Reduction of Infarct Volume and Mortality by Thrombolysis in a Rat Embolic Stroke Model

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Background and Purpose: Thrombolytic therapy with recombinant tissue plasminogen activator was tested in a rat embolic stroke model.

Methods: The rat carotid territory was embolized with arterial-like microthrombi formed under pressure. Hemispheric cerebral blood flow before and after embolization was measured by the intraarterial Xenon-133 injection method. Fifteen minutes after embolization, 24 rats were treated with 3 mg/kg or 10 mg/kg tissue plasminogen activator, and 27 were treated with saline. Carotid angiography displayed the rate of occlusion of the cerebral arterial supply before and after treatment. Brains were fixed and evaluated neuropathologically and infarct volume was measured.

Results: Cerebral blood flow was reduced 70–86% after embolization. The comparison of pretreatment and posttreatment angiography showed significant (p=0.0005) reperfusion in the treated rats. Thrombolytic therapy significantly reduced the infarct volume from 55.1% to 24.4% of embolized hemisphere volume (p=0.007) and increased the survival rate from 0.48 to 0.96 (p=0.0004). Fifty-three percent of the embolized rats recanalized completely after thrombolytic treatment and developed almost no infarction (median volume 2.8%), and all survived. No hemorrhagic complications were observed.

Conclusions: Early thrombolytic therapy induced recanalization and reduced mortality and infarct volume after embolic stroke in this model. (Stroke 1992;23:1167–1174)

KEY WORDS • cerebral blood flow • cerebral ischemia • thrombolytic therapy • rats
and thermostatically-controlled heating of the operating table.

The right femoral artery and vein were catheterized with a polyethylene PP 25 tube, and the arterial line was filled with 0.5 ml saline with 5 units/ml heparin and clamped. The venous line was kept patent by continuous flow of saline at a rate of 0.5 ml/hr. Mean arterial blood pressure and arterial PaO₂, PaCO₂, and pH were measured twice (Radiometer ABL 2, Copenhagen). One animal was excluded from the study because of PO₂ saturation twice below 90%.

Preparation of the Emboli

A 1-ml insulin disposable syringe with a 28-gauge (inner diameter, 0.164 mm) needle was filled with 50 μl saline containing 2.5 units of thrombin (Topostasin, Roche Laboratories, Nutley, N.J.). Then, in 44 rats, 150 μl arterial blood was drawn in another syringe of the same type. After less than 20 seconds, the two syringes were interconnected from tip to tip with a polyethylene PP 10 tube, and the suspension was moved approximately 70 times from one syringe to the other during 3 minutes. The syringes were left standing for 30 minutes until embolization. In another 20 animals, the emboli solution was prepared in the same manner with 200 μl arterial blood instead of 150 μl.

Carotid Operation Procedure

The right external carotid artery and its branches were exposed and the pterygopalatine, thyroid, and occipital arteries ligated. A polyethylene PP 25 catheter was inserted through a transverse arteriotomy of the external carotid artery with the tip 2 mm distal to the bifurcation and fixed with ligatures; care was taken not to injure the intima. Clotting in the catheter was avoided by continuous flow of heparinized (5 units/ml) saline through the line (the animals obtained up to 10 units of heparin during the entire procedure).

Embolization and Cerebral Blood Flow (CBF) Measurements

Just before and after embolization, CBF was measured using an intracarotid bolus injection of 0.15–0.20 ml saline containing 5–10 mCi/ml Xenon-133 (Amersham). Clearance was recorded by external detection with a collimated NaI(Th) crystal placed over the right MCA area. The preembolic clearance was recorded as the initial 15-second slope, then the preformed emboli.
Angiography

Immediately after and 2 hours after embolization, angiography was performed via the carotid catheter by bolus injection of 0.2 ml heparinized (5 units/ml) iohexol (Omnipaque, 300 mg I/ml, Nycomed, Denmark). An x-ray tube (Philips SRO 03/100) with a small focus spot 0.15 mm² and a large focus spot 1.5 mm² was used. Exposure data were as follows: 70 kV, 14 mA, and 0.4 seconds. A focus-object distance of 31.5 cm and focus-film distance of 141.5 cm gave a linear magnification of 4.5. Angiograms were made on mammographic high-resolution Kodak NRM-1 films and were evaluated in a blinded manner by a neuroradiologist according to the following score: 0, patent arteries; 1, distal MCA branch occlusion; 2, main stem MCA occlusion; 3, internal carotid artery occlusion (Figure 1).

