A Mouse Model of Hemorrhagic Transformation by Delayed Tissue Plasminogen Activator Administration After In Situ Thromboembolic Stroke

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Background and Purpose—Thrombolytic treatment with tissue plasminogen activator (tPA) improves outcome of patients with stroke who can be treated within 3 hours of symptom onset. However, delayed treatment with tPA leads to increased risk of hemorrhagic transformation and can result in enhanced brain injury. The purpose of this study is to validate a reproducible mouse model of hemorrhagic transformation associated with delayed administration of tPA.

Methods—Mice were anesthetized and thrombin was injected into the middle cerebral artery to induce the formation of a clot as described by Orset et al. To induce reperfusion, tPA (10 mg/kg) was intravenously administered 20 minutes or 3 hours after thrombin injection.

Results—Thrombin produced a clot in 83.1% of the animals, which caused focal ischemia determined 24 hours after the injection. Different degrees of bleeding were found in the middle cerebral artery occlusion group, including hemorrhagic infarction type 1 (HI-1) in 46.2%, hemorrhagic infarction type 2 (HI-2) in 30.8% and parenchymal hemorrhage type 1 in 23.0%. Administration of tPA 20 minutes after the occlusion produced an effective reperfusion in 62.5% of the animals and reduced both infarct volume and appearance of severe hemorrhage (10% nonhemorrhage, 80% HI-1 and 10% HI-2). However, administration of tPA 3 hours after the occlusion led to effective reperfusion in 47.1% of the animals, did not reduce infarct volume, caused hemorrhagic transformation (25% HI-1, 37.5% HI-2, and 37.5% parenchymal hemorrhage type 1), and increased hemorrhage and brain swelling.

Conclusions—We have set up a reproducible mouse model of hemorrhagic transformation associated with delayed administration of tPA similar to that observed in humans. (Stroke. 2011;42:196-203.)

Key Words: blood–brain barrier ■ brain swelling ■ clot ■ edema ■ thrombin

Thrombolytic treatment with tissue plasminogen activator (tPA) is the main approved therapy that improves outcome in patients with acute ischemic stroke. Unfortunately, the therapeutic window is only 3 hours from symptom onset.1 Some studies have suggested that there may still be patients able to benefit from thrombolysis even beyond 4.5 hours using the “mismatch” imaging concept.2,3 However, recent studies4,5 did not confirm those results, supporting the concept that delayed treatment with tPA is associated with an increased risk of blood–brain barrier breakdown, edema, and hemorrhagic transformation (HT), which can result in enhanced brain injury. Due to these complications together with the narrow therapeutic window, only a small percentage of patients with ischemic stroke (<5%) benefit from tPA-induced thrombolysis. Therefore, it becomes urgent to investigate mechanisms underlying risk of reperfusion injury not only to improve safety of tPA, but also to search for alternatives to this treatment.

Experimental models of stroke present several problems, even more evident when the model aims to emulate HT. In this context, most authors have used either normal or spontaneous hypertensive rats in which the occlusion is performed by using an intraluminal filament,6,7 injection of blood clots,8 or in situ clot formation by using rose Bengal.9 First, there are numerous differences in cerebral pathophysiology and reperfusion injury when mechanical and embolic clot-based models are compared. In addition, although clot-based models are closer to what happens in patients, a limitation is the lack of reproducibility and uniformity in the size and location of the infarcts, because placement and ultimate lodgement of the multiple emboli are not controlled. In addition, photochemically induced emboli are platelet-rich and lack fibrin and,
therefore, are not accessible to thrombolysis with tPA (for review, see Willing). Recently, a mouse model based on an in situ thromboembolic occlusion of the middle cerebral artery (MCA) was described, which allows recanalization and improves stroke outcome after early administration of tPA. Using this approach, the purpose of this study is to establish a reproducible mouse model of HT associated with delayed administration of tPA.

Materials and Methods

General
Adult male Swiss (Jackson Labs, Bar Harbor, Maine) mice weighing 25 to 35 g were used in this study. Experimental ischemia was carried out as described. Mice were anesthetized and mouse α-thrombin (2 UI) was injected into the MCA to induce a clot (Figure 1). A clot was defined as stable when laser Doppler flowmetry displayed a drastic fall of brain perfusion that remained stable during 60 minutes (mean reduction of 70% to 80%) and that was accompanied by a change in the color of the artery.

