Influence of Tissue Plasminogen Activator and Heparin on Cerebral Ischemia in a Rabbit Model

L.P. Carter, MD; A.N. Guthkelch, FRCS; J. Orozco, MD; and O. Temeltas, MD

Background and Purpose: The aims of this study were to verify that tissue-type plasminogen activator given either 1 or 2 hours after experimental embolic stroke in rabbits diminishes the volume of resulting ischemic brain and to ascertain the effect of the simultaneous administration of heparin.

Methods: We embolized the middle cerebral artery of rabbits by injecting preformed autologous arterial ("white") thrombus into one internal carotid artery. Treatment with 2 mg/kg tissue-type plasminogen activator, alone or in combination with heparin, was commenced either 1 or 2 hours after embolization. The animals were killed 5 hours after treatment commenced, and their brains were examined for evidence of ischemia and hemorrhage.

Results: Administration of tissue-type plasminogen activator significantly diminished the size of the resulting brain ischemia. Administration of heparin, with or instead of tissue-type plasminogen activator, did not result in a significant decrease in the volume of cerebral ischemia, but it also did not lead to hemorrhagic transformation of the stroke.

Conclusions: In the rabbit model, administration of tissue-type plasminogen activator within 2 hours diminished the volume of brain rendered acutely ischemic by embolic stroke. Since the simultaneous administration of heparin during this same period did not result in any instances of hemorrhagic transformation, tissue-type plasminogen activator may have some place for use in such circumstances to mitigate a tendency to further embolic or thrombotic events. (Stroke 1992;23:883-888)

KEY WORDS • cerebral ischemia • heparin • plasminogen activator, tissue-type • rabbits

The management of cerebral embolism remains a major problem in occlusive cerebrovascular disease. An intriguing form of therapy is to dissolve the embolus using thrombolytic agents, with the aim of achieving reperfusion of the devascularized brain tissue distal to the site of obstruction. There have been anecdotal reports of success in humans, though not without occasional serious complications. The preliminary results of an open, multicenter trial suggest that tissue-type plasminogen activator (t-PA) therapy can often result in partial, and occasionally in complete, recanalization of the blocked vessel.

To investigate in a systematic fashion the possibilities of thrombolytic therapy, several animal models have been developed in which the middle cerebral artery (MCA) is embolized by the injection of one or more fragments of thrombus either directly into the surgically exposed internal carotid artery (ICA) in rabbits or rats or indirectly via the common femoral artery.

The thrombolytic agents studied include streptokinase, urokinase, t-PA, and Fb-Fb-CF, an analogue of t-PA prepared by recombinant techniques. However, streptokinase proved ineffective in causing clot lysis in the cerebral arteries of a rabbit model, whether given immediately or 3 hours after embolization. Moreover, the use of either streptokinase or urokinase in the treatment of acute cerebral infarction in humans was sometimes followed by the development of hematoma intracranially or elsewhere, as well as being without demonstrable therapeutic benefit. Unlike streptokinase and urokinase, t-PA does not cause systemic anticoagulation because its affinity for plasminogen is high only when both molecules are bound to fibrin. Therefore, t-PA may be less likely to cause hemorrhagic complications if used in the treatment of acute occlusive stroke.

Zivin et al originally showed that intravenous t-PA was effective in causing thrombolysis in the main arteries of a rabbit's brain if given immediately after cerebral embolism had been produced by the injection of multiple small blood clots into the ICA. Other investigators have since employed either a rabbit or a rat model, administering t-PA or an analogue at varying intervals after embolization of the MCA with either single or multiple thrombi of various sizes. It appears that thrombolysis can be achieved in rabbits which have received t-PA more than 2 hours after cerebral embolism. However, the thrombi used in these studies were in most instances less than 24 hours old.
and all were "red" thrombi prepared by preclotting autologous or heterologous venous blood. In the study reported here, which in many respects follows the methods reported by Benes et al.\(^1\) and Rigamonte et al.,\(^2\) the emboli were prepared from white thrombus at least 2 days old since this is the embolic material that is regularly found in human cerebral embolism. However, unlike the two last-mentioned groups, we administered t-PA by intravenous infusion rather than by direct intracarotid injection since direct intra-arterial therapy in humans\(^3\) requires special expertise and equipment.