Recombinant Tissue-Type Plasminogen Activator (rt-PA) Administration

In the experimental group, eight rats embolized with 200-μl emboli suspension received 3 mg/kg rt-PA (Actilyse, Boehringer Ingelheim, Ridgefield, Conn.). Eight rats embolized with 200-μl emboli suspension and eight embolized with 250 μl emboli suspension received 10 mg/kg rt-PA. Intravenous infusion started 15 minutes after embolization and was administered during 30 (3 mg/kg dose) or 45 (10 mg/kg dose) minutes using a Harvard infusion pump. Twenty-seven control animals received an equal amount of isotonic saline instead of rt-PA. Thirteen died immediately after embolization and consequently did not receive infusion. After the second angiography, all animals received 10 ml i.p. isotonic saline, and femoral and neck wounds were closed after ligations of vessels. The anesthesia was reverted with 0.1 mg i.m. naloxone (Narcanti, Du Pont Pharmaceuticals, Wilmington, Del.). After recovery from anesthesia, the rats neurological status was evaluated according to the method of Bederson et al.10 Rats were held gently by the tail 1 meter above the floor and observed for forelimb flexion. Normal rats extended both forelimbs toward the floor and were assigned grade 0. Rats with flexion of the forelimb contralateral to the injured hemisphere were graded 1, rats with reduced resistance to lateral push toward the paretic side were graded 2, and rats with spontaneous circling toward the paretic side were graded 3.

The animals were then placed in an incubator with 75% humidity and a temperature of 28°C for approximately 10 hours. Animals unable to drink received intraperitoneally 10 ml of equal parts of isotonic saline and isotonic glucose 10 and 20 hours after embolization to prevent dehydration.

Preparation of Brain Tissue and Measurement of Infarct Size

Four to 6 days after the operation, the rats were once again graded according to the Bederson score and were subsequently anesthetized and killed by cardiac perfusion fixation with a 4% phosphate buffered (pH 7.2) formalin solution. In the perfusion-fixed rats and in those that died before this procedure, the brains were carefully removed, postfixed and dehydrated during 5

<table>
<thead>
<tr>
<th>Table 1. Blood Gas and Arterial Blood Pressure Values</th>
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<td>Before embolization</td>
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<td>Control (n=27)</td>
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<td>(93-103)</td>
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<td>Treated (n=23)</td>
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All results are displayed as median values, with 25th and 75th percentiles shown in parentheses. Values of pH, PaO₂, PaCO₂, and mean arterial blood pressure (MABP) are expressed in mm Hg.
days, embedded in paraffin, and cut into sections 4 μm section thick. In each brain, approximately 17 horizontal sections with a distance of 0.4 mm between each were obtained and stained with hematoxylin-eosin. Using a Leitz TAS plus image analyzer (Figure 2), affected hemisphere and infarct volumes were calculated as areas multiplied with the distance between sections. Infarct areas included areas with 100% neuronal death. Results of infarct and hemisphere volumes were expressed as an average of two independent measurements performed without knowledge of the treatment regimen.

Calculations and Statistical Analyses
Cerebral blood flow was measured twice, immediately before and after embolization. Animal weight was also obtained twice, at the induction of anesthesia and just before perfusion fixation. Body weight reduction and CBF reduction were calculated as the following ratio: first—second value/first value.

Nonparametric statistical analyses of our data were performed because all our data were either nominal or could be placed on rank ordinal or ratio–interval scales with no apparent normal distribution of values. Mann-Whitney U test was used for unpaired observations, Wilcoxon matched-pairs for paired observations, Fisher’s exact probability test for survival rate, and the Spearman rank correlation test for paired ranked observations.

Results
No differences were found in any parameters between the groups of rats treated with 3 mg/kg and 10 mg/kg rt-PA; therefore, the groups were pooled. Blood gas values are shown in Table 1. No significant differences between surviving, dying, treated, or control groups were found. The animals hypoventilated slightly during this anesthesia with spontaneous respiration. Thirteen rats (10 of 43 embolized with 200 μl suspension and three of 20 embolized with 250 μl suspension) that died

Figure 3. Top panel: Micrograph of an emboli suspension showing an elongated microembolus composed of fibrin (large arrow) and platelets, a few erythrocytes (small arrow) and leukocytes (bended arrow). May-Grünwald-Giemsa stain. Original magnification, ×270. Bottom panel: Cross section of middle cerebral artery and embolus occluding its trunk (arrow). Due to technical artefact during tissue preparation, the embolus is not occupying the whole luminal space, as it probably did in vivo. Hematoxylin-eosin stain. Original magnification, ×125.
immediately after embolization were excluded. The emboli (Figure 3, top panel) were heterogenous in size and shape, although the majority were elongated, irregularly outlined, less than 0.2 mm in cross diameter, and resembled arterial "white" thrombi, rich in platelets and fibrin, intermingled with a few erythrocytes and leukocytes; emboli were present in many brain arteries (Figure 3, bottom panel). Rats embolized with the larger dose of emboli (Table 2) had larger CBF reduction after embolization ($p=0.0025$, Mann-Whitney test). There was no difference in CBF reduction between treated and control rats. In all rats CBF reduction after embolization was between 43% and 94% (total range) of initial CBF, except for one animal in the control group embolized with 200 $\mu$l of emboli with a CBF reduction of only 15% (this animal was the only control animal without infarction).