For reperfusion, tPA (10 mg/kg) was intravenously administered 20 minutes or 3 hours after thrombin injection. We defined a reperfusion as effective when blood flow was recovered (in the range of 60% to 100% of basal values) and remained stable within the first 60 minutes after tPA injection.

Details consistent with good laboratory practice, methods, etc, are provided in the Supplemental Data (which includes an addition to the “Methods” section, a Table, and a Figure; available at http://stroke.ahajournals.org).

Experimental Groups and Exclusion Criteria
Animals were classified in 5 different groups: (1) MCA occlusion (MCAO), in which vehicle was intravenously administered 20 minutes or 3 hours after thrombin injection; and (2 and 3) MCAO+tPA–20 minutes and MCAO+tPA–3 hours, in which tPA was intravenously administered 20 minutes or 3 hours, respectively, after thrombin injection; (4) spontaneous-reperfusion, a group of mice in which perfusion was transiently arrested but spontaneously returned to basal values in the absence of tPA within 60 minutes after thrombin injection; and (5) no-reperfusion, a group of mice that did not present reperfusion after tPA injection. Additional details regarding exclusion criteria, methods for allocation to treatment group, etc, are provided in Figure 2 and Supplemental Data.
Outcome Measures
Neurological deficit was assessed by using a modified neurological severity score and infarct size and brain swelling were determined as described in the Supplemental Data.

Assessment of HT
Hemorrhages were macroscopically classified in 5 groups: (1) nonhemorrhage; (2) hemorrhagic infarction type 1 (HI-1), defined as small petechiae, generally along the boundary of the infarct; (3) hemorrhagic infarction type 2 (HI-2), with more confluent petechiae within the damaged area; (4) parenchymal hemorrhage type 1 (PH-1), characterized by blood clots in <30% of the injured parenchyma; and (5) parenchymal hemorrhage type 2 (PH-2) with clots in >30% of the infarct. Additional histological examination was performed after Nissl and diaminobenzidine staining. Hemorrhage was also quantified by area measurement on images of 2,3,5-triphenyl tetrazolium chloride-stained sections and by a spectrophotometric assay (details and references consistent with Figure 3. Effect of early or late administration of tPA on infarct outcome. Infarct volume (A), areas (B), and neurological deficit (C) were determined 24 hours after ischemic insult in MCAO, MCAO+tPA–20 minutes, and MCAO+tPA–3 hours groups. Data are mean±SD; n=6 to 15; *P<0.05 versus MCAO group. Photographs of brain slices from representative experiments.
HT and statistical analysis are provided in the Supplemental Data).

Results

Effect of tPA on Infarct Volume Caused by Thrombin Injection

Injection of thrombin into the MCA generated a clot as previously described,11 which induced an infarct (MCAO, infarct volume of 11.7%±2.0%, n=13; Figure 3A–B; Table 1) and neurological damage 24 hours after the injection (n=6; Figure 3C; Table 1). A thrombin-induced stable clot was formed in 83.1% (Figure 2) of the animals associated with a rapid and sustained reduction of brain perfusion during 60 minutes (mean reduction of 70% to 80%; Figure 4A).

Administration of tPA 20 minutes after thrombin injection was associated with higher incidence of vessel recanalization, reduction in infarct volume (MCAO+tPA–20 minutes, 33% of reduction; n=10; P<0.05 versus MCAO; Figure 3A–B; Table 1), and better functional outcome 24 hours after MCAO (n=6, P<0.05; Figure 3C; Table 1). Early treatment with tPA led to an effective reperfusion, as shown by a recovery of initial cerebral blood flow, which remained stable within the first 60 minutes after tPA injection (Figure 4B), in 62.5% of the animals (Figure 2).

Administration of tPA 3 hours after clot formation led to effective reperfusion (the blood flow returned to basal levels and remained stable during the first 60 minutes after tPA injection; Figure 4C) in 47.1% of the animals (Figure 2). However, it did not affect infarct volume (MCAO+tPA–3 hours, n=8; P>0.05 versus MCAO; Figure 3A–B; Table 1) or functional outcome 24 hours after MCAO (n=5, P>0.05; Figure 3C; Table 1).

