Efficacy of Alteplase in a Mouse Model of Acute Ischemic Stroke
A Retrospective Pooled Analysis

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Background and Purpose—The debate over the fact that experimental drugs proposed for the treatment of stroke fail in the translation to the clinical situation has attracted considerable attention in the literature. In this context, we present a retrospective pooled analysis of a large data set from preclinical studies, to examine the effects of early versus late administration of intravenous recombinant tissue-type plasminogen activator.

Methods—We collected data from 26 individual studies from 9 international centers (13 researchers; 716 animals) that compared recombinant tissue-type plasminogen activator with controls, in a unique mouse model of thromboembolic stroke induced by an in situ injection of thrombin into the middle cerebral artery. Studies were classified into early (<3 hours) versus late (≥23 hours) drug administration. Final infarct volumes, assessed by histology or magnetic resonance imaging, were compared in each study, and the absolute differences were pooled in a random-effect meta-analysis. The influence of time of administration was tested.

Results—When compared with saline controls, early recombinant tissue-type plasminogen activator administration was associated with a significant benefit (absolute difference, −6.63 mm³; 95% confidence interval, −9.08 to −4.17; F = 76%), whereas late recombinant tissue-type plasminogen activator treatment showed a deleterious effect (+5.06 mm³; 95% confidence interval, +2.78 to +7.34; F = 42%; P = <0.00001). Results remained unchanged after subgroup analyses.

Conclusions—Our results provide the basis needed for the design of future preclinical studies on recanalization therapies using this model of thromboembolic stroke in mice. The power analysis reveals that a multicenter trial would require 123 animals per group instead of 40 for a single-center trial. (Stroke. 2016;47:1312-1318. DOI: 10.1161/STROKEAHA.116.012238.)

Key Words: magnetic resonance imaging ■ middle cerebral artery ■ stroke ■ thrombolytic therapy ■ tissue-type plasminogen activator

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Intravenous recombinant tissue-type plasminogen activator (r-tPA), administered within 4.5 hours after stroke onset, is the only pharmacological treatment approved for acute ischemic stroke. However, it can only be administered to a minority of patients, achieves early arterial recanalization in <50% of cases, and has deleterious effects, including intracerebral hemorrhage, thus underlying the need for developing new acute strategies to be used for the treatment of stroke.

Testing potential acute therapies in animal models is presently the most common strategy for the development of new drugs for use in stroke. However, many approaches that showed efficacy in experimental stroke models have either not been translated or failed when tested in clinical trials. This translational roadblock is commonly attributed to inherent weaknesses of preclinical studies that include, lack of clinical relevance of the stroke models, monocentric design, and small sample sizes. Thus, improving the validity and reproducibility of preclinical studies is warranted. Some authors advocate for the use of multicenter preclinical studies and much effort was expended to develop new experimental models that mimic more closely the pathophysiology of stroke. Reporting of systematic reviews and meta-analysis of preclinical stroke studies from groups such as Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies (http://www.dcn.ed.ac.uk/camarades/default.htm: About) has increased.

These approaches allow one to take into account the fact that individual studies may have used small sample sizes, and to compare data from >1 type of experimental stroke model. In 2007, we developed a thromboembolic model of stroke in mice, which seems physiologically relevant and is now used by several groups to evaluate the effects of r-tPA either alone or where necessary in combination with putative neuroprotective drugs. However, no large-scale validation of this model is available to date. Therefore, we evaluated the effects of early (<3 hours) and late (≥23 hours) r-tPA administration in this stroke model in a retrospective pooled analysis of individual data from 9 international research centers.

