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Delayed Recanalization Promotes Functional Recovery in Rats Following Permanent Middle Cerebral Artery Occlusion

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Abstract

Most large vessel stroke patients have permanent occlusion, for which there are no current treatment options. Recent case studies have indicated delayed recanalization, that is recanalization outside of the 6-h treatment window, may lead to improved outcome. We hypothesized that delayed recanalization will restore cerebral blood flow, leading to improved function in rats. Male SD rats were subjected to pMCAO or sham surgery. Delayed recanalization was performed on either day 3, 7, or 14 after pMCAO in a subset of animals. Cerebral blood flow was monitored during suture insertion, during recanalization, and then at sacrifice. Neurological function was evaluated for 1 week after delayed recanalization and at 4 weeks post-ictus. After sacrifice, cerebral morphology was measured. Compared to no treatment, delayed recanalization restored cerebral blood flow, leading to sensorimotor recovery, improved learning and memory, reduced infarct volume, and increased neural stem/progenitor cells within the infarction. The data indicate that earlier delayed recanalization leads to better functional and histological recovery. Yet, even restoring cerebral blood flow 14 days after pMCAO allows for rats to regain sensorimotor function. This exploratory study suggests that delayed recanalization may be a viable option for treatment of permanent large vessel stroke.

Keywords

Delayed recanalization; Permanent middle cerebral artery occlusion; Stroke; Ischemia

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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Introduction

Of the 650,000 ischemic stroke patients in the USA annually, 40–50% of them have large vessel strokes [1], with up to 15% treated with tPA [2] and up to 4% treated with embolectomy [1], resulting in recanalization rates of 11–40% for tPA recipients [3, 4] and 75% for patients receiving embolectomy [3]. Thus, about 270,000 patients have permanent occlusion large vessel stroke, for which there is currently no standard treatment [5].

Recent clinical studies have reported improved outcome for permanent stroke patients when treated with delayed recanalization, that is recanalization beyond the 6-h therapeutic window [6, 7]. The first published case studies on delayed recanalization of permanent stroke were performed 4 months [6] and 80 days after onset [7]. In the latter report, following endovascular recanalization, the patient's outcome improved, and the artery remained patent 6 months later. More recently, the DAWN Trial found that delayed recanalization (up to 24 h) via embolectomy decreased disability and improved functional independence [8].

Interestingly, while delayed recanalization has been examined in patients [6–8], it is not considered as a treatment for large vessel permanent occlusion stroke patients. This may be in part due to a lack of understanding of the optimal treatment window and its complications. Thus, we sought to study delayed recanalization in rodents. We hypothesized that delayed recanalization will promote functional recovery after permanent middle cerebral artery occlusion (pMCAO) in rats.

Material and Methods

The experiments were approved by the Loma Linda University IACUC, conducted in compliance with the *NIH Guidelines for the Use of Animals in Neuroscience Research*, and are reported in compliance with the ARRIVE (Animal Research: Reporting in Vivo Experiments) guidelines.

Experimental Groups, Randomization, and Sample Size

One hundred and twelve adult male Sprague-Dawley rats (240–260 g, Harlan Laboratories) were used. Rats were randomly assigned (stratified, electronically generated) to either sham ($n = 16$), pMCAO ($n = 48$), pMCAO with delayed recanalization on day 3 ($n = 16$), pMCAO with delayed recanalization on day 7 ($n = 16$), or pMCAO with delayed recanalization on day 14 ($n = 16$). These sample sizes were determined based on literature [9, 10]. To test for statistical significance, we required eight animals per group for the Morris water maze, open field, and rotarod tests. Since the pMCAO model has a mortality rate of approximately 50% [9, 11], our sample size was sixteen per group. The pMCAO group has three sub-cohorts of 16 animals each for comparison with the delayed recanalization groups.

Permanent MCAO

One day before pMCAO, rats were briefly anesthetized with isoflurane (2.5% sustained) and a burr hole was created over the MCA territory and then sealed with bone wax. On the day of pMCAO, rats were anesthetized with a ketamine/xylazine mixture (80/20 mg/kg, IP) then

administered atropine (10 mg/kg, SC). The scalp incision was re-opened, the bone wax was removed, and cerebral blood flow was measured via a laser Doppler flow probe (OxyFlo probe, MNP100XP, AdInstruments Inc., Colorado Springs, CO, USA) for 1 min to obtain baseline perfusion (PowerLab PL3504 and LabChart Pro, AdInstruments Inc., Colorado Springs, CO, USA).