A group of mice (16.9% of the animals) showed spontaneous reperfusion (Figures 2 and 4D). The infarct size in this group was similar to that found in the MCAO+tPA–20 minutes group (spontaneous-reperfusion, n=6; P>0.05 ver-

| Table 1. Summary of the Results |
|------------------|------------------|------------------|------------------|------------------|
|                  | MCAO             | MCAO+tPA–20 minutes | MCAO+tPA–3 hours | Spontaneous Reperfusion | No Reperfusion |
| Infarct volume, %| 11.7±2.0         | 7.8±1.3            | 13.2±2.3         | 6.6±3.6           | 11.8±3.0        |
| Neurological deficit, points | 5.1±0.6        | 3.2±0.4            | 5.4±0.5          | 3.3±0.3           | 5.2±1.3         |
| Edema, %         | 1.9±0.2          | 2.6±2.2            | 6.1±2.0          | 1.9±0.9           | 2.0±3.1         |
| Hemorrhagic area, mm² | 0.44±0.47      | 0.18±0.1           | 1.80±2.2         | 0.11±0.06         | 1.10±1.1        |
| Blood volume, nL | 5.5±13.5         | 0±0                | 30.1±38.6        | 4±5.6             | 29.2±46.4       |

Figure 4. Effect of early or late administration of tPA on cerebral blood flow. Representative cerebral blood flow tracings of MCAO (A), MCAO+tPA–20 minutes (B), MCAO+tPA–3 hours (C), and spontaneous-reperfusion groups (D) as measured by laser Doppler flowmetry. Cerebral blood flow is expressed as percent of basal level.
Table 2. Physiological Parameters*

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<td>240 Minutes After Thrombin</td>
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</table>

*Parameters were measured in mice before and after tPA administration. Values are mean±SD (n=4).
MABP indicates mean arterial blood pressure; Ht, hematocrit; Hb, hemoglobin.

Finally, a group of mice without reperfusion was found in 45.5% of the animals treated with tPA (Figure 2). In this case, the infarct size was similar to that found in the MCAO group (no-reperfusion, n=15; P>0.05 versus MCAO; Table 1). The neurological damage was also similar (n=5, P>0.05 versus MCAO; Table 1). In any case, all the animals from this group had the MCAO recanalized when the wound was reopened 24 hours after MCAO and before euthanasia.

Physiological parameters were not significantly different among the groups studied, apart from some degree of hypotension at 240 minutes after occlusion, which was previously described and was independent of tPA administration (Table 2).12,13 The values of activated partial thromboplastin time, as indicator of coagulation pathway, were not significantly different between control animals and after tPA administration (ratios of activated partial thromboplastin time: 0.93±0.22 versus 2.18±1.50, respectively, n=4 to 7, P>0.05).

Effect of tPA on Hemorrhage and Brain Swelling
Mild signs of bleeding were found in all the animals from the MCAO group when measured 24 hours after thrombin injection in both macroscopic or histological examinations (Figures 5 and 6A, D, respectively). HI-1 was present in 46.2%, HI-2 in 30.8%, and PH-1 in 23.0% of the animals. No PH-2 was found in this group (Figure 5).

Administration of tPA 20 minutes after clot formation (MCAO+tPA–20 minutes group) significantly decreased HI-2 percentage at the same time as increasing the HI-1 fraction when compared with the MCAO group (P<0.05 versus MCAO; Figure 5). However, the mean hemorrhage area was similar to that found in the MCAO group (n=10, P>0.05 versus MCAO, Figure 7A; Table 1) and the blood volume was undetectable (n=6, P>0.05 versus MCAO, Figure 7B; Table 1). Mild signs of bleeding were also observed by histological examination (Figure 6B, E).

Interestingly, administration of tPA 3 hours after clot formation (MCAO+tPA–3 hours group) increased the occurrence of PH-1 and HI-2, reducing the percentage of HI-1 (Figure 5). Furthermore, delayed administration of tPA increased hemorrhage area (n=8, P<0.05 versus MCAO and MCAO+tPA–20 minutes), blood volume (n=5, P<0.05 versus MCAO+tPA–20 minutes; Figure 7A–B; Table 1), brain swelling (n=8, P<0.05 versus MCAO and MCAO+tPA–20 minutes; Figure 7C; Table 1), and blood extravasation (Figure 6C, F).

