Safety Profile of Transcranial Near-Infrared Laser Therapy Administered in Combination With Thrombolytic Therapy to Embolized Rabbits

Paul A. Lapchak, PhD; Moon-Ku Han, MD; Karmen F. Salgado, BSc; Jackson Streeter, MD; Justin A. Zivin, MD, PhD

Background and Purpose—Transcranial near-infrared laser therapy (TLT) is currently under investigation in a pivotal clinical trial that excludes thrombolytic therapy. To determine if combining tissue plasminogen activator (tPA; Alteplase) and TLT is safe, this study assessed the safety profile of TLT administered alone and in combination with Alteplase. The purpose for this study is to determine if the combination of TLT and thrombolysis should be investigated further in a human clinical trial.

Methods—We determined whether postembolization treatment with TLT in the absence or presence of tPA would affect measures of hemorrhage or survival after large clot embolism-induced strokes in New Zealand white rabbits.

Results—TLT did not significantly alter hemorrhage incidence afterembolization, but there was a trend for a modest reduction of hemorrhage volume (by 65%) in the TLT-treated group compared with controls. Intravenous administration of tPA, using an optimized dosing regimen, significantly increased hemorrhage incidence by 160%. The tPA-induced increase in hemorrhage incidence was not significantly affected by TLT, although there was a 30% decrease in hemorrhage incidence in combination-treated rabbits. There was no effect of TLT on hemorrhage volume measured in tPA-treated rabbits and no effect of any treatment on 24-hour survival rate.

Conclusion—In the embolism model, TLT administration did not affect the tPA-induced increase in hemorrhage incidence. TLT may be administered safely either alone or in combination with tPA because neither treatment affected hemorrhage incidence or volume. Our results support the study of TLT in combination with Alteplase in patients with stroke. (Stroke. 2008;39:3073-3078.)

Key Words: intracerebral hemorrhage ■ ischemic stroke ■ neuroprotection ■ tissue plasminogen activator ■ transcranial laser therapy

Transcranial laser therapy (TLT) involves photobiostimulation and the subsequent biological effects of infrared laser therapy, which are wavelength-specific but are not due to thermal effects of TLT.1-3 Photon energy in the 808 nm region of the electromagnetic spectrum is nonionizing and, therefore, poses none of the hazards associated with ultraviolet light. However, it has been demonstrated that irradiation of specific infrared wavelengths is able to penetrate deeply into the brain.4 Possible mechanisms of action involved in neuroprotection after photobiostimulation are increases adenosine triphosphate formation after energy absorption by mitochondrial chromophores such as cytochrome C oxidase.5,6 An enzyme complex that plays a central role in the bioenergetics of cells by delivering protons across the inner mitochondrial membrane, thereby driving the formation of adenosine triphosphate by oxidative phosphorylation. Currently, the putative mechanism for TLT in stroke involves the stimulation of mitochondria, which then leads to preservation of tissue in the ischemic penumbra and enhanced neurorecovery.

The clinical development of TLT was initiated based on the results from preclinical development studies in rodents and rabbits.7-9 The rodent studies using a rat middle cerebral artery occlusion model showed that laser therapy administered 24 hours after the start of occlusion resulted in significant behavioral improvement measured 28 days after the stroke,10 an effect of TLT that may be independent of neuronal restoration or survival. In addition, rabbit studies showed that TLT could improve behavioral performance for up to 21 days after embolization if treatment was initiated up to 6 hours after a stroke.8,9 Thus, preclinical studies in 2 species using 2 methods of ischemia induction established that TLT could significantly improve behavioral outcome.

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The TLT device, a 808 nm Class IV laser, was recently evaluated in a Phase II clinical study using a laser probe that was applied to 20 points on the human skull for 2 minutes at each position. Because the study used a power density of 10 mW/cm², an estimated energy density of 1.2 J/cm² was delivered to the brain. The prospective, double-blind, randomized Neurothera Effectiveness and Safety Trial (NEST-1) trial enrolled within 24 hours of ischemic stroke onset over 120 patients presenting with National Institutes of Health Stroke Scale scores of 7 to 22. Both the National Institutes of Health Stroke Scale and the modified Rankin Scale showed a greater percentage of patients in the TLT treatment group with successful outcomes compared with controls. The main conclusion of the study was that TLT showed reasonable safety and sufficient effectiveness for a further, definitive trial.

