coefficient (r). A 2-tailed value of p < 0.05 was considered significant.

RESULTS

Physiological Parameters

Rectal temperatures both before and after ischemia and at 24 hours postreperfusion values were similar among the groups at all time points (p = 0.68) (data not shown). Baseline body weights also showed no differences (p = 0.16). An average loss of body weight of 8% (from 38 ± 5 g to 35 ± 4 g, p = 0.01) was detected at 24 hours, with no inter-group difference at this time point (p = 0.13).

Transient Focal Brain Ischemia (Criterion 1)

A 20-minute period of transient ischemia was initially based on studies in rats (14–16); shorter periods were studied thereafter. Experimental groups of 6 mice each experienced MCAo of 20, 15, 12.5, 10, 7.5, 5, and 2.5 minutes; 4 sham-operated animals served as controls. Intraluminal MCAo carries risks of inadequate occlusion and reperfusion that can be detected reliably by CBF measurements using LDF (17).
Pilot studies showed that during right-sided MCAo, a substantial decrease (≥75%) in ipsilateral CBF values from the baseline indicated successful occlusion. A minimum recovery of greater than or equal to 50% after suture withdrawal indicated adequate reperfusion (data not shown). To ensure occlusion and reperfusion by LDF (Criterion 1), we included only mice with appropriate LDF-based CBF values. In successful MCAo cases, CBF values from the right hemisphere dropped on average to 15% of the baseline value (Fig. 1). After suture withdrawal, CBF values recovered substantially and reached on average 83% of the baseline value at the end of 30 minutes postreperfusion and 96% at 24 hours. Mice with inadequate MCAo (n = 9) and inadequate reperfusion (n = 25) were excluded. Baseline right and left hemisphere CBF values were similar (p > 0.61) among groups. Left hemisphere CBF values persisted at baseline levels throughout the experiment (p = 0.99). Sham-operated controls displayed no significant CBF changes during the experiment (p = 0.96).

**Neurological Deficit and MRI (Criteria 2 and 3)**

Of the 6 animals with 20 minutes of ischemia, only 1 showed a neurological deficit at 24 hours, but 5 developed infarcts that were clearly visible on MRI (1 small hippocampal infarct, 2 medium-sized subcortical infarcts, and 2 large confluent infarcts). In the 15-minute ischemia group, 2 of the 6 animals showed no infarct, 1 animal had a small cortical infarct, and 3 had medium-sized cortical and subcortical infarcts (Fig. 2). Only 1 mouse in this group exhibited a neurological deficit. In the 6 mice of the 12.5-minute MCAo group, no neurological deficits were detected, and only 1 mouse had a small cortical infarct (Fig. 2). All mice in the 10-, 7.5-, 5-, and 2.5-minute MCAo groups and the control group had no MRI lesion and normal neurological status.

**Three-Day Follow-Up Group**

Because ischemic lesions can appear late after brief periods of focal cerebral ischemia (18), a separate group of 6 mice with 12.5-minute MCAo (a borderline duration inducing either infarction or not) had an extended reperfusion period up to 3 days (3-day follow-up group). As with the 12.5-minute MCAo group with 24 hours of reperfusion, none of the animals showed neurological deficits at 24 hours and a normal neurological status persisted up to Day 3. No MRI lesions were identified at 24 hours or on Day 3.

**Routine Histology**

The extent of ischemic changes correlated with the duration of the MCAo (r = 0.95, p < 0.001) (Fig. 3). There were scattered hypereosinophilic neurons (score, 1) in the hippocampus of 2 of 4 sham-operated control mice and in 2 of 6 mice in the 2.5-minute MCAo group. Selective neuronal necrosis (score, 2) was a consistent feature in the ischemic frontoparietal cortex starting at 5 minutes of MCAo; only 1 mouse in the 2.5-minute MCAo group had selective neuronal necrosis in the cortex. There was infarction (score, 3) in 1 mouse in the 12.5-minute MCAo group. This group had similar H&E findings to those of the 3-day follow-up group (p = 0.53), indicating the absence of delayed changes. Mice in the 12.5-, 15-, and 20-minute MCAo groups had higher scores compared with the controls (p = 0.003) (Fig. 4A).