The spontaneous-reperfusion group showed percentages of bleeding similar to those found in the MCAO+tPA–20 minutes group (20% nonhemorrhage, 80% HI-1; n=5, P>0.05 versus MCAO+tPA–20 minutes). The mean values of hemorrhage area (n=5, P>0.05 versus MCAO+tPA–20 minutes).
minutes; Table 1) and blood volume (n=4, P>0.05 versus MCAO+tPA–20 minutes; Table 1) were also similar to the early tPA reperfused group. In contrast, the no-reperfusion group showed values similar to the MCAO group (42.9% HI-1, 21.4% HI-2, 35.7% PH-1, n=14, P>0.05 versus MCAO; hemorrhage area of 1.1±1.1 mm², n=14, P>0.05 versus MCAO; blood volume of 29±46 nL/hemisphere, n=7, P>0.05 versus MCAO; Table 1). Moreover, all animals of this group had the vessel reperfused 24 hours after surgery.

Discussion
So far, tPA is the main approved treatment for acute ischemic stroke treatment but with a very narrow therapeutic window due in part to the risk of HT and decreased efficacy beyond 3 hours. We hereby describe a model of HT caused by delayed tPA administration in an in situ thromboembolic stroke model in mice. Its features, resembling human clinic, suggest that this model may be very useful for the study of the mechanisms underlying this severe complication and to investigate new targets to improve stroke treatment.

Recently, Orset et al.11 described a useful stroke model based on in situ clot formation with several advantages such as its similarity to the human clinical situation with involvement of the whole neurovascular unit, the possibility to undergo reperfusion in response to early treatment with tPA, and its relatively good reproducibility. Therefore, this model appeared very suitable for the study of HT, that is, the bleeding into an area with pre-existing ischemic tissue damage,14 which often results as an adverse effect of thrombolytic therapy. For that, we assayed the effect of the intravenous administration of tPA at 20 minutes and 3 hours after occlusion.

First of all, we have found that thrombin-induced MCAO (MCAO group) caused a rapid fall in cerebral blood flow rate associated with the formation of a clot at the injection site. This clot was stable for at least the 60-minute duration of Doppler flow-rate recording and was associated 24 hours later with the appearance of an infarcted region in brain cortex, concomitant to a neurological deficit. The infarct volume showed good reproducibility within the territory supplied by the MCA. In this condition, brain tissue also showed modest values of hemorrhage area and blood volume. Of note, although this condition was associated in approximately half of the animals studied with benign, hardly noticeable bleeding15 (classified as HI-1), the other half showed either mild bleeding, classified as HI-2, or even PH-1. In addition, intravascular thrombin injection did not produce any detectable side effects, as previously described,16 likely due to its in vivo short half-life17 and to the dose used in this study.

In agreement with Orset et al.,11 administration of tPA 20 minutes after thrombin-induced occlusion (MCAO+tPA–20 minutes) induced recovery of cerebral blood flow rate up to 60% of initial values at least 10 minutes after the infusion onset and a better stroke outcome as shown by reduced infarct volume and better neurological scores. In addition, there was a reduction in both blood volume and hemorrhage area when compared with the MCAO group. More importantly, brain tissues showed just benign bleeding (HI-1, 80%) or even no bleeding at all (10%) with just 10% of HI-2. These data indicate that early vessel recanalization is able to rescue still viable ischemic brain tissue despite the appearance of some petechial bleeding. This might explain evidence from the clinical practice that has demonstrated that early thrombolytic treatment improves stroke outcome2,18,19 and that HI is mostly associated with neurological improvement and recanalization in patients receiving tPA.20,21 Although Orset et al.11 could not detect any evidence of hemorrhage, a discrepancy that might arise from the use of different assessment methods among other reasons, our results are basically in agreement with

Figure 6. Effect of early or late administration of tPA on hemorrhage. Representative Nissl combined with diaminobenzidine staining of brain sections from MCAO (A, D), MCAO+tPA–20 minutes (B, E), and MCAO+tPA–3 hours groups (C, F). Scale bars, 25 μm.
theirs, being now our major finding the difference between early and delayed administration of tPA.