A pivotal Phase III multicenter clinical trial, NEST-2, is currently underway. This is an acute ischemic stroke study within 24 hours of stroke onset and excludes patients who have received thrombolytic therapy and patients with evidence of intracranial hemorrhage (ICH). These 2 patient populations have been excluded because it is uncertain whether there would be an adverse effect of combining TLT with thrombolytic therapy or whether TLT would exacerbate ICHs after thrombolytic treatment.

Before the use of TLT in combination with a thrombolytic such as tissue plasminogen activator (tPA), the only US Food and Drug Administration-approved treatment for patients with acute ischemia patients who present within 3 hours of an ischemic stroke, it is necessary to determine if combination therapy will affect outcome measures of embolism-induced hemorrhage and survival. To evaluate the safety profile of the combination therapy, we used the rabbit large clot embolic stroke model, an animal model of middle cerebral artery occlusion that is sensitive to intravenously induced hemorrhage and survival. To address the effects of TLT on both thrombolytic-induced hemorrhage rate and on hemorrhage volume, we adopted a design in which the thrombolytic would be administered 60 minutes after embolization and then TLT would be applied subsequent to thrombolytic treatment. Our results show that TLT and tPA may be a safe combination therapy that should be further studied in a clinical trial setting.

Materials and Methods

Animals and Animal Welfare

Male New Zealand white rabbits weighing 2 to 2.5 kg were purchased from Rabbit Source, Ramona, Calif. Rabbits were supplied food (alfalfa cubes) and water ad libitum while under quarantine in an enriched environment for at least 5 days before experimental use. Surgery was done in a sterile controlled environment with a room temperature between 22.8°C and 23.2°C. All surgical, embolization, and histological procedures were done as described previously. The Department of Veterans Affairs and Institutional Animal Care and Use Committee approved the surgical and treatment procedures used in this study. Extreme care was used throughout the study to minimize pain and discomfort. Per the Institutional Animal Care and Use Committee-approved protocol, rabbits were euthanized if they were in pain, showed extreme discomfort, or if they were unable to reach food or water.

Surgery and Embolization

Rabbits were anesthetized with isoflurane through a face mask, 5% in 3 L/min at induction, and 3% in 3 L/min as a maintenance dose. The right internal carotid artery was exposed, and the external carotid artery and the common carotid artery were ligated. If any other branches were seen originating from the internal carotid artery, these were also ligated. A Becton, Dickinson and Company plastic catheter oriented toward the brain was inserted into the common carotid and secured with ligatures. The incision was closed around the catheter so that the distal end was accessible outside. The catheter was filled with 0.2 mL of heparinized saline (33 U/mL) and plugged with injection caps. The animals were allowed to recover from anesthesia for at least 2 hours before embolization.

Emboli were prepared by withdrawing 2 to 4 mL of arterial blood from a single donor rabbit. Because the studies had a 24-hour end point, there was no concern for immunogenicity of blood clots prepared from a single donor rabbit that were subsequently used in the experiment for a group of rabbits on a particular experiment day. The blood was mixed with a trace quantity of 57Co-labeled plastic NEN-Trac microspheres (New England Nuclear Inc) and allowed to clot for at least 3 hours at 39°C. The clot was cut into small cubes weighing approximately 2.5 to 3.5 mg and they were suspended in phosphate-buffered saline containing 0.1% bovine serum albumin. The amount of radiolabel present in each blood clot was measured using a mobile gamma counter. Before embolization, each animal was placed in a Plexiglas restrainer and the injection cap of the catheter was removed to allow the rabbit’s blood to fill the catheter and wash out the heparinized saline. The line was then filled with heparin-free normal saline. One of the clot cubes was placed inside the injection port of the catheter and rapidly injected with 3 mL of saline flush followed immediately by a 5-mL flush. Care was taken during both flushes to be sure that no air bubbles were present in the catheter or syringe. If the animal did not have a behavioral stroke reaction, which could include nystagmus, kicking, rolling, loss of balance, hemiparesis, seizure, or coma, then another blood clot was injected in the same way 3 minutes after the first embolization. If there was no behavioral reaction to either embolization, no further blood clots were administered. After the embolization process was completed, the catheter was ligated close to the neck and the rest of the catheter and injection port were heat-sealed. Animals that died before euthanasia but received treatment(s) were included in this study; the brains were fixed and sectioned as described subsequently. The surviving animals were euthanized 24 hours postembolization with 1 to 1.5 mL of Beuthanasia-D through the marginal ear vein. The brains were removed and immersion fixed in 4% paraformaldehyde for at least 1 week and then examined by a blinded observer.