**Materials and Methods**

**Selection Criteria, Search Strategy, and Data Collection**

Eligible studies for inclusion in this analysis were those that (1) used the thromboembolic stroke model described below according to a Standard Operating Procedure, (2) compared human r-tPA (Alteplase) treatment alone with a control saline group, whatever the time-window of treatment after stroke onset or the dose of r-tPA used, and (3) evaluated efficacy on lesion volumes measured either by magnetic resonance imaging (MRI) or histology at 24 hours post stroke onset. There were no restrictions on the strain of mice, or the dose of r-tPA used, during the protocol. Relevant studies were identified by a systematic search of the scientific literature of studies published from 2007 to 2013 and by collecting data from studies that we were aware of but not yet published. Thus, at the time of this meta-analysis, 9 international centers were identified and made their data available for this study. The inclusion criterion was a reduction of cerebral blood velocity to at least 60% of the baseline value before initiating treatment. No animals were excluded from the final analysis because of premature death—related to technical complication—or as a result of the drug itself, during or after administration. However, 85 animals were excluded from the global analysis (47 saline controls and 15 early and 31 late r-tPA treated) because either an excessively high-dose of thrombin was used (3 U) in noncompliance with the Standard Operating Procedure or an unmatched control group was used (ie, early saline versus late r-tPA treated). For each study, we collected raw data that included the identification of each experimenter, mouse strain, sex, experimental treatment (including the dose of thrombin used), the dose of r-tPA administered, the time of administration of r-tPA after stroke onset, and the lesion volume (Table I in the online-only Data Supplement). Early r-tPA administration was defined when the injection was performed within the first 3 hours, and late r-tPA administration was defined when r-tPA was injected after 3 hours post stroke onset.

**Animals and Ethics**

Depending on the research centers, experiments were performed on groups of male mice (C57/Bl6) weighing 40 g and 20 and 30 g, respectively (Charles Rivers Laboratory; Janvier Laboratory, Jackson Laboratories, Harland Laboratories, and the Center Universitaire de Resources Biologiques de Caen—Normandie Universite). All animals were housed under standard conditions with a 12-hour light/dark cycle and access to food and water ad libitum.

Studies were performed in accordance with the mandate of either the European Community Council Directive of November 24, 1986 (86/609/EEC) or the National Institutes of Health guide for the Care and Use of Laboratory Animals. Animal procedures were approved by the regional Ethical Committees for Laboratory Animal Experiments for each center. All efforts were made to minimize the possible suffering of the animals.

**Thromboembolic Stroke Model**

Cerebral ischemia was induced as described previously, and all centers followed a Standard Operating Procedure (Standard Operating Procedure; Materials and Methods in the online-only Data Supplement). In brief, the mice were anesthetized with isoflurane (induction 4%–5%, maintenance 1%–2%) in eight of nine centers. In 1 center, mice were anesthetized with a ketamine/xylazine mixture (IP, 50 and 6 mg/kg, respectively). A small craniotomy was performed, the dura was excised, and the middle cerebral artery was exposed. The pipette (glass micropipette, tip size 30–50 μm) was introduced into the lumen of the artery and 1 or 2 μL of murine α-thrombin (Hematologic Technologies) was injected to induce a clot, in situ. One center used human α-thrombin (0.75 UI/μL; Hematologic Technologies). Different doses of thrombin were used by the different centers (0.75–3 UI). However, we considered that a dose ≥2 UI/μL of thrombin was too high to allow reperfusion after r-tPA treatment. Thus, 23 animals were excluded from the global analysis because 3 UI of thrombin was used. To allow stabilization of the clot, the pipette was removed 10 minutes after the injection of thrombin. Thrombolysis was initiated via the tail vein (200 μL) of human recombinant tPA (r-tPA; Boeringher Ingelheim, Alteplase; 10% in bolus, 90% in perfusion >40 minutes) at different doses (0.9, 5, and 10 mg/kg, IV) and at different times after stroke onset (from 20 minutes to 4 hours). Control mice received saline under identical conditions. Rectal temperature was maintained at 37±0.5°C throughout the surgical procedure using a feedback-regulated heating system. Cerebral blood flow velocity was used as an index of the occlusion and was measured using either laser Doppler within the middle cerebral artery territory or Doppler Speckle on the dorsal surface of the skull during 60 to 120 minutes.

**Outcome Assessment**

The primary outcome was the lesion volume measured 24 hours after stroke onset either by histological staining or MRI analysis. Brains were cryosectioned, and slices (20 μm) were stained interchangeably using cresyl violet, thionine, or hematoxylin/eosin. One section in 10 (10- or 20-μm thick) was stained and analyzed (covering the entire lesion). Regions of interest were determined through the use of a stereotaxic atlas for the mouse and an image analysis system (ImageJ software) was used to measure the infarct. MRI images were obtained from...
T2-weighted RARE sequences with either a 7T Bruker pharmascan MRI (echo time [TE]/repetition time [TR]=51.3 ms/2500 ms) or a 9.4T Bruker biospec (TR/TE=3300/60 ms). Lesion areas were quantified on T2-weighted images with ImageJ software (version 1.45r; National Institutes of Health).