pMCAO was performed as previously described [9, 10, 12]. Briefly, rats were placed supine, an incision in the neck was made, and then the external and common carotid arteries were exposed. Then, the external carotid artery was ligated and cut to create a stump, the internal carotid artery was exposed, and the pterygopalatine artery was ligated. Artery clips were placed on the common carotid and internal carotid arteries, and an incision was made on the external carotid artery stump. A monofilament suture with a silicon-coated tip was inserted into the external carotid artery stump, and then advanced into the middle cerebral artery (after removal of the artery clip on the internal carotid artery) until resistance was felt (~ 18–20 mm). The suture was tied in place (using a 7–0 nylon suture), the artery clip on the common carotid artery removed, the neck wound closed, and the animal flipped to prone for measurement of the cerebral blood flow (minimum 1 min collection). After cerebral blood flow measurement, the probe was removed, bone wax was used to seal the burr hole, and the scalp was sutured. The animal was placed into a recovery chamber. Sham animals underwent all surgical procedures, including insertion of the monofilament suture, but the monofilament suture was removed immediately after reaching the middle cerebral artery (where resistance was felt).

Delayed Recanalization

In the cohorts of animals allocated to the delayed recanalization groups, the suture was removed from the middle cerebral artery 3, 7, or 14 days after pMCAO. Briefly, on the day of suture removal, rats were anesthetized with isoflurane (4% induction, 2.5% sustained, in oxygen (1.0 L/min)), the scalp incision was opened, bone wax was removed, and cerebral blood flow was measured (pre-recanalization). The animals were then placed supine, and the neck incision re-opened. The external, common, and internal carotid arteries isolated, exposing the monofilament suture protruding from the external carotid artery stump. The monofilament suture was gently retracted from the middle cerebral artery until the silicon tip was near the external carotid artery stump. Artery clips were placed on the internal carotid and common carotid arteries. The nylon tie (on the external carotid artery stump) securing the monofilament suture was loosened, and the monofilament suture was completely removed from the artery. The external carotid artery was ligated with the nylon suture, the neck wound sutured, and the animal placed prone for measurement of cerebral blood flow (post-recanalization). After cerebral blood flow was measured, bone wax was applied, and the head incision was closed. The rats were allowed to recover. The Supplemental Video shows the major steps of delayed recanalization.

Neurobehavioral Testing

All animals were tested 24 h after pMCAO (or sham surgery) using a modified Garcia neuroscore, beam walking score, and forelimb placement test. Details on how to perform each neurological test can be found in the Supplementary Material.

Functional Recovery after Delayed Recanalization

Sham animals were tested using the modified Garcia neuroscore, beam walking, and forelimb placement test on days 4, 6, 8, 10, 12, 14, 15, 17, 19, 21, and 32.

The untreated pMCAO rats were sub-divided into three groups of 16. One group had neurological testing performed on days 4, 6, 8, and 10 post-pMCAO. Another group had functional testing on days 8, 10, 12, and 14 post-pMCAO. The final group had neurobehavioral testing on days 15, 17, 19, and 21 post-pMCAO. These sub-cohorts are used for comparison with the rats subjected to delayed recanalization on day 3, 7, and 14 after pMCAO, respectively.

The rats receiving delayed recanalization on day 3 after pMCAO were subjected to functional testing on days 4, 6, 8, and 10 after pMCAO. The group with delayed recanalization on day 7 was subjected to neurological testing on days 8, 10, 12, and 14 after pMCAO. The animals with delayed recanalization on day 14 had their behavior tested on days 15, 17, 19, and 21.

Functional Performance 4 Weeks After pMCAO

Morris water maze was performed on days 28–31 post-pMCAO, and open field, rotarod, modified Garcia neuroscore, beam walking, and forelimb placement tests were performed on day 32 after pMCAO.

Cerebral Blood Flow, Sacrifice, and Histological Analysis

Upon completing neurobehavioral testing on day 32 post-pMCAO, rats were anesthetized with isoflurane (2.5% sustained). The head incision was opened, bone wax removed, the laser Doppler probe positioned, and cerebral blood flow measured. The rats were then deeply anesthetized, transcardially perfused with PBS and formalin, and the brains removed and prepared for cryosectioning (fixation, dehydration). Nissl staining was performed on five 30 µm thick slices, and analyzed for infarct volume (% of whole brain) [13], white matter volume, ventricular volume, and hippocampus area using ImageJ.

Ten micrometer thick slices were stained for neurons (NeuN), astrocytes (GFAP), endothelial cells (RECA1), microglia (Iba1), and neural stem (Nestin+/SOX-2+) and neural progenitor cells (Nestin-positive and SOX-2-negative). The infarction core and peri-infarction tissue within the ipsilesional hemisphere were imaged using three sections per animal group. Each cell type was counted and averaged, and expressed as cells/field as previously described [14].