**TUNEL Positivity**

The total TUNEL positivity indices were significantly different among the groups with higher values occurring in the 20- and 15-minute MCAo groups (p = 0.003) (Fig. 4B). Regional TUNEL positivity indices showed no differences for the hippocampal (p = 0.64) and frontoparietal cortical (p = 0.20) regions, but there was a significant difference among the groups for the caudoputamen, (p < 0.001) (Fig. 4C). A longer duration of reperfusion (3 days vs 24 hours) resulted in no increase in the TUNEL positivity of animals with 12.5 minutes of MCAo (p = 0.22), again confirming no delayed changes. Furthermore, there was a correlation between the total TUNEL positivity index and the duration of ischemia (r = 0.92, p < 0.001).

**DISCUSSION**

We found that MCAo of 10 minutes or less induced by the intraluminal suture method is a reliable method of inducing a mouse model of TIA. To achieve relevance to the clinical condition, we required the model to fulfill all 3 predetermined criteria. The first required an objective assessment of MCAo and reperfusion that was necessary because the intraluminal MCAo method is occasionally associated with inadequate occlusion or premature reperfusion (17), and successful reperfusion does not always follow withdrawal of the suture (13). Consequently, our first criterion provided definite control over the duration of the ischemic event. The second clinical and third neuroimaging criteria were required to approximate the novel clinical definition of human TIA (5). The symptoms of most TIA patients resolve by the time they reach the hospital. Therefore, clinicians must rely on the history of the event that, in most cases, involves motor paresis (19). For the second criterion, we used sensorimotor evaluation rather than behavioral tests that are sensitive to more complex abilities. The third criterion aimed to rule out infarction by DWI and T2-weighted imaging. Both neurological and MRI outcomes were evaluated at 24 hours because by this time, any infarct would be complete, that is, we intended for the imaging to take place outside the time frame in which reversible DWI abnormalities may occur (15, 20). Middle cerebral artery occlusion of 10 minutes or less fulfilled all 3 criteria; the 12.5-minute MCAo was a critical threshold for inducing permanent changes equivalent to infarction with definite histopathologic ischemic changes. Durations longer than 12.5 minutes resulted in higher rates of infarction on MRI and in histological specimens, thus proving unsuitable for modeling TIA.

Postmortem studies are impossible in TIA patients, but pathologic changes after TIA can be addressed with an animal model. Histopathologic analysis at 24 hours after the ischemia allowed sufficient time for any permanent injury to emerge. Middle cerebral artery occlusion as brief as 2.5 minutes induced slight histopathologic changes suggesting cell death and apoptosis; the longer the MCAo duration, the more abundant were these changes. Ischemic effects milder
than infarction (i.e. scattered or selective neuronal necrosis) were not visible on MRI, but MRI was positive in all cases with histological evidence of infarction. Interestingly, the presence of infarction at 24 hours was not always associated with neurological deficits, likely because our neurological assessment was sensitive only to gross sensorimotor deficits. Similarly, such mild deficits are often difficult to detect in humans, a phenomenon known as MRI-positive TIA in patients with a long-lasting (>30 minutes) reversible ischemic event (3).