Statistical Analyses

Our primary analysis was to determine whether the efficacy of r-tPA differed according to the time-window of treatment and consisted of a pooled analysis of mean differences in infarct volume between r-tPA and saline (control), with stratification by treatment time-window (classified into <3 and ≥3 hours). For each experiment, we calculated the mean (±SD) difference in infarct volume between the r-tPA and the control group. The weighted mean difference was obtained using a random-effect meta-analysis; the weight given to each experiment being equal to the inverse of the variance of the difference. We then assessed whether the effect of r-tPA on infarct volumes differed between early and late r-tPA, using an interaction test. We also examined whether the result differed according to various experimental characteristics (eg, the mouse strain (Swiss versus C57Bl6), method of outcome assessment (MRI versus histology), and dose of thrombin used 0.75, 1, or 1.5 U/µL). This analysis was performed with RevMan 5.3 software. Finally, we performed a sensitivity analysis after exclusion of data from the largest center (Caen).

Results

We collected data from 26 experimental studies performed between 2007 and 2013 (from 13 experimenters in 9 different laboratories; Table I in the online-only Data Supplement). In total, data from 716 mice were available for the study. As previously explained, we excluded 85 animals. Thus, 623 animals (291 saline treated and 332 r-tPA treated) were included in the final analysis (Table I in the online-only Data Supplement). In the r-tPA group, 235 animals had early r-tPA treatment (<3 hours: from 20 to 40 minutes post ictus) and 97 late treatment (≥3 hours: 180 and 240 minutes post ictus; given 200 µL IV whatever the dose used 0.9, 5, or 10 mg/kg). As such, data were evaluated in 19 early administration studies and in 9 late r-tPA treatments.

In the pooled analysis, the early r-tPA was associated with a significant reduction in the final infarct volume (absolute difference, −6.63 mm³; 95% confidence interval, −9.08 to −4.17; P =<0.0001; F=76%; Figure 1),19–28 whereas the late r-tPA treatment showed a deleterious effect (+5.06 mm³; 95% confidence interval, +2.78 to +7.34; P =<0.0001; F=42%; Figure 1), with a statistically significant qualitative interaction (P2 =<0.0001).

A similar beneficial effect was observed for the early r-tPA treatment when considering the 7 studies performed outside the Caen laboratory: absolute difference=−10.61 mm³; 95% confidence interval, −14.80 to −6.43; P =0.008; F=66% (Figure 2).20–25 Looking at the 2 studies performed outside of our laboratory that applied late r-tPA treatment, there was

![Figure 1](https://stroke.ahajournals.org/)

**Figure 1.** Pooled analysis of lesion volumes expressed in mm³, comparing the mean values of saline (control) and recombinant tissue-type plasminogen activator (r-tPA)–treated animals. Total is the number of animals per group. CI indicates confidence interval.
still no beneficial effect (absolute difference = +3.07 mm³; 95% confidence interval, −1.63 to +7.77; \( P_{\text{sig}} = 0.6; I^2 = 0\% \); Figure 2). Again, the interaction with the time-window was still significant (\( P_{\text{int}} < 0.0001 \)).

Interaction at the subgroups level (Table)—which includes mouse strain (Swiss mice versus C57/Bl6 mice); method of evaluation to determine the lesion volume (ie, histology versus MRI analysis); whether studies were published, whether the studies were performed in a blinded manner; expertise of the experimenters; or whether the studies reporting hemorrhages are included; and dose of r-tPA administered—had no influence on the effects of r-tPA (Figure 1).

### Discussion

In this retrospective study of a large pooled analysis of multicenter preclinical data (based on a thromboembolic stroke model), we demonstrated that early (<3 hours) administration of r-tPA after cerebral ischemia is associated with a significant reduction in lesion volume, whereas late administration (\( \geq 3 \) hours) has no, or a deleterious, effect.

Although pooled analyses of data are common in clinical studies, such analyses are rare in preclinical research and no pooled analysis exists on r-tPA in ischemic stroke in animals. Yet, such an approach is of major importance because most of therapeutic strategies with beneficial effects in experimental stroke models failed when evaluated in humans, or have not been translated into a clinical trial, because of lack of support from industry or clinicians. It is therefore crucial to provide drug companies and clinicians with reliable stroke models that represent the clinical situation as much as possible.