Data Collection, Data Processing, and Statistical Analysis

All raw data was collected and analyzed by a blinded investigator (to group assignment). Data is presented as the median, interquartile range, and minimum/maximum (Box and Whiskers plot) unless specified otherwise. Normality was tested for all data using Sigmaplot 11.0 (SysStat, Germany), all tests were two-sided, and no further adjustment for multiple comparisons was done for the overall number of tests. All statistical comparisons were

analyzed using GraphPad Prism 6 (La Jolla, CA, USA) and confirmed using SigmaPlot. A p value of less than 0.05 was considered statistically significant.

Survival in this study was assessed using a Kaplan-Meier curve and analyzed by Mantel-Cox and Gehan-Breslow-Wilcoxon tests. Cerebral blood flow was normalized to the average baseline perfusion. One-way ANOVA with Tukey post-hoc was used to test for statistical significance between the groups for each time point (occlusion, pre- and post-recanalization, and sacrifice). The infarct volume, white matter and ventricle volumes, hippocampus area, and cell counts were analyzed using one-way ANOVAs with Tukey post-hoc tests.

The single time point data (day 1 and 32) for the modified Garcia neuroscore, beam walking score, and forelimb placements were analyzed using the Kruskal-Wallis test with Dunn's post-hoc. The longitudinal data (modified Garcia neuroscore, beam walking, and forelimb placement) were analyzed using Scheirer-Ray-Hare tests with Dunn's post-hoc. The Morris water maze data was analyzed using one-way ANOVA with Tukey post-hoc (average velocity, probe test) and two-way ANOVA with Tukey post-hoc (learning blocks). The rotarod and open field data were analyzed using one-way ANOVAs with Tukey post-hoc tests.

Results

Herein, the results for all outcomes with the major statistical findings are presented. A detailed, full report of the statistical analysis and results for each measure can be found in the Supplementary Material.

Exclusion and Inclusion

Ten animals were excluded from the study due to surgical complication (Sham $n = 1$), lack of an infarction after pMCAO (pMCAO $n = 1$, pMCAO with delayed recanalization at day 14 $n = 1$), no drop in cerebral blood flow during occlusion (less than a 15% drop in cerebral blood flow from baseline values) (pMCAO $n = 2$), and subarachnoid hemorrhage (pMCAO $n = 4$, pMCAO with delayed recanalization on day 3 $n = 1$). One animal in the pMCAO with delayed recanalization on day 3 group was excluded from the Morris water maze test because it failed to keep its head above the surface of the water, but this animal was included in all other outcomes.

All other animals were included in the study. The average drops in cerebral blood flow (standard deviation) were 84% (7.7%), 80% (12.6%), 80% (6.1%), and 76% (11.9%) for untreated pMCAO, delayed recanalization (day 3), delayed recanalization (day 7), and delayed recanalization (day 14), respectively.

Survival Following pMCAO

No mortality was observed for sham animals. Untreated animals allocated into the pMCAO group had a mortality of 53.7%. Whereas the mortality rates for rats receiving delayed recanalization on day 3, 7, and 14 were 46.7, 43.8, and 53.3%, respectively. No statistical significance was observed for the survival among any of the groups (Mantel-Cox test: $p = 0.8837$, Gehan-Breslow-Wilcoxon test: $p = 1.0$) (Fig. 1a).

Cerebral Blood Flow Measurement

All animals subjected to pMCAO were confirmed to have a drop in cerebral blood flow (greater than 50% from baseline) during suture insertion (Fig. 1b); no significant difference was observed among the groups. After recanalization, cerebral blood flow returned to baseline values for all rats receiving delayed recanalization. At sacrifice, the cerebral blood flow was similar among all pMCAO animals receiving delayed recanalization which was significantly higher than that of untreated pMCAO rats.

Functional Recovery After Delayed Recanalization

Twenty-four hours after pMCAO, all rats subjected to pMCAO had significantly lower modified Garcia neuroscores, beam walking scores, and left forelimb placements than those of sham animals, indicating similar severity of ischemic injury among all pMCAO animals (Fig. 2).