**Effect of Thrombolytic Therapy**

The main results are displayed in Table 2. There was no difference in the occlusion rate of 22 treated and 24 control rats comparing the first angiograms according to the scale described in "Materials and Methods." Only slight or nonsignificant spontaneous recanalization appeared in control animals, but highly significant recanalization was achieved by the thrombolytic therapy ($p=0.0005$ in 21 treated animals) assessed by comparing the pretreatment and posttreatment angiograms of each animal. Comparing the posttreatment angiograms, the patency rate was significantly higher in the rt-PA treated groups than in the control groups ($p=0.036$, Mann-Whitney test).

Recovering from anesthesia, the damaged animals were lethargic, showing asymmetrical posture, flexion of the forelimb contralateral to embolization, circling, tilting of the head, and pallor of the eyeball. In a few animals, episodes of motor seizures were observed. The most severely damaged animals were unable to take fluid and food in the postoperative period. As seen in Table 2, the treatment increased the survival rate (from 0.48 in all controls to 0.96 in all treated animals; $p=0.0004$, Fisher's exact test). Animals of the control groups had severe clinical damage and obtained a high Bederson score even 4-6 days after embolization or died. The treated rats had a much better clinical recovery than control rats, comparing Bederson scores at day 5 ($p<0.00005$, Mann-Whitney test) (dead animals were not included in the calculation). All deaths were observed in the first 48 hours after ischemic event.

Neuropathological examination showed infarction in all rats except in three treated and one control rat. The right hemisphere ipsilateral to embolization was affected with loss of all cell types (neurons, astrocytes, oligodendrocytes, and endothelial cells) and presence of numerous macrophages, often with distended cytoplasm. There were no signs of inflammation. All infarcts (Table 2) were anemic. Some of the infarcts were irregularly shaped, and almost all infarcts were clearly demarcated with a neuropathological borderline of only 5–10 cells in width, with sporadic neurons with eosinophilic cytoplasm. In all animals with infarction, the MCA area was involved without exception, the region of the posterior cerebral artery was infarcted with some variability, and the anterior cerebral artery area was never infarcted. Brains of six dead control rats
were lost because of technical reasons (mostly, the animals were found late after death with autolyzed brains), and their hemisphere volume and infarct size values were substituted by medians of the same values obtained of all other dying animals from the same group. The treatment reduced the infarct size from 43.7% of hemisphere volume to 11.3% \( (p=0.016) \) in the group embolized with 200 \( \mu \)l emboli suspension and from 66% to 27.5% \( (p=0.0006) \) in the group embolized with 250 \( \mu \)l emboli suspension (comparing all 23 treated and 27 control rats, the significance rose to \( p=0.0002 \), all by Mann-Whitney tests). Treatment reduced edema formation in the affected hemisphere: 23 treated rats with a median embolized hemisphere volume of 265 mm\(^3\) were compared with 26 control rats with a median embolized hemisphere volume of 399 mm\(^3\) \( (p=0.0001, \text{Mann-Whitney test}) \). If no substitution of infarct sizes and embolized hemisphere volumes of dying animals was performed (values shown in Table 2), both the median infarct size and the significance of the rt-PA-mediated reduction of infarct size were reduced in the 200-\( \mu \)l embolized control group because some of the presumed most heavily damaged and dead rats from that group were not included in the calculation. The significance of the difference between infarct volumes of all 21 control (median infarct volume, 51.1%) and all 23 treated (median infarct volume, 24.4%) rats decreased to \( p=0.007 \).

Twenty-eight rats that on the second angiography had patent arteries or only MCA branch occlusion developed smaller infarcts than 19 rats with more severe occlusion \( (p=0.0099, \text{Mann-Whitney test}) \). The overall correlation between the clinical score at day 5 and the infarct volume of the 35 surviving rats \( (p=0.0096) \) and among surviving rats in the treated groups \( (p=0.003; \text{both Spearman tests}) \). The body weight reduction (median 10.2%) correlated with the infarct volume \( (p=0.0027, \text{Spearman test}) \). The total range of the volume of the affected hemisphere in rats that died was larger \( (378-429 \text{ mm}^3) \) than in survivors \( (189-319 \text{ mm}^3) \) \( (p<0.00005, \text{Mann-Whitney test}) \).