In addition, some of our animals received heparin as well as t-PA because in clinical practice patients who receive thrombolytic agents for either coronary or cerebral artery occlusion often are given an anticoagulant so as to diminish the danger of spread of thrombosis.

The specific aims of the study were 1) to confirm that the administration of t-PA to animals suffering cerebral embolism due to 2-day-old white thrombus resulted in smaller volumes of ischemic brain than in control and 2) to investigate the effects of administering an anticoagulant simultaneously with a thrombolytic agent as a prophylaxis against the occurrence of new ischemic events. We hypothesized that, on the one hand, this combination might further diminish the amount of brain tissue showing signs of ischemia but, on the other hand, it might increase the liability to hemorrhagic transformation of the stroke or to intraparenchymal hemorrhage. Only one publication so far has addressed this issue in experimental animals.\(^2\)\(^5\)

**Materials and Methods**

The experimental protocol was approved by the University of Arizona Institutional Animal Care and Use Committee (control no. 90-0075). A total of 35 New Zealand White rabbits of either sex and weighing about 3 kg were used and randomized into groups of five, this being judged likely, on the basis of some preliminary observations, to be the minimum number required to give statistically valid results.

Emboli were produced as in the Barrow Neurological Institute model.\(^1\)\(^2\)\(^3\) Under short-term intravenous anesthesia comprising a mixture of ketamine, xylazine, and acetylpromazine, the central artery of the pinna was punctured using an 18-gauge needle 1.5 cm long. Through this was introduced into the arterial lumen the stylet of a 22-gauge spinal needle 10 cm long, the tip of which had been roughened and slightly bent. The tip of this stylet was advanced until it projected beyond the tip of the needle and was then gently rotated several times so as to scarify a 2-cm length of the intima of the central artery of the pinna. The stylet and needle were then withdrawn. A ligature was passed percutaneously around the artery just proximal to the needle puncture and was tied sufficiently tightly to reduce the blood flow within the artery by about 50%. The rabbits were returned to their cages and given routine care for the next 2 days. Thrombosis of the artery was signaled by the appearance of swelling and induration of its wall distal to the needle puncture, with discoloration of the overlying skin.

Two days later the animals were anesthetized again using 1 ml/kg of a tranquilizing mixture containing 1 mg/ml acetylpromazine and 10 mg/ml xylazine. Ketamine was not used because it is an N-methyl-D-aspartate receptor antagonist and is known to alter cerebral oxygen requirements. The rabbits were then placed on a heating blanket, intubated, and maintained on oxygen supplemented with up to 2% halothane if required during actual surgery. Monitoring of the electroencephalogram (EEG) was commenced using needle electrodes introduced in the frontal and occipital regions on either side of the scalp and a ground electrode at the base of the nose. The femoral artery was exposed below the inguinal ligament and catheterized with a 19-gauge plastic catheter so as to monitor the mean arterial pressure (MAP) throughout the experiment; MAP was maintained at about 65 torr by adjusting the concentration of inspired halothane and the rate of intravenous infusion. Pressor agents were not used. The PCO\(_2\) was maintained between 30 and 40 torr and the rectal temperature at 38±0.5°C. The partial thromboplastin time (PTT) was measured by the fibrometer method at the beginning of the surgery, at the time of embo
dization, and at 2-hour intervals thereafter.

The thrombosed segment of the central artery of the pinna was excised. The lumen of the artery was opened, and a piece of thrombus was extracted and trimmed under a dissecting microscope to exactly 0.7 mm long and 0.5 mm in diameter. The thrombus was then suspended in Dulbecco's phosphate buffered saline and drawn up into a transparent 19-gauge plastic catheter attached to a 1-ml syringe.