After delayed recanalization on day 3, rats receiving delayed recanalization exhibited significantly better performance in the modified Garcia neuroscore than that of untreated pMCAO animals on all days post-recanalization (Fig. 3a). One day after recanalization (day 4), rats performed significantly better in the modified Garcia neuroscore than they performed before recanalization and continued to display functional recovery. Untreated pMCAO rats did not show functional recovery. Additionally, in the beam walking and forelimb placement tests, pMCAO rats treated with delayed recanalization on day 3 displayed functional recovery whereas untreated pMCAO rats did not (Figs. 4a and 5a).

pMCAO animals receiving delayed recanalization on day 7 displayed significantly better behavioral capability in the modified Garcia neuroscore compared to that of untreated pMCAO rats on all days following recanalization (Fig. 3b). Rats treated with delayed recanalization on day 7 also showed functional recovery after treatment. Untreated pMCAO rats showed marginal recovery from pMCAO. The same trends for untreated pMCAO rats and pMCAO rats receiving delayed recanalization on day 7 were observed in the beam walking and forelimb placement tests (Figs. 4b and 5b).

Recanalization on day 14 in rats led to improved behavioral performance on the modified Garcia neuroscore compared to that of untreated pMCAO rats on all days after recanalization (Fig. 3c). Rats treated with delayed recanalization on day 7 also exhibited functional recovery in the neuroscore, while untreated pMCAO rats demonstrated slight recovery. In the beam walking and forelimb placement tests, pMCAO rats treated with delayed recanalization on day 14 displayed functional recovery whereas the untreated pMCAO rats did not (Figs. 4c and 5c).

Neurofunctional Performance at 28–32 Days After pMCAO

Four weeks after pMCAO, untreated pMCAO animals had worse performance on the modified Garcia neuroscore, beam walking test, and forelimb placement test compared to those of sham (Fig. 6). Animals treated with delayed recanalization on day 3 or 14 were significantly different from untreated pMCAO rats on the modified Garcia neuroscore. In the beam walking test, only delayed recanalization on day 7 provided any significant

improvement compared to that of untreated animals. The left forelimb placements indicated that delayed recanalization on day 3 or 7 lead to improved performance compared to that of pMCAO rats.

For the rotarod test, untreated pMCAO rats had significantly smaller latency to fall times compared to that of sham in the accelerating tests (Fig. 7a). Treatment of pMCAO rats with delayed recanalization improved the latency to fall times in both accelerating tests. The locomotion time in the open field test shows that untreated pMCAO rats spend less time exploring compared to sham, and that delayed recanalization recovers the animal's propensity for exploring novel areas (Fig. 7b).

In the Morris water maze, pMCAO caused significantly worse performance for the escape latency test compared to that of sham (Fig. 7c). Only delayed recanalization on day 3 displayed task learning similar to that of sham and significantly different from untreated pMCAO rats. Delayed recanalization on day 3 also improved memory of the target quadrant in the probe test, which was significantly impaired in untreated pMCAO rats (Fig. 7d).

Cerebral Morphology 32 Days After pMCAO

All animals subjected to pMCAO had significantly greater infarct volumes compared to sham rats (Fig. 8a–b). Delayed recanalization on day 3 leads to significantly lower infarct volume than untreated pMCAO rats; however, delayed recanalization on either day 7 or 14 exhibit infarction volumes statistically indistinguishable from that of untreated pMCAO rats. While all pMCAO rats have significantly lower white matter volumes compared to sham animals, delayed recanalization on day 3 significantly increases the white matter volume compared to no treatment (Fig. 8c). pMCAO animals receiving delayed recanalization on either day 7 or 14 display white matter volumes statistically lower than those receiving delayed recanalization on day 3. Untreated pMCAO rats have significantly higher ventricular volumes compared to sham, which is reversed by delayed recanalization on either day 3 or 7; delayed recanalization on day 14 did not reduce ventricular volume compared to no treatment. Finally, untreated pMCAO rats had significantly lower hippocampus area (compared to sham rats), whereas delayed recanalization leads to hippocampus areas which are not significantly different from that of sham.

To determine if there were surviving cells within the infarcted tissue, we stained for neurons, astrocytes, endothelial cells, and microglia. In the infarcted tissue, at 32 days post-pMCAO, no neurons or astrocytes were observed in any of the pMCAO rats (Fig. 9). However, rats treated with delayed recanalization presented with endothelial cells and microglia residing within the infarcted tissue (Fig. 10). To investigate possible repair within the infarction, we probed for neural stem cells (Nestin-positive and SOX-2-positive) and neural progenitor cells (Nestinpositive, SOX-2-negative). While no neural progenitor or stem cells were observed in the infarction of untreated pMCAO rats, pMCAO rats receiving delayed recanalization display neural progenitor and stem cells (Fig. 11). Finally, we sought to quantify the number of cells within the infarcted tissue. Using stereological analysis, cell counting indicated that within the infarction core, delayed recanalization lead to significantly higher counts (compared to untreated pMCAO animals) of microglia (delayed recanalization on day 3, 7, and 14), endothelial cells (delayed recanalization on day 3, 7, and 14), Nestin-

positive cells (delayed recanalization on day 3 and 7), and SOX-2-positive cells (delayed recanalization on day 3 and 7) (Fig. 12). At the edge of the infarction (peri-infarct), delayed recanalization on day 3 displayed higher counts of microglia and SOX-2 (compared to untreated pMCAO rats).