As the benefit of r-tPA is well established in humans, it appeared to us interesting to demonstrate that this benefit is also clear in an appropriate animal model of ischemic stroke. Usually, preclinical studies have small sample sizes, and there is often a substantial heterogeneity in the stroke models used. Although we focused on a specific model of thromboembolic stroke and increased the sample size, we still observed a certain degree of heterogeneity across studies. This heterogeneity may be explained by variations in the animal strain, in the method of assessment, or in interindividual technical aspects despite a well-standardized model. However, our sensitivity analyses were highly consistent with the main finding (ie, a time–effect relationship between r-tPA administration and infarct volume).

The inclusion of a large sample population (623 animals) may have helped contribute to the validation of the model. Our group developed and characterized an embolic stroke model in mice, in which cerebral ischemia is induced by a local injection of thrombin directly into the middle cerebral artery. This leads to the immediate formation of a clot, cerebral blood flow disruption, and subsequent cortical infarction.29 Several other experimental stroke models exist and have been used for years in various animal species. However, those that use electrocoagulation, ligatures, or a filament are not appropriate in which to test thrombolytic drugs. Other researchers use the autologous injection of a clot, or microemboli, via the internal carotid artery to induce stroke, but such methods evince poor reproducibility and uniformity in the location of the lesion29 and result in a high mortality rate.30,31 Accordingly, despite successful r-tPA–induced reperfusion, it is not surprising to observe opposite effects of r-tPA treatment on infarct size depending on the extent to which the models reflect the contribution of fibrinolysis, blood–brain barrier alterations, or neurotoxicity.

![Figure 2](http://stroke.ahajournals.org/DownloadedFrom)
Reporting of systematic reviews and meta-analysis of preclinical stroke studies is increasing. In the present study, we evaluated the effects of r-tPA in a model of thromboembolic stroke with a large sample population and examined the effects of r-tPA dose, time of administration, animal strain, research center, and method for calculating the infarction volume in the mouse. Nonetheless, there are some potential limitations in our analysis. Inherent differences exist between animal and human studies and applying the same method of meta-analysis to preclinical data is not straightforward. Although we had the individual data available, we finally opted for a pooled analysis of group (research center) studies. Indeed, in each study, there is no heterogeneity in the animal model that has the same characteristics at baseline and consequently excludes adjustment for confounding factors. The main source of nonuniformity was the experiment (the study) itself. However, we also performed the same analyses with generalized linear models and found the same interaction with time (data not shown). In addition, although we initially used 10 mg/kg r-tPA, as is usually recommended in rodents, the current analysis shows that a dose as low as 0.9 mg/kg (the dose used in clinical studies) is sufficient to produce a beneficial effect with early r-tPA treatment.

Although the original publication was based on data obtained from Swiss mice, the present data show similar results when using C57/Bl6 animals. The use of different time-windows, different doses, and 2 strains of mice together

### Table. Interaction Between Early and Late r-tPA Treatments in Different Subgroups

<table>
<thead>
<tr>
<th>Mouse strains</th>
<th>No. of Studies</th>
<th>Mean Difference (95% CI)</th>
<th>I² (%)</th>
<th>Test for Interaction, P Value</th>
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<tbody>
<tr>
<td>Swiss</td>
<td></td>
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<tr>
<td>Early r-tPA</td>
<td>14</td>
<td>−6.31 (−9.03 to −3.58)</td>
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<tr>
<td>Late r-tPA</td>
<td>7</td>
<td>5.38 (2.93 to 7.83)</td>
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<td>C57/B1</td>
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<tr>
<td>Early r-tPA</td>
<td>5</td>
<td>−8.18 (−14.90 to −1.46)</td>
<td>64</td>
<td>&lt;0.02</td>
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<td>Late r-tPA</td>
<td>2</td>
<td>2.42 (−3.12 to 7.96)</td>
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### Evaluation methods

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<tr>
<th>Histology</th>
<th>No. of Studies</th>
<th>Mean Difference (95% CI)</th>
<th>I² (%)</th>
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<tr>
<td>Early r-tPA</td>
<td>9</td>
<td>−9.92 (−12.49 to −7.35)</td>
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<th>MRI</th>
<th>No. of Studies</th>
<th>Mean Difference (95% CI)</th>
<th>I² (%)</th>
<th>Test for Interaction, P Value</th>
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<tbody>
<tr>
<td>Early r-tPA</td>
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<td>−3.76 (−6.28 to −1.25)</td>
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<td>&lt;0.0001</td>
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<tr>
<td>Late r-tPA</td>
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<td>4.41 (1.41 to 7.41)</td>
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### Published vs unpublished studies