The right internal jugular vein and right carotid arterial system were exposed through a vertical incision midway between the larynx and the angle of the mandible. The internal jugular vein was cannulated, and a saline infusion was commenced. The branches of the external carotid artery were identified and controlled by clips or ligatures. (The origin of the ICA is more variable in rabbits than in humans. Occasionally the occipital artery originates from the common carotid artery proximal to the ICA, but the ICA can of course be distinguished because it has no branches in the neck.) Temporary clips were applied to the common carotid artery and ICA. The termination of the external carotid artery was opened, and the tip of the catheter containing the embolus was inserted and passed in a retrograde fashion to the point of origin of the ICA, where it was secured by two ligatures passed around the external carotid artery and by a suture at skin level. The temporary clip on the ICA was removed, and the embolus was injected. As soon as the embolus had disappeared up the ICA, the clip on the common carotid artery was removed so as to restore normal intracerebral circulation. The catheter in the external carotid artery was left in place for the duration of the experiment and was used occasionally to check that carotid pressure was comparable with femoral artery pressure as the experiment proceeded. The skin incision was closed with a few silk sutures.

From this point on halothane administration was discontinued, the rabbits breathing room air. A second 1 ml/kg dose of the tranquilizing mixture was given, followed by further doses at approximately 90-minute intervals as necessary. Blood pressure, heart rate, respiratory rate, rectal temperature, and EEG continued to be monitored regularly. Blood gases were sampled at
least three times during every experiment, and assisted ventilation was resumed if the PCO₂ rose above 40 torr. The first 20 rabbits were randomized into one of four treatment groups, of which the first received only intravenous saline via the internal jugular vein. The second group received an intravenous bolus of 200 mg/kg heparin 1 hour after embolization followed by a 200 mg/kg/hr infusion until the time of sacrifice. The third group received 2 mg/kg t-PA intravenously commencing 1 hour after embolization, the injection lasting 30 minutes. The fourth group received both heparin and t-PA.

Four of these animals were studied by nuclear magnetic resonance (NMR) imaging, scans being taken every 20 minutes from the time of embolization until sacrifice. Two of these rabbits received only saline, one received t-PA, and one received both t-PA and heparin. The remaining 15 animals were randomized to receive heparin, t-PA, or both as above, but administration was not begun until 2 hours after embolization. All rabbits were killed 5 hours after commencement of treatment. Their brains were immediately removed and immersed for 35 minutes in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) at 38°C so as to delineate any areas of ischemic brain. (TTC stains normal brain tissue deep crimson but is not taken up by ischemic tissue.) The brains were then taken out of the TTC solution, photographed, and immersed in ice-cold saline for a few minutes to render them sufficiently firm for cutting. Serial coronal slices 2 mm thick were cut using a specially designed rabbit brain slicer. The slices were immersed again in 2% TTC solution for a further 35 minutes and photographed (Figure 1).

A planimeter was used to measure the unstained area of ischemia on each slice, the volume of ischemic brain being computed from these areas together with the thickness of the slices on the basis of a conic section model. Finally, representative microscopic sections were cut from each slice, stained with hematoxylin and eosin, and examined microscopically. A search was made, by an observer unfamiliar with the treatment given or the planimetric estimations, for vacuolization and neuronal hyperchromatism as indicators of early infarction. The severity of each indicator, graded arbitrarily from 0 to 4, was assessed on a section taken from each brain slice. Because in practice no infarct extended beyond four slices, the minimum score was 0 and the maximum score was 32. Evidence of hemorrhagic change was also sought.

Results

The mean PTT of all the rabbits, measured prior to surgery, was 31.3 seconds, which agrees well with the reported value for this species (31.0 seconds).26 In those animals that received heparin, with or without t-PA, the PTT was maintained at >300 seconds until they were killed. Administration of t-PA had no influence on subsequent PTT values (p=0.876), nor did statistical analysis using step-wise regression show any relation between the pretreatment PTT and the eventual volume of brain ischemia, whatever treatment was given (R²=0.006, p=0.782).

Passage of the embolus from the plastic catheter into the ICA was directly observed in almost every case and was often accompanied by slowing or flattening of the EEG on the side of embolization. Of the rabbits studied by NMR, changes suggestive of early stroke became visible about 2 hours after embolization in both those receiving only saline, but not in the two animals that had received t-PA 1 hour after embolization.