Discussion

Herein, we hypothesized that delayed recanalization would improve functional performance in rats subjected to pMCAO. Using pMCAO in rats, we observed functional recovery in rats treated with delayed recanalization compared to untreated pMCAO rats. One month after pMCAO, rats receiving delayed recanalization lead to improved sensorimotor function and less anxiety-like behaviors, as well as improved learning and memory compared to untreated pMCAO animals. As far as we know, this is the first report investigating delayed recanalization as a treatment for pMCAO in rodents.

A critical evaluation of stroke outcome is mortality, and it would be anticipated that a promising therapy for pMCAO would reduce mortality; however, delayed recanalization did not. This is because most animals subjected to pMCAO died within 3 days. Therefore, these animals were not able to be treated using delayed recanalization. Brain edema, which peaks 1–3 days post-ictus [15] (before the chosen recanalization times), may be the cause of the observed high mortality.

While delayed recanalization did not affect mortality rates, in surviving rats, it restored cerebral blood flow, thereby promoting healing and functional recovery. Untreated pMCAO rats exhibited minor recovery of sensorimotor deficits associated with injury. However, rats treated with delayed recanalization had substantially better performance on sensorimotor tests compared to untreated pMCAO rats, as well as showing functional recovery after recanalization. Interestingly, Yu et al. tested delayed recanalization in a patient with chronic basilar artery stroke and observed improved outcomes after recanalization [7]. These findings suggest that reperfusion of the ipsilesional hemisphere may improve the function of surviving tissue, thereby leading to better outcome. Finally, within the infarcted tissue of rats receiving delayed recanalization, there was a greater number of endothelial cells and microglia, as well as the presence of neural progenitor and neural stem cells.

Long-Term Functional Performance after Delayed Recanalization

Sensorimotor—One month after pMCAO, untreated rats continued to display functional deficits in the modified Garcia neuroscore, beam walking, and forelimb placement tests. Typically, these neurological tests are only sensitive enough to evaluate acute function, since rodents have the innate ability for spontaneous functional recovery [16]. However, pMCAO rats may lose the ability to spontaneously recovery sensorimotor deficits after brain injury [10, 12, 17]. But restoration of cerebral blood flow by delayed recanalization, even as delayed as 14 days after injury, allows for recovery of sensorimotor function in rats. The regained sensorimotor function may be caused by restoring blood flow to the penumbra. Li and Murphy identified three zones of tissue affected by MCAO: ischemic core, reversibly damaged penumbra, and a structurally intact, hypoperfused penumbra [18].

Herein, only the rats receiving delayed recanalization on day 3 exhibited significantly lower infarct volumes compared to untreated rats; delayed recanalization on day 7 had infarct volumes which were close to significance ($p = 0.0623$ vs pMCAO). However, despite no significant reduction in infarct volume for the delayed recanalization on day 7 and 14 groups, both groups exhibited improved behavioral/functional performance on sensorimotor and anxiety tests. A potential reason for this observation is that increased endothelial cells and microglia, and/or neural stem/progenitor cells, aids in functional recovery. It is possible that a longer recovery period after delayed recanalization can lead to repair of the infarcted tissue. Another potential reason for the improved sensorimotor function in the rats treated with delayed recanalization (day 7 and 14) is that rats have a remarkable ability to spontaneously recover functional deficits, in particular sensorimotor deficits [16, 19–21]. Although rats with pMCAO typically do not exhibit spontaneous recovery [10, 12, 17], the restoration of blood flow may be a trigger for recovery of sensorimotor deficits [22]. This should be further explored in a future study.

Learning and Memory—The ability for pMCAO rats to learn and remember the Morris water maze task was severely impaired in untreated animals. Rats receiving delayed recanalization on day 3 displayed improved learning and memory. These findings may be explained by reduced infarct volume, higher white matter volume, smaller ventricular volume, or increased hippocampus area.