Indeed, we have now found that delayed tPA administration (MCAO + tPA–3 hours) exhibits remarkable differences with its early counterpart; although tPA was effective in recovering cerebral perfusion, infarct outcome was not affected by this treatment, because both infarct volume and neurological scores were not significantly different from the MCAO group. However, especially noteworthy was the finding of a remarkable increase in both hemorrhage area and blood volume in the brain tissue of these mice. Accordingly, only one fourth of the animals treated showed benign bleeding (HI-1); this treatment was associated in the rest of the individuals with either HI-2 or to PH-1, indicating that delayed tPA causes HT of the ischemic infarct in this model. Furthermore, we have found that administration of tPA at 3 hours produces brain swelling indicating brain edema, in agreement with the concept that delayed administration of tPA can disrupt the blood–brain barrier.25 The development of HT after delayed tPA treatment found in our model is consistent with previous studies in other experimental models.14,23–27 In contrast with our model in mice, the therapeutic window for tPA in humans might be longer in some cases (4.5 hours).1 Several reasons may account for this, but very likely it is due to a faster metabolism in mice.

Interestingly, we have found that some animals (16.9%) underwent spontaneous reperfusion after thrombin injection, which could be followed by both recovery in cerebral perfusion and by visualization of the recanalization. In these cases, both infarct volume and bleeding were very close to those found in the early-reperfused group. In addition, we also found a set of tPA-treated animals (45.5%) that did not experience reperfusion regardless of treatment with tPA. It is worth highlighting that these 2 situations resemble the clinical situation,28 thus identifying our model as one of the most adequate ones for the study of the adverse effects of thrombolytic treatments.

Our model presents important advantages; it provides a reproducible and predictable infarct volume within the territory supplied by the MCA, it has a low mortality rate, and it resembles the findings in human patients, in which the longer the administration of tPA, the more frequent and severe the bleeding.28,29 Another positive feature is that the model has been developed in mice, thus facilitating studies of pathophysiological mechanisms using animals with genetic manipulations. For all these reasons, this model appears very suitable for the study of HT after delayed tPA.

Despite all this, our model presents some limitations: (1) clot is induced by a direct thrombin injection; although we have not detected any remarkable effect of this treatment, we cannot discard that some biochemical parameters might be affected by this protease, especially those implicated in edema30,31 and apoptosis or cell damage32,33; (2) in line with the previous issue, the clot induced by thrombin is a fibrin-rich embolus that contains a low number of cells and platelets11; this indicates that the model is suitable for the study of thrombolytic treatment after ischemic infarcts of a cardioembolic nature, but further studies are required to ascertain its validity as a model in the case of atherothrombotic infarct types; and (3) the delayed administration has been set at 3 hours after the ischemic occlusion; however, this is still a safe time when it translates to humans, very likely due to species differences.

**Conclusion**

We have set up a reproducible mouse model of HT associated with delayed administration of tPA, similar to that noted in humans. This method may be useful for the study of risk of reperfusion injury and to improve the safety and efficacy of thrombolytic therapy.
Acknowledgments

We thank Prof Eng H. Lo for his insightful discussion of the manuscript.

Sources of Funding

This work was supported by grants from Spanish Ministry of Science and Innovation SAF2009-08145 (M.A.M.) and SAF2008-03122 (L.L.) and the Spanish Ministry of Health RENEVAS RD06/0026/0005 (M.A.M.), RD06/0026/0013, and FIS-P1006788 (A.D.) and RD07/0026/2002 (J.S.). I.G.-Y. is a fellow of the Spanish Ministry of Health (FIS), M.S. is funded by S-BIO-0170/2006 (I.L.), and J.G.Z. is supported by the Programme Albahn, scholarship No. E07D400805CO.

Disclosures

None.

References


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Stroke. 2011;42:196-203; originally published online November 24, 2010; doi: 10.1161/STROKEAHA.110.600452

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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MATERIAL AND METHODS
Animals
Adult male Swiss mice weighing 25-35 g were used in this study (Jackson Labs, Bar Harbor, Me). All experimental protocols adhered to the guidelines of the Animal Welfare Committee of the Universidad Complutense (following EU directives 86/609/CEE and 2003/65/CE). Animals were housed individually under standard conditions of temperature and humidity and a 12h light/dark cycle (lights on at 08:00) with free access to food and water.