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<th>Published</th>
<th>No. of Studies</th>
<th>Mean Difference (95% CI)</th>
<th>I² (%)</th>
<th>Test for Interaction, P Value</th>
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<tr>
<td>Early r-tPA</td>
<td>9</td>
<td>−8.77 (−12.74 to −4.80)</td>
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<td>&lt;0.00001</td>
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<td>Late r-tPA</td>
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<td>6.26 (3.52 to 9.00)</td>
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<table>
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<th>Unpublished</th>
<th>No. of Studies</th>
<th>Mean Difference (95% CI)</th>
<th>I² (%)</th>
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<td>Early r-tPA</td>
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<td>−4.70 (−6.78 to −2.62)</td>
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<td>Late r-tPA</td>
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<td>2.73 (−0.67 to 6.14)</td>
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<th>Blind vs not blind studies</th>
<th>No. of Studies</th>
<th>Mean Difference (95% CI)</th>
<th>I² (%)</th>
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<tr>
<td>Blind</td>
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<td>−6.35 (−9.04 to −3.66)</td>
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<td>4.19 (1.61 to 6.78)</td>
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<th>Caen vs others</th>
<th>No. of Studies</th>
<th>Mean Difference (95% CI)</th>
<th>I² (%)</th>
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<td>−4.69 (−7.09 to −2.69)</td>
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<td>5.31 (2.76 to 7.85)</td>
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<td>−10.61 (−14.80 to −6.43)</td>
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<tr>
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<th>Influence of training</th>
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<tr>
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<td>−6.45 (9.37 to −3.52)</td>
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<tr>
<td>Late r-tPA</td>
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<td>6.98 (4.10 to 9.86)</td>
<td>53</td>
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### Table. Continued

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<thead>
<tr>
<th>No. of Studies</th>
<th>Mean Difference (95% CI)</th>
<th>I² (%)</th>
<th>Test for Interaction, P Value</th>
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<tbody>
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<td>Lower 25% trained</td>
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<td>Early r-tPA</td>
<td>−8.98 (−13.26 to −4.70)</td>
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<tr>
<td>Late r-tPA</td>
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<table>
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<th>Doses of tPA</th>
<th>No. of Studies</th>
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<th>Test for Interaction, P Value</th>
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<td>Early r-tPA</td>
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<td>5.48 (3.12 to 7.84)</td>
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<td>2.60 (−2.41 to 7.61)</td>
<td>…</td>
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</table>

CI indicates confidence interval; MRI, magnetic resonance imaging; and r-tPA, recombinant tissue-type plasminogen activator.
with histological analysis is in agreement with some of the recommendations made by the Stroke Treatment Academic Industry Roundtable (STAIR) group. Furthermore, saline was used in all control groups instead of the vehicle containing -arginine, which is used in clinical trials. Nevertheless, recent experimental studies demonstrated no significant effect of -arginine when compared with saline in a stroke model in rabbits. The outcome we used was infarct volume as a measure of stroke size. This work was supported by Spanish grants PI12/00710 and PI12/00720; National Institutes of Health grants (NS049263 and NS055104); grants from the INSERM (French National Institute for Health and Medical Research), the Foundation for Medical Research (FRM/NoDEQ2014032955), and the Regional Council of Lower Normandy.

**Disclosures**

None.

**References**


Efficacy of Alteplase in a Mouse Model of Acute Ischemic Stroke: A Retrospective Pooled Analysis


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SUPPLEMENTAL MATERIAL
Supplementary Material & Methods

Ethic:
Animal procedures were approved by the local ethical Committees for Laboratory Animal Experiments. Nevertheless, some discrepancies may exist between the ethical approval systems of the different countries. All experiments from Caen were performed under the same agreement (5 years validity): C1411800. In Madrid, the experiments were agreed by the University ethical committee (no number available). In Manchester, all procedures were performed under relevant personal and project licences and adhered to the Animals (Scientific Procedures) Act, UK (1986). Others centres presented specific protocol agreement: Boston: 2003N000303; Lund: N/03-01/13/03/01-18; Lyon: 237; Mannheim: 35-9185.81/G-25/09; Pamplona: 014/08; Paris: P2.CM.152.10