Clinical Implications of Delayed Recanalization

Interestingly, while this is the first-time delayed recanalization has been attempted in an experimental model of pMCAO, delayed recanalization has been tested in stroke patients since before 1977. Hopkins et al. evaluated the therapeutic benefit of intracranial bypass for vertebrobasilar insufficiency in a study of 45 patients [23]. Although the initial conclusion was that bypass surgery improved outcome related to recanalization, the authors subsequently argued against intracranial bypass. The first two case reports of delayed recanalization with stents were those by Terada et al. and Yu et al. which examined treatment at 4 months and 80 days post-ictus, respectively [6, 7]. The latter report found that the basilar artery remained patent at 6 months after recanalization [7]. While these two case studies argue that delayed recanalization months after permanent large vessel stroke can lead to neurological improvement, most clinical studies focus on recanalization within 1 day of stroke. Several clinical studies have reported no benefit to recanalization after 6–7 h post-stroke [24], yet the recent findings of the DAWN trial suggest that delayed recanalization decreases disability and improves functional independence 3 months after treatment (embolectomy up to 24 h post-ictus). Furthermore, the DAWN trial results indicate that the number of patients able to live an independent lifestyle after severe stroke may increase by as much as 270% [8]. Despite conflicting reports about the benefit of recanalization within 1 day after stroke, the reports by Terada et al. and Yu et al. suggest that the current paradigm of “time is brain” may be more targeted at strokes with little or no penumbra to be salvaged (i.e., no diffusion-perfusion MRI mismatch). However, an alternative explanation is that patients with severe brain edema (or other secondary injury) may not benefit from recanalization until the brain edema resolves; it is possible, that after secondary injury resolves, delayed recanalization can promote healing and functional recovery. Based on the

findings from the aforementioned clinical studies, and the observations reported in this experimental study, delayed recanalization may be therapeutically beneficial for patients with permanent large vessel stroke, and future studies are warranted.

Considerations for the Use of Delayed Recanalization

Although delayed recanalization restores cerebral blood flow to the ischemic tissue, recanalization after long-term occlusion may lead to additional pathological events and complications, such as vasospasm or vessel rupture [6]. Furthermore, both experimental and clinical studies investigating the time window for reperfusion have found that recanalization should be achieved within 6–7 h post-ictus or the infarction volume will significantly increase [24–26]. However, while several studies suggest that recanalization needs to be performed early to provide a benefit, these studies did not investigate recanalization days or weeks after the initial stroke insult. Indeed, recanalization between 8 and 24 h post-stroke may be contraindicated, potentially because brain edema and other secondary injury mechanisms are beginning to weaken the cerebrovasculature [27–32]. However, brain edema, which peaks 1–3 days after stroke, typically resolves within 1–2 weeks [33]. Therefore, delayed recanalization provided after resolution of secondary injury may be therapeutically beneficial. The findings of Terada et al. [6], Yu et al. [7], and the DAWN trial [8] provide clinical support that there is, at least, a population of permanent large vessel stroke patients who may benefit from delayed recanalization. As such, it is critical that delayed recanalization be further studied in experimental models.

In this study, the cerebral blood flow results on day 32 suggest that chronic vasospasm was not present in the groups treated with delayed recanalization. However, vasospasm may occur after delayed recanalization and may be a transient complication; since vasospasm was not a measured outcome in this study, future studies of delayed recanalization need to evaluate this deleterious event. As for blood vessel rupture, intracerebral and/or intraventricular hemorrhage was observed in nine of 28 surviving pMCAO animals (used for histological evaluation). Intracerebral hemorrhage was observed in two untreated, one day 3 recanalization, one day 7 recanalization, and two day 14 recanalization rats. Intraventricular hemorrhage was observed in one day 7 recanalization and two day 14 recanalization animals. Future studies will be conducted to determine if delayed recanalization causes acute or chronic cerebral vasospasm and significant intracerebral/intraventricular hemorrhage.

Limitations and Future Studies

The current study has a few limitations. First, the days of delayed recanalization were chosen pseudo-arbitrarily. Recanalization on day 3 was chosen, because an experimental study of distal pMCAO showed that the peak of brain edema was between 24- and 72-h post-ictus [33]. Day 7 was chosen, since brain edema after distal pMCAO in rodents is significantly reduced compared to the third day post-injury [33]. Day 14 was chosen for recanalization, since brain edema had resolved 2 weeks after distal pMCAO in rodents [33]. Future studies can be conducted to monitor brain swelling/edema and/or penumbra vs core infarct using MRI (diffusion-perfusion mismatch) and treat pMCAO with delayed recanalization at specific MRI indicators rather than specific days post-pMCAO; diffusion-perfusion mismatch [34] would be the target of delayed recanalization. Future studies using

laser speckle imaging, two-photon microscopy, stereological fluorescence staining, and MRI will be performed for detailed evaluation of these zones and their changes related to delayed recanalization.