Surgical procedures
Middle cerebral artery (MCA) occlusion
Experimental ischemia was carried out as described previously\textsuperscript{1}. Mice were anesthetized in a chamber ventilated with 2.5% and then, maintained at 1.5-2% isoflurane in a 30%/70% mixture of O\textsubscript{2}/NO\textsubscript{2}. Body temperature was maintained at 36.5-37ºC using a feedback-controlled heating blanket. A catheter was inserted into the tail vein to allow the intravenous administration (200 µL) of vehicle or t-PA.

Animals were placed in a stereotaxic device to prevent any movement, the skin between the right ear and eye was cut, the temporal muscle was retracted and the temporal and parietal bones were exposed. Then a small craniotomy was performed over the artery bifurcation, the meninges were removed and the MCA, with its parietal and frontal branches, was exposed.

Previously, a microneedle was made with hematologic glass capillaries (REF 555/5; Karl Hecht KG Assistant, Germany) using a vertical puller (PC-10; Narishige, Japan), and was pneumatically filled with 2 UI/µl mouse alpha-thrombin (4,045 NIH/mg; Haematologic Technologies Inc., USA). Finally, the pipette was introduced carefully and against the flow into the lumen of the artery bifurcation and 1 µl of mouse alpha-thrombin (2 UI) was injected to induce the formation of a clot \textit{in situ} (Figure 1). The pipette was removed 10 minutes later, time at which the clot had stabilized.
A clot was defined as stable when laser Doppler flowmetry showed a rapid and drastic fall of brain perfusion that remained stable during 60 min (mean reduction of 70-80%) and that was accompanied by a change in the color of the artery.

**Measurement of cerebral perfusion**

With the purpose of measuring the cerebral perfusion by laser Doppler flowmetry (PeriFlux System 5000; Perimed AB, Sweden), a small hole was performed over the parietal branch and a fiberoptic probe (418-1; Perimed AB, Sweden) was inserted through it. The blood flow was recorded before the injection of thrombin and during 1 hour after artery occlusion or after the beginning of the t-PA infusion (see Supplementary Figure 1).

**t-PA-induced thrombolysis**

To induce reperfusion, t-PA (10 mg/Kg; Actilyse; Boehringer Ingelheim, Germany; pharmaceutical presentation containing t-PA as well as arginine, dilute phosphoric acid and polysorbate 80 as excipients), was intravenously administered (10% bolus, 90% perfusion during 40 min) at 20 min or 3 hours after the injection of thrombin. The control group received the same volume of vehicle (saline). We defined a reperfusion as effective when the blood flow was recovered (in the range of 60-100% of basal values) and remained stable within the first 60 minutes after t-PA injection.

**Experimental groups and exclusion criteria**

All the groups were performed and quantified in a randomized fashion by investigators blinded to treatment groups. Animals were allocated by randomization (coin toss) to 5 different groups (see Supplementary Figure 1): a) **MCAO**, in which vehicle was intravenously administered 20 minutes or 3 hours after thrombin injection (n=13); b) **MCAO + t-PA 20min**, in which t-PA was intravenously administered 20 minutes after thrombin injection (n=10); c) **MCAO + t-PA 3h**, where t-PA was intravenously administered 3 hours after thrombin injection (n=8); d) **Spontaneous reperfusion**, a group of mice in which perfusion was transiently arrested but spontaneously returned to basal values in the absence of t-PA within 60 minutes after thrombin injection (n=6), and e) **No**
reperfusion, a group of mice that did not present reperfusion after t-PA injection (n=15). The last two groups were established after the analysis of the results, and were not initially designed.

Some mice were ruled out of further analysis according to the following criteria: a) when MCAO caused an infarct that affected the striatum, probably due to an occlusion of the common trunk of the MCA but also of the lenticulostriatal arteries, b) when mice suffered an extra-parenchymal hemorrhage, probably due to surgical damage of the artery and c) mice with recanalization or occlusion of only one branch of the MCA.

All the studies (determination of infarct size, brain swelling and HT) were performed by investigators blinded to the different treatments.

**Neurological deficit evaluation**

Prior to sacrifice, 24 hours after surgical procedure, neurological deficit was assessed (n=6) by two independent observers, blinded to animal pharmacological treatment, using a modified neurologic severity score (mNSS)\(^2\). Supplementary Table I shows a description of the mNSS. Neurologic severity score comprises motor (muscle status, abnormal movement), sensory (visual, tactile and proprioceptive) and reflex tests. In the scale, the severity of injury is defined by the score graded on a range of 0 to 14 (normal score 0, maximal deficit score 14). One point was awarded either for the inability to perform, or for abnormal task performance, or for the lack of a tested reflex.