Randomization and blinding:
All experiments included in the analysis were randomly performed. Thus, randomization was performed by a person out of the procedures that assigned animals to the different treatments (saline, tPA etc…) before the surgery in 8 out of 9 centres. Only one centre used computer-generation of random number for each animal included in the experiment. Blind evaluation of the lesion volumes were performed in 21 out of 26 experiments. Measurement of the lesion volumes (histology or MRI) was performed by an investigator blind to treatment.

Experimenter:
Experiments were achieved by investigators (n=13) skilled for the surgical procedure. It is important of note that all experimenters are experts in experimental surgery in the field of stroke models. The mean number of animals performed with this model is of 38 (median =30 animals) before inclusion in a study. Due to heterogeneity of experimenter skill, the training animals performed before any inclusion in a study varied between 10 to 70 animals. Furthermore, no correlation was observed between the skills and the outcome (Table 1).

Standard Operating Procedure (SOP):
SOP of thromboembolic cerebral ischaemia induced by injection of thrombin into the right middle cerebral artery of the anaesthetized mouse.

Goal of the model:
- To induce a reversible focal cerebral ischaemia representative of the clinical situation
- Clot can by lysed by thrombolytic therapy
- Effect of rtPA administration to ameliorate the ischaemic outcome
- No mortality
- Reproducible

1. Pipette preparation
- Micropipettes are made from an haematologic sampling pipette (Assistent 555/5, Germany) using a pipette puller.
- The pipette is inserted in the pipette puller (heating level around 70, is determined to make 2 pipettes from 1 haematologic pipette).
- Using a scalpel, the tip of the pipette (~20µm external diameter) is cut in order to make it sharp and 1µL graduations are made if necessary (15 mm of pipette length = 1µL).
• The pipette is mounted on the micromanipulator of the stereotaxic device and connected to a 10ml syringe using tubing.
• In order to fill the pipette with thrombin, the tip is plunged in the thrombin sample (1 U/µL, from HTI, USA) and negative pressure is applied with syringe.
• When the pipette is filled with 1µL of thrombin, the syringe is disconnected from the tubing.
• Until use, the pipette and its contents can be stored at 4°C (maximum of 12 hours).

2. Surgical procedures
• Mice (male Swiss, C57B6 J, 25-30 g, Janvier, France) are anaesthetized with isoflurane (5% for induction in an induction box and 2% during maintenance using a face mask with a gas mixture of O₂: N₂O; 1:2).
• The mice are not intubated and breathe spontaneously.
• The mouse is positioned on a rat stereotaxic device (rat frame with 45° ear bars, Stoelting, UK).
• During all surgical procedures the body temperature is maintained at 37°C using a homeothermic blanket system with a retro-controlled heating pad and rectal probe.

3. Tail vein catheterization
• The tail is turned and fixed using adhesive, in order to present a vein on the dorsal plane.
• A small incision of the tail (0.5 cm long) is made using a scalpel (blade n°11).
• The vein is dissected with micro-scissors.
• The vein is incised to allow for catheter insertion (filled with saline non-heparinized).
• To verify the patency of catheterization a small volume of saline (20 to 50µL) is injected.
• The catheter (30G) is fixed on the tail with adhesive tape and connected to a syringe filled with saline.

4. Clot emplacement
• The stereotaxic device is positioned perpendicularly to the manipulator (See Note 1).
• An incision of the skin (2 cm) between the right eye and the right ear is made and the temporal muscle retracted (See Note 2).
• Micro-clips (micro-bulldog clamp, WPI, UK) are positioned on the skin at the extremity of the incision to allow a better access to the field of view.
• The masseter muscle is dissociated, using micro-scissors, from the edge of the skull and cut vertically in 2 sections.
• Sutures (7/0) are placed in both extremities to reflect the muscle in order to visualize the parietal part of the skull.
• The stereotaxic device is inclined for a better approach (See Note 3).
• The bifurcation of the middle cerebral artery (MCA) is located through the translucent skull and a small craniotomy (0.8-1 mm Ø) is performed using a saline-cooled dental drill (See Note 4).
• The residual bone is removed using a needle (25G) and the dura is excised using the same needle.
• The Doppler probe (fiber optic, Oxford Optronix, UK) is positioned on the distal part of the MCA to monitor blood velocity in the artery. A small quantity of oil is placed around the probe to enhance the signal.
• The pipette containing the thrombin is mounted on the micromanipulator and connected to a syringe filled with air (See Note 5).
• The pipette is positioned near the artery before final insertion.
• After the tip of the pipette is inserted into the MCA lumen (Figure 2.a), thrombin is injected by applying a positive air pressure with the syringe (See Note 6).
• The pipette is removed 10 min after the injection of thrombin (See Note 7) to allow for the stabilization of the clot.
• Following thrombolysis, reperfusion of the artery begins within 15-20 min.