**FIGURE 3.** Postmortem assessments at 24 hours postreperfusion. Representative histopathologic findings in the lateral caudoputamen are shown by hematoxylin and eosin (left) and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) right staining. Middle cerebral artery occlusion (MCAo) for the indicated durations was followed by a gradual increase in ischemic changes. Nonsignificant TUNEL positivity (as in the control) is present with 2.5 minutes of MCAo; ischemia for 10 minutes led to an increase in TUNEL-positive cells and selective neuronal necrosis characterized by scattered shrunken (arrows) and hyper eosinophilic (arrowhead) neurons. Ischemia for 15 minutes induced pan-necrosis, with loss of tissue structure and vacuolization of the neuropil. Scale bars = 50 μm.
Experimental evidence suggests that whereas severe cerebral ischemia leads to necrosis and infarction, mild ischemia or brief periods of transient ischemia induce mainly selective neuronal necrosis and apoptosis (21). Garcia et al. (14) addressed this aspect in extensive studies of rats with induced 10 to 25 minutes of MCAo followed by several days of reperfusion. One third of the animals showed no evidence of brain injury, whereas the others showed pathological changes that varied from selective neuronal necrosis to infarction. That study lacked objective confirmation of MCAo or reperfusion and used no brain imaging, however. Our present results show that selective neuronal necrosis is an invariant component of the injury in mice with MCAo longer than 5 minutes, whereas shorter periods of MCAo showed various impacts ranging from no findings to scattered neuronal necrosis or, rarely, selective neuronal necrosis. Although these changes were insignificant compared with those among the controls, the results show that the fate of brain tissue may vary after even very brief TIA episodes. Apoptotic cell death, as a consequence of TIA-like brief transient focal ischemia, seems to evolve with a progressive and delayed course associated with a worsened long-term outcome (22, 23). In the present study, microscopic ischemic changes induced by 12.5-minute MCAo were similar at 24 hours and on Day 3 by both H&E and TUNEL staining. There was no evidence of further damage up to 72 hours after reperfusion.

Our study clearly shows that the threshold for infarction after MCAo was around 12.5 minutes in mice. To the best of our knowledge, no systematic study has yet addressed whether different animal species have distinct thresholds. At present, the mechanisms of necrosis and apoptosis seem to be universal among mammals. Fragmental data suggest that the threshold for developing visible infarcts increases phylogenetically, but this issue will remain uncertain until similar studies are carried out in different species.

Brief episodes of focal transient cerebral ischemia (most commonly studied in rats) serve mainly as an ischemic preconditioning trigger. The structural changes after such insults were recently reviewed (24), but studies of ischemic preconditioning in mice are relatively limited. Stagliano et al. (25) induced 5-minute MCAo in C57Bl/6 mice 1 or 3 times (separated by 10-minute reperfusion periods) and detected scattered neuronal damage in 20% of the mice. Fifteen minutes of MCAo seems sufficient to induce infarction in this strain (26–28). In their early work, however, Connolly et al. (28) found that only 15% of C57Bl/6 mice developed visible infarcts with 15 minutes of ischemia. Another group of experiments investigated brief focal brain ischemia in relation to DWI changes, but those were performed in rats. We previously showed that 8- and 15-minute MCAo episodes lead to acute DWI changes in rats, with rapid and complete reversal upon reperfusion (15). Animals that experienced 8-minute ischemia were either normal or showed selective neuronal necrosis within the caudoputamen 3 days later. This was a consistent finding in the 15-minute ischemia group, but the animals were not reimaged. Later, another study showed reappearance of the recovered DWI signal abnormalities in rats that had undergone 30 minutes of MCAo (18). In the present study, we detected no abnormalities on DWI and T2-weighted images at 24 hours in all mice subjected to 10-minute ischemia and in most with 12.5-minute ischemia, as previously shown (16). Microscopic ischemic changes were consistent as scattered neuronal cell death and selective neuronal necrosis. There were no delayed MRI lesions when the follow-up period was extended to 3 days.

Because it has long been considered to be a benign condition, TIA has not been extensively studied. It is now widely accepted to be a clinical entity that signals forthcoming stroke and is also suspected to have long-term deteriorating effects. One reason for the paucity of studies of TIA has been the lack of an optimized animal model. We identified 10 minutes (or less) of ischemia induced with the intraluminal suture MCAo method to be an appropriate mouse model for TIA. This model induces no permanent neurological deficit or MRI abnormalities in accordance with the definition of human TIA. The use of LDF guidance or comparable methodology is necessary to ensure occlusion and reperfusion and thus the reliability of the model. When applying our model, strain or vendor differences in the susceptibility of the mice to ischemia should also be considered.

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