Additionally, we observed increased microglia number in animals treated with delayed recanalization. Herein, we did not determine the phenotype of the microglia present in the infarcted tissue; anti-inflammatory microglia are capable of repair and recovery, thus increased numbers of these microglia would be beneficial. In future studies, we will identify the specific microglia phenotype(s) within the infarcted tissue after delayed recanalization.

Interestingly, although infarct volumes for delayed recanalization at day 7 and 14 were similar to untreated pMCAO rats, these delayed recanalization groups had functional recovery. As mentioned above, the possibility exists that restoration of cerebral blood flow allows recovery of neurological function [22]. However, we cannot rule out the possibility that the rats treated with delayed recanalization at day 7 and 14 had a bias for recovery because of better initial behavioral performance 24 h post-pMCAO.

Vascular injury/damage, such as vessel rupture and vasospasm, is a concern for delayed recanalization. Long-term ischemia can cause vessel weakening which may rupture upon recanalization. Additionally, vasospasm in response to either prolonged ischemia or blood flow restoration by delayed recanalization is a secondary complication which may occur. These events were not explicitly examined after delayed recanalization. In future studies, we will use video imaging to investigate any potential deleterious consequences of delayed recanalization (e.g., vasospasm, BBB disruption, and hemorrhagic transformation). We will also quantify hemorrhages within the brain tissue and ventricles after delayed recanalization.

Additionally, untreated animals did not undergo sham recanalization surgery at the time points corresponding to recanalization (i.e., isoflurane was not given to untreated pMCAO animals at 3, 7, or 14 days post-MCAO). While it is unlikely that 10–15 min of isoflurane exposure would have any significant benefit with respect to cerebral perfusion, functional recovery, or long-term cerebral morphology, this needs to be explored in future studies.

There is the possibility that the degree of cerebral blood flow reduction during occlusion could be a crucial factor in determining outcome, including secondary injury post-ischemia. In future studies of permanent MCAO and delayed recanalization, it is important to determine the risk cerebral blood flow drop plays in hemorrhagic transformation and other post-occlusion complications (e.g., infarction size, vasospasm, mortality). For example, are animals with cerebral blood flow reductions of more than 90% at greater risk for hemorrhagic transformation?

Finally, there are some translational limitations of this study. First, only young male rats were used in this study; future studies require delayed recanalization to be performed in aged animals, as well as female rats. The former may present with complications for delayed recanalization due to weakened vasculature. Second, the intraluminal suture model for pMCAO was used herein. Future studies should be performed using the embolic stroke model of MCAO without the use of tPA; although spontaneous recanalization may occur in the embolic stroke model, this will provide a better clinical mimetic of patients arriving past

the 4.5-h time window for tPA treatment. The embolic model of pMCAO will be difficult to recanalize in rats, but non-human primates may be used. Third, delayed recanalization should be investigated in rats with comorbidities (e.g., hypertension, diabetes/hyperglycemia, hypercholesterolemia) which may also present with weakened cerebral vasculature.

Summary

Herein, delayed recanalization was investigated as a potential therapy to treat permanent large vessel occlusion. In the pMCAO rat model, delayed recanalization lead to reperfusion, recovery of sensorimotor deficits, improved learning/memory, reduced infarction volume, and increased cell counts within the infarcted tissue. The findings of this study suggest that delayed recanalization is a potentially viable surgical intervention for treatment of large vessel permanent occlusion in stroke patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
a Kaplan-Meier survival curve. Dashed lines indicate day 1 and day 3. $n = 15-41/\text{group}$. **b** Cerebral blood flow change during suture insertion ($n = 15-41/\text{group}$), pre-recanalization (7-12/group), post-recanalization ($n = 7-12/\text{group}$), and at the time of sacrifice ($n = 7-19/\text{group}$). # $p < 0.05$ vs pMCAO, N.S. indicates no significance

Fig. 2.

a Modified Garcia neuroscore 1 day after pMCAO ($n = 12-34/\text{group}$). **b** Beam walking score 24 hours after pMCAO ($n = 12-34/\text{group}$). **c** Forelimb placement test 1 day post-ictus ($n = 12-34/\text{group}$). * $p < 0.05$ vs Sham