The total volumes of ischemic brain in each rabbit varied from 45 to 3,003 mm³ (Figure 2). Table 1
TABLE 1. Study Design, Showing Mean±SD Volume of Ischemic Brain for Each Treatment Group

<table>
<thead>
<tr>
<th>Tissue-type plasminogen activator</th>
<th>Heparin</th>
<th>After 1 hour</th>
<th>After 2 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1,500.9±1,123.7</td>
<td>313.3±277.3</td>
<td>758.2±402.4</td>
</tr>
<tr>
<td>After 1 hour</td>
<td>1,200.4±754.4</td>
<td>172.6±178.3</td>
<td>-</td>
</tr>
<tr>
<td>After 2 hours</td>
<td>763.5±834.6</td>
<td>-</td>
<td>429.6±495.0</td>
</tr>
</tbody>
</table>

n=5 rabbits in all nonvacant cells.

indicates the design of the study and shows the mean±SD volume for each treatment group, but it can be seen that the SDs are large and roughly proportional to the means, indicating skewed and heteroskedastic distributions of volume of ischemia within the groups. These problems were controlled by taking the logarithm of the volume of ischemic tissue (logvol) (Figure 3), thus reducing the relative magnitudes of the SDs. Table 2 shows the mean±SD logvol for each treatment group.

Analysis of variance (Table 3) shows the single-degree-of-freedom a priori contrasts within the overall analysis of logvol. Compared with the saline-treated controls, there was a significant decrease in the mean logvol of ischemic brain in the rabbits that received t-PA (p=0.008) but no significant decrease in those that received heparin (p=0.094). There was no further significant decrease in the logvol of ischemic brain when heparin was added to t-PA treatment (p=0.680), nor were the ischemic changes significantly greater in the animals in which t-PA treatment was commenced 2 hours rather than 1 hour after embolization (p=0.080).

Histological examination of microscopic sections taken from the serial 2-mm slices of the rabbits' embolized cerebral hemispheres showed areas of vaculization and neuronal hyperchromatism corresponding in distribution to the areas that had been left unstained by TTC. No areas of hemorrhage were seen in any specimen. The extent and severity of the histological changes correlated quite well with the volumes of ischemic brain as measured by planimetry of the TTC-stained sections (r=0.6796).

Discussion

In patients the source of a cerebral embolus is most frequently thrombus adhering to an atherosclerotic lesion occurring in either the heart or the carotid arterial system.27 Thus, although the use of arterial thrombus in our model somewhat complicated the experimental procedure, we, like others,6,23 considered this to be acceptable because the resulting emboli more closely resembled those found in humans than if we had employed emboli prepared from clotted venous blood. However, whereas Benes et al6 injected t-PA directly into the previously embolized ICA, which presumably produced a very high concentration of t-PA at the site of embolism, we gave t-PA by the intravenous route, on the basis that the highly sophisticated interventional neuroradiological techniques for thrombolysis, such as those described by Zeumer et al24 are unlikely to be available for most stroke victims in the immediate future.

In fact, 2 mg/kg t-PA given over 30 minutes by intravenous injection commencing either 1 or 2 hours after embolization was effective in reducing the volume of ischemic brain in our rabbit MCA embolism model.

TABLE 2. Study Design, Showing Mean±SD Logarithm of Volume of Ischemic Brain for Each Treatment Group

<table>
<thead>
<tr>
<th>Heparin</th>
<th>Tissue-type plasminogen activator</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.0010±0.4991</td>
</tr>
<tr>
<td>After 1 hour</td>
<td>2.8921±0.5969</td>
</tr>
<tr>
<td>After 2 hours</td>
<td>2.6236±0.5517</td>
</tr>
</tbody>
</table>

n=5 rabbits in all nonvacant cells.

TABLE 3. Analysis of Variance Showing Single-Degree-of-Freedom Contrasts for Logarithms of Volumes of Ischemic Brain in Rabbits