5. Ischaemia and reperfusion
• Twenty minutes after the thrombin injection, rtPA (10 mg/kg) is injected intravenously using an infusion pump (200µL, 10% bolus, 90% infusion over 40 min) connected to the tail catheter. Other neuroprotective drugs may be administered according to the desired protocol.
• Throughout the experiment, the wound is hydrated using saline and blood velocity is monitored continuously by the Doppler probe.
• After the rtPA infusion, a small piece of synthetic dura (Dura substitute, Gore, USA) is placed over the craniotomy and glued (sciano-acrylate) to the skull. The temporalis muscle is then repositioned over the synthetic dura, the wound sutured (7/0), and the tail catheter removed.
• Twenty-four hours later, the mice are killed by decapitation under isoflurane (5%) delivered in medical air. The brain is rapidly removed, frozen in isopentane, and stored at -80 °C.

6. Advantages:
• **Thrombolytic therapy can be used**
The model offers the possibility to induce thrombolysis following the appropriate treatment. This is a big advantage over the intraluminal thread model where a filament is introduced in the same manner as the autologous blood clot) but in which a pharmacologically-induced reperfusion is not possible.

• **Control of the clot formation**
This model permits the direct visual emplacement of the clot to be controlled by the experimenter. The clot can be introduced exactly in the same position each time and should spontaneous reperfusion occur this also can be recorded (mice in which the thrombin does not remain in place for the entire duration of the experiment can be excluded) in order to achieve a homogeneous group of mice for inclusion into the experimental protocol.

• **Infarcts are highly reproducible**
This is possible due to the poor vascular network in mice and the direct control of the clot emplacement.

• **No mortality**
The purely cortical infarct is not sufficient to induce mortality in the mouse.

• **Transient ischaemic accident is possible**
The duration of the occlusion is controlled by the operator. Small injections of thrombin, which do not stay lodged in the MCA can be introduced once or repeatedly in order to mimic a transient ischaemic attack.

7. Disadvantages:
• **Craniotomy**
The main drawback of this model is the fact that the cranium is not sealed after the surgical intervention. However, for those of you who wish to improve upon the present model, the application of a small section of synthetic dura matter could be glued to the skull prior to suturing the wound. If this procedure is carried out immediately following the injection of the
thrombin, then there is no way by which one can confirm that the clot has remained in place unless CBF is monitored continuously.

- **Secondary micro-clot formation following disruption of the initial thrombus**
  Once again, this is a situation which is often associated with embolic stroke in humans.

- **Limited behavioral disturbances due to the specificity cortical lesion**
  Indeed, one should not be surprised if one cannot differentiate between occluded and sham-operated animals after a few days. Impairment of motor function is not readily evident and behavioral changes require complex cognitive tests to show neuro-behavioral dysfunction.

4. Notes

1. All the procedures should be performed in the stereotaxic device.
2. The operating microscope should have a high magnification, at least x50.
3. The stereotaxic device should be positioned in a perpendicular way to the field of view of the microscope during the introduction of the thrombin.
4. To enhance the observation of the MCA through the parietal part of the skull, it should be hydrated with a topical application of warm saline.
5. The pipette tip should be sharpened prior to use, thus reducing the problems during its introduction into the lumen of the MCA (less hemorrhagic complications, less damage to the wall of the artery).
6. The thrombin injection should not be performed in one step but in different steps by disconnecting and reconnecting the syringe to the pipette tubing.
7. The use of thrombin is species dependent (1 U thrombin for a C57B6 J mouse)
## Table I. Data collection

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<th>Experiment ID</th>
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