Fig. 3. Functional Recovery after delayed recanalization assessed using the modified Garcia neuroscore. **a** Recanalization on day 3 ($n = 6-15$ /group). **b** Recanalization on day 7 ($n = 7-15$ /group). **c** Recanalization on day 14 ($n = 6-15$ /group). * $p < 0.05$ Sham vs pMCAO, and $p < 0.05$ Sham vs pMCAO + delayed recanalization, # $p < 0.05$ pMCAO vs pMCAO + delayed recanalization

Fig. 4. Functional recovery after delayed recanalization assessed using the beam walking test. **a** Recanalization on day 3 ($n = 6-15/\text{group}$). **b** Recanalization on day 7 ($n = 7-15/\text{group}$). **c** Recanalization on day 14 ($n = 6-15/\text{group}$). * $p < 0.05$ Sham vs pMCAO, and $p < 0.05$ Sham vs pMCAO + delayed recanalization, # $p < 0.05$ pMCAO vs pMCAO + delayed recanalization

Fig. 5.

Functional recovery after delayed recanalization assessed using the forelimb placement test.

a Recanalization on day 3 ($n = 6-15/\text{group}$). **b** Recanalization on day 7 ($n = 7-15/\text{group}$). **c** Recanalization on day 14 ($n = 6-15/\text{group}$). * $p < 0.05$ Sham vs pMCAO, and $p < 0.05$ Sham vs pMCAO + delayed recanalization, # $p < 0.05$ pMCAO vs pMCAO + delayed recanalization

Fig. 6. Neurological function on day 32. **a** Modified Garcia neuroscore. **b** Beam walking score. **c** Left forelimb placement test. $n = 12-34/\text{group}$. $*p < 0.05$ vs Sham

Fig. 7.

Behavioral performance on rotarod, open field, and Morris water maze 4 weeks after injury.

a Rotarod ($n = 7-19/\text{group}$). **b** Open field ($n = 7-19/\text{group}$). **c** Morris water maze memory test ($6-19/\text{group}$) (median with interquartile range is plotted). **d** Morris water maze probe test ($6-19/\text{group}$). Dotted line indicates random chance of time (25%) spent in target quadrant. Rotarod, open field, Morris water maze probe test: * $p < 0.05$ vs Sham, # $p < 0.05$ vs pMCAO, and $p < 0.05$ vs pMCAO + delayed recanalization (day 3). Morris water maze memory test: @ $p < 0.05$ Sham vs pMCAO, † $p < 0.05$ Sham vs pMCAO + delayed recanalization (day 3), ‡ $p < 0.05$ Sham vs pMCAO + delayed recanalization (day 7) and Sham vs pMCAO + delayed recanalization (day 14), § $p < 0.05$ pMCAO vs pMCAO + delayed recanalization (day 3), § $p < 0.05$ pMCAO + delayed recanalization (day 3) vs pMCAO + delayed recanalization (day 7) and pMCAO + delayed recanalization (day 3) vs pMCAO + delayed recanalization (day 14)

Fig. 8. Cerebral morphology 32 days after pMCAO. **a** Representative Nissl stained brains. **b** Infarct volume (%). **c** White matter and ventricular volumes (mm³). **d** Hippocampus area (mm²). $n = 7/\text{group}$. * $p < 0.05$ vs Sham, # $p < 0.05$ vs pMCAO, and $p < 0.05$ vs pMCAO + delayed recanalization (day 3)

Fig.9.

Neurons and astrocytes are absent within the infarction. Neurons (NeuN, green) and astrocytes (GFAP, red) are not observed in the infarct core in untreated pMCAO rats, or pMCAO rats treated with delayed recanalization. Scale bar: 20 μ m

Fig. 10. Delayed recanalization increases endothelial cells and microglia. Endothelial cells (RECA1, green) and microglia (Iba1, red) increase in number for pMCAO rats treated with delayed recanalization compared to untreated pMCAO rats. Arrowheads indicate some RECA1-positive cells. Arrows indicate some Iba1-positive cells. Scale bar: 20 μm

Fig. 11.

Delayed recanalization on day 3 promotes proliferation of neural stem cells and neural progenitor cells. Within the ischemic core, delayed recanalization induced proliferation of neural progenitor cells (Nestin-positive, green) and neural stem cells (Nestin-positive and SOX-2-positive (red)). Arrowheads indicate Nestin-positive staining. Arrows indicate SOX-2-positive staining. Scale bar: 20 μ m

Fig. 12. Cell counting within the infarct core and peri-infarct zones. $n = 2-4/\text{group}$. # $p < 0.05$ vs pMCAO, and $p < 0.05$ vs pMCAO + delayed recanalization (day 3), @ $p < 0.05$ vs pMCAO + delayed recanalization (day 7)