<table>
<thead>
<tr>
<th>Factor</th>
<th>F ratio</th>
<th>Significance of F</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-PA</td>
<td>8.05</td>
<td>0.008</td>
</tr>
<tr>
<td>Heparin</td>
<td>3.01</td>
<td>0.094</td>
</tr>
<tr>
<td>t-PA×heparin</td>
<td>0.17</td>
<td>0.680</td>
</tr>
<tr>
<td>Interval×t-PA</td>
<td>3.29</td>
<td>0.080</td>
</tr>
<tr>
<td>Interval×heparin</td>
<td>0.01</td>
<td>0.935</td>
</tr>
<tr>
<td>Interval×t-PA×heparin</td>
<td>0.39</td>
<td>0.540</td>
</tr>
</tbody>
</table>

t-PA, tissue-type plasminogen activator.
Chehrazi et al\textsuperscript{22} found that the radiographic image of a tin-tagged embolus disappeared and the volume of ischemic brain was significantly reduced when their animals received a 0.5 mg bolus of t-PA followed by a 1 mg/kg/hr infusion over 2 hours, treatment commencing 30 minutes, 2 hours, or 4 hours after embolization, but there is a trend in their data that suggests that the earlier the t-PA administration was commenced, the smaller the resulting infarct. With higher doses of t-PA (3 mg or 5 mg/kg), thrombolysis occurred when t-PA was administered 8 or even 24 hours after embolization, but neurological damage was not prevented.\textsuperscript{8} Reperfusion of the embolized, ischemic rabbit brain was significantly hastened by giving 0.8 mg/kg of the t-PA analogue Fb-Fb-CF 90 minutes after embolization,\textsuperscript{13} and similar results have been obtained in a rat model.\textsuperscript{10} Recently Kaplan et al\textsuperscript{13} reported that in rats simultaneously occlusion of the MCA and common carotid artery for more than 3-4 hours results in no smaller a volume of focal ischemia than when the occlusion is continued for 24 hours, suggesting that after 3-4 hours "recirculation or pharmacological interventions will provide little benefit."

The absence of hemorrhagic change in the ischemic areas of the brains of our rabbits accords with the findings of Benes et al\textsuperscript{6} (using rabbits treated with a 1 mg t-PA bolus 30 minutes after embolization, then 1 mg/kg/hr infusion for 2 hours and killed 6 hours later) and of Chehrazi et al\textsuperscript{22} (dosage as above and killed immediately after completion of treatment), as well of Slivka and Pulsinelli\textsuperscript{29} (whose animals survived 30 hours after receiving a single bolus of streptokinase 1 hour postembolization). These latter authors also found that hemorrhagic transformation of the stroke was common when treatment was delayed for 24 hours with 7-9 hours' subsequent survival. Del Zoppo et al\textsuperscript{29} working with baboons, found no increased liability to intracerebral hemorrhage in treated animals over controls when t-PA treatment was given after 3 hours of MCA occlusion followed by 30 minutes of reperfusion. On the other hand, Lyden et al\textsuperscript{8} found intracerebral hemorrhages in rabbits receiving 3-5 mg/kg t-PA over 30 minutes, whether this was commenced at 10 minutes, 8 hours, or 24 hours after embolization, while Phillips et al\textsuperscript{14} saw hemorrhagic transformation of strokes in rabbits receiving a bolus of 0.8 mg/kg t-PA analogue either 15 or 90 minutes after embolization. These findings leave it uncertain to what extent the total dose of t-PA, its speed of administration, and the length of subsequent survival contribute to the finding of hemorrhagic change within the infarct of animals treated with thrombolytic agents.

In the clinical situation the underlying causes of thromboembolic stroke often remain operative for an indefinite period after an acute stroke has occurred, so that it may be desirable to institute anticoagulant therapy. Our results do not show any immediate statistically significant advantage for simultaneous heparin therapy in terms of the ultimate volume of ischemic brain. The absence of evidence of intracranial hemorrhages, however, suggests that the administration of an anticoagulant simultaneously with a thrombolytic agent, soon after the occurrence of a stroke, does not increase the danger of this complication, at least in a rabbit model. This result confirms the recent observation of Clark et al\textsuperscript{22} that the simultaneous administration of heparin and t-PA 90 minutes postembolization did not increase the incidence of hemorrhage in a rabbit cerebral embolus model in which the animals survived 24 hours. At present it seems possible that a lapse of 3-4 hours represents the period during which thrombolytic therapy is likely to be effective in reducing the extent of eventual cerebral ischemia after an occlusive stroke, \textsuperscript{26} but further studies are needed to define the time limits within which there is no increased risk of hemorrhagic transformation of such a stroke following thrombolytic therapy with or without anticoagulation.

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