**Discussion**

Our model demonstrates reduction of mortality by thrombolytic therapy with rt-PA in an acute embolic stroke model. The model documents arterial reperfusion resulting in a reduction of infarct volume as a direct effect of thrombolytic treatment. The embolization is caused by thrombotic clots resembling natural arterial thrombi.

Doses of rt-PA in the range of 1–5 mg/kg in rabbit models and 1.2–1.5 mg/kg in rat models have been reported to reperfuse occluded cerebral arteries, proven by in vivo\(^{12-18}\) and/or postmortem\(^{19}\) angiography, investigation of vessels at autopsy,\(^{20}\) autoradiography,\(^{21}\) clearance of radioactivity from hemisphere embolized with radiolabeled clots,\(^{22}\) and CBF measurements,\(^{12,19,23}\) although in some of these experiments usage of whole-blood clots with a high tendency to spontaneous recanalization\(^{7}\) could have lead to false conclusions in cases of insufficient inclusion of nontreated control animals. Recombinant t-PA in a dose of 0.3 mg/kg body weight infused over 30 minutes failed to reconstitute embolized occluded intracranial arteries in a rat model.\(^{21}\) We used a higher dose of rt-PA than previously reported by others because rt-PA in rats probably has only 10% (in rabbits, approximately 60%) of the efficacy of that in humans.\(^{24,25}\) This placed our doses in the range of those used in humans, ruling out the possibility that insufficient reperfusion and no limitation of infarction were caused by insufficient dosage regimen of rt-PA.

The conclusions from the present study are that an increase of the volume of emboli resulted in angiographically verified occlusion of larger arteries and more pronounced CBF reduction after embolization. Thrombolytic therapy reperfused occluded arteries, and did so with more ease when the volume of emboli and rate of angiographic occlusion were small.

The efficacy of thrombolytic therapy in stroke in terms of limitation of infarct extent presently lacks sufficient documentation. In animal models, neuropathologic microscopic examination of brain cells and tissue with volumetric measurement of infarct sizes, which may be considered one of the most reliable parameters, was done by Zivin et al\(^{26-27}\) and Phillips et al,\(^{15}\) who found no difference in infarct volume between thrombolytic-treated and control animals. In the studies of Benes et al,\(^{20}\) Chehrazi et al,\(^{13}\) and Bednar et al,\(^{23}\) possible infracts were demonstrated by very early 2,3,5-triphenyltetrazolium chloride staining, which early after the ischemic event is not a reliable parameter of loss of neurons but might indicate transient damage.\(^{28,29}\) These problems have been solved in the present model.\(^{7}\)

A number of papers demonstrate improvement in different neurophysiological parameters such as neurologic clinical score,\(^{16,26,27}\) decreased ratio of inorganic phosphate/phosphocreatine measured by \( ^{31}\text{P} \) spectroscopy,\(^{20}\) decreased lactate, pyruvate and water content of tissue, improved electrocorticogram\(^{19}\) after rt-PA administered in doses in the range of 1–5 mg/kg starting up to 4 hours after embolization in rabbit models\(^{12-18,22,23,26,27,30,19}\) and 1.5–2 mg/kg starting up to 2 hours after embolization in rat models.\(^{19,22}\)

In the animals treated with rt-PA, apart from slight oozing of blood during and shortly after operation, we observed no hemorrhagic complications. Because this phenomenon was rare in thrombolytic-treated as well as in controls, this model is probably not sensitive to hemorrhagic transformation of infarction. This is in accordance with other investigators,\(^{18,31}\) even when such a large dose of rt-PA was administered. Whether longer delay between embolization and rt-PA infusion will result in hemorrhagic complications remains to be investigated.

We found in all animals that died before sacrifice a massive enlargement of the injured hemisphere with shift of the midline, and we considered that the animals died of mass effect due to edema.

Our findings that animals with small occlusion or no occlusion on the posttreatment angiography had smaller
infarcts than the animals with more pronounced occlusion and the fact that treated animals with complete recanalization were almost free of infarction supports the hypothesis that early reperfusion leads to limitation of the infarct size. If the emboli had been easier to lyse, a larger proportion of the animals might have recanalized completely with minimal brain damage. Our conclusions from the present study are that the thrombolytic therapy is both life saving and infarct reducing. The reduction of mortality was more pronounced in the heavily embolized animals, and we proved an infarct-limiting effect of the thrombolytic therapy that was more pronounced in the animals embolized with a smaller volume of emboli.

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References
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