**Determination of infarct size and brain swelling**

Twenty-four hours after MCAO, mice were anesthetized again and the wound was reopened to observe whether the vessel was still occluded. After that, animals were sacrificed by an overdose of sodium pentobarbital to assess infarct outcome and were subjected for 10 minutes to transcardial perfusion with phosphate buffer 0.1 M pH 7.4 to eliminate intravascular blood for further analysis (hemoglobin spectrophotometry). Brain was removed and cut into 1-mm thick coronal slices and stained with 2,3,5-triphenyltetrazolium chloride (1% TTC in 0.1 phosphate buffer). Photographs of each coronal section were made with a digital camera (Nikon Coolpix 990; Nikon corp., Tokyo, Japan), and analyzed using ImageJ 1.41o (NIH, Bethesda, Washington).
Infarct area was delineated and determined (in mm$^2$) by counting the number of pixels within the outline. Infarct volume (in mm$^3$) was calculated as the sum of the orthogonal projections of each damaged area over the section thickness. In order to exclude the brain edema effects, infarct area was corrected by the ratio of the entire area of the ipsilateral hemisphere to that of the contralateral one.

In addition, brain swelling was calculated according to the following formula: (ipsilateral hemispheric volume - contralateral hemispheric volume) / contralateral hemispheric volume x 100$^3$).

**Assessment of hemorrhagic transformation**

**Macroscopic hemorrhage classification**

On the digital images, hemorrhages were classified by type and extension, according to previous publications $^4$-$^6$ in 5 groups: a) Non hemorrhage (NH); b) hemorrhagic infarction type 1 (HI-1), defined as small petechiae, generally along the boundary of the infarct; c) hemorrhagic infarction type 2 (HI-2), with more confluent petechiae within the damaged area; d) parenchymal hemorrhage type 1 (PH-1), characterized by blood clots in less than 30% of the injured parenchyma; e) parenchymal hemorrhage type 2 (PH-2) with clots in more than 30% of the infarct. Each animal was assigned to one group according to the most severe bleeding.

**Histological hemorrhage examination**

Twenty-four hours after MCAO, mice were anesthetized again and sacrificed as described above. Brains were taken out and frozen in isopentane. Then, sets of five 50-µm serial sections were obtained, and the first section of every set was stained with cresyl violet (Nissl staining) and diaminobenzidine (DAB). Diaminobenzidine reacts with peroxidases in red blood cells, and facilitates precise identification of HT, because DAB highlights hemorrhages and leaves non-hemorrhagic areas unstained.$^7$

**Hemorrhage area**

All noticeable hemorrhages, both petechial and parenchymal ones, were quantified on the images of TTC-stained sections as previously described$^8$. The
boundary of each bleeding was drawn using ImageJ 1.41o (NIH, Bethesda, Washington). The hemorrhage area (mm$^2$) was calculated by a summation of the area of every bleeding.

**Hemoglobin spectrophotometry:**
Intracerebral hemorrhage was quantified (n=6) with a spectrophotometric assay previously described$^9, 10$, but modified. Briefly, after transcardial perfusion with phosphate buffer to eliminate intravascular blood, each hemispheric brain tissue was homogenized for 30 seconds in 250 µl of phosphate buffer 0.1 M pH 7.4, followed by sonication on ice for 1 minute and centrifugation at 30,000 g for 30 minutes. The supernatant (10 µl) was reacted with Drabkin’s reactive (20 µl, SIGMA, Spain), for 30 minutes at room temperature and finally centrifuged again at 16,000 g for 30 more minutes. Drabkin’s reactive converts all hemoglobin forms to cyanometahemoglobin, which presents a maximum absorbance peak near 420 nm. Then, the optical density in this last supernatant was measured at 420 nm with a Nanodrop spectrophotometer$^11$. The relationship between the absorbance and the amount of blood was calculated with a standard curve made by adding a known volume of blood to the contralateral hemispheres. These measurements were performed after TTC staining, because it does not affect the spectrophotometric hemoglobin assay$^9, 10$.

**Statistical analysis**
Results are expressed as mean ± S.D. of the indicated number of experiments; statistical analysis involved one-way analysis of variance (ANOVA, or the Kruskal-Wallis test when the data were not normally distributed) followed by individual comparisons of means (Bonferroni or Dunn´s method when the data were not normally distributed) and comparisons between the groups of mice with macroscopic hemorrhage were performed with Fisher’s exact test. $P<0.05$ was considered statistically significant.
REFERENCES


10. Asahi M, Asahi K, Wang X, Lo EH. Reduction of tissue plasminogen activator-induced hemorrhage and brain injury by free radical spin


**Supplementary Table I.** Modified neurologic severity score for mouse (mNSS)²

<table>
<thead>
<tr>
<th>Behavioral test</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. Motor tests</strong> (muscle status-hemiplegia)</td>
<td></td>
</tr>
<tr>
<td>(normal=0; maximum=6)</td>
<td></td>
</tr>
<tr>
<td>1.1. Raising the mouse by the tail</td>
<td></td>
</tr>
<tr>
<td>- Flexion of forelimb</td>
<td>0-6</td>
</tr>
<tr>
<td>- Flexion of hindlimb</td>
<td></td>
</tr>
<tr>
<td>- Head moving more than 10° to the vertical axis</td>
<td></td>
</tr>
<tr>
<td>within 30 seconds</td>
<td></td>
</tr>
<tr>
<td>1.2. Placing the mouse on the floor</td>
<td></td>
</tr>
<tr>
<td>- Normal walk</td>
<td>0</td>
</tr>
<tr>
<td>- Inability to walk straight</td>
<td>1</td>
</tr>
<tr>
<td>- Circling toward the paretic side</td>
<td>2</td>
</tr>
<tr>
<td>- Falling down to the paretic side</td>
<td>3</td>
</tr>
<tr>
<td><strong>2. Beam balance tests</strong> (normal=0; maximum=6)</td>
<td>0-6</td>
</tr>
<tr>
<td>- Balances with steady posture</td>
<td>0</td>
</tr>
<tr>
<td>- Grasps side of beam</td>
<td>1</td>
</tr>
<tr>
<td>- Hugs the beam and one limb falls down from the</td>
<td>2</td>
</tr>
<tr>
<td>beam</td>
<td></td>
</tr>
<tr>
<td>- Hugs the beam and two limbs fall down, or spins on</td>
<td>3</td>
</tr>
<tr>
<td>beam (&gt;30 s)</td>
<td></td>
</tr>
<tr>
<td>- Attempts to balance on the beam but falls off</td>
<td>4</td>
</tr>
<tr>
<td>(&gt;20 s)</td>
<td></td>
</tr>
<tr>
<td>- Attempts to balance on the beam but falls off</td>
<td>5</td>
</tr>
<tr>
<td>(&gt;10 s)</td>
<td></td>
</tr>
<tr>
<td>- Falls off: No attempt to balance or hang on to the</td>
<td>6</td>
</tr>
<tr>
<td>beam (&lt;10 s)</td>
<td></td>
</tr>
<tr>
<td><strong>3. Reflexes absent</strong> (normal=0; maximum=2)</td>
<td>0-2</td>
</tr>
<tr>
<td>- Pinna reflex (a head shake when touching the</td>
<td>1</td>
</tr>
<tr>
<td>auditory meatus)</td>
<td></td>
</tr>
<tr>
<td>- Corneal reflex (an eye blink when touching the</td>
<td>1</td>
</tr>
<tr>
<td>cornea with cotton)</td>
<td></td>
</tr>
</tbody>
</table>
Supplementary Figure 1. Design of the study. Animals were allocated to their respective groups by randomization. Five different groups were studied: a) **MCAO**, in which vehicle was intravenously administered 20 minutes or 3 hours after thrombin injection (Thr); b) **MCAO + t-PA 20min**, in which t-PA was intravenously administered 20 minutes after thrombin injection; c) **MCAO + t-PA 3h**, where t-PA was intravenously administered 3 hours after thrombin injection; d) **Spontaneous reperfusion**, a group of mice in which perfusion was transiently arrested but spontaneously returned to basal values in the absence of t-PA within 60 minutes after thrombin injection, and e) **No reperfusion**, a group of mice that did not present reperfusion after t-PA injection. Blood flow was recorded by laser Doppler flowmetry, before the injection of thrombin and during 1 hour after artery occlusion or after the beginning of the t-PA infusion.