Intracerebral Hemorrhage and Radioactivity Quantitation

The brain was cut into 10 coronal slices, each having 2 faces. We noted the presence and type of each hemorrhage and the number of section faces showing hemorrhage. Three major types of ICH can be identified: hemorrhagic infarction consists of red speckling of an area usually surrounded by soft infarcted tissue; punctate hemorrhages are small isolated red marks within tissue that do not extend through the tissue like a blood vessel would; parenchymal hemorrhages are large homogenous masses of blood within tissue. For infarcts, we noted the presence of ischemia showing damage. Infarction is grossly visible as pale, softer tissue surrounded by pink, normal brain tissue on the brain sections. After evaluation of the brain tissue, the total radioactivity in the brain was measured by placing the slices into a portable gamma counter and the amount of radiolabel present in the brain was compared with that contained in the labeled blood clot before embolization. Table 1 presents the fraction of counts received in brain for the 4 treatment groups injected in this study. If fewer than 10% of the total counts were found in the brain, it was assumed that the labeled blood clot had not reached the brain and the animal was excluded from the study.
Table 1. Stroke Reactions and Isotope Recovery*

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Behavioral Reaction at Embolization</th>
<th>Radioactive Counts in Brain Postmortem (% injected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20/23 (86.9%)</td>
<td>35.71±6.94 (23)</td>
</tr>
<tr>
<td>TLT</td>
<td>20/23 (86.9%)</td>
<td>34.38±5.14 (23)</td>
</tr>
<tr>
<td>TLT+IPA</td>
<td>20/20 (100%)</td>
<td>36.74±6.59 (20)</td>
</tr>
</tbody>
</table>

*Behavioral reaction and radioactive count recovery data in the brain for the 4 groups included in the study. Results are presented as percentage population for behavioral reaction and survival rate and mean±SEM (n) for radioactive counts. There were no statistically significant differences between any treatment group and the control group (P>0.05).

Treatment

We randomly allocated catheter-implanted rabbits, all of which had shaved heads, to 4 different treatment groups before the embolization procedure. The rabbit large clot embolic stroke model was primarily used to determine whether TLT in the absence or presence of tPA administration would alter hemorrhage incidence (rate) or volume after embolic strokes in rabbits.

Near-Infrared Transcranial Laser Therapy

Rabbits were placed in a Plexiglas restrainer for the duration of the treatment and the probe was placed in direct contact with the skin overlaying the skull. An Acculaser low-energy laser fitted with an OZ Optics Ltd fiberoptic cable and laser probe measuring 2 cm in diameter was used (wavelength of 808±5 nm).8,9,22,27 Pilot instrument design studies showed that these specifications would allow for near-infrared light penetration of the rabbit skull and brain to a depth of approximately 2.5 to 3 cm and that the light beam would encompass the majority of the brain if placed on the skin surface posterior to bregma on the midline.8 Using the specifications described previously, the average energy delivered to the brain was 0.9 to 1.2 J. In our previous laser studies,8,9 we showed that the laser-induced improvements in behavior were present 48 hours after embolization and were also measurable up to 21 days after treatment when we used a power density in the range of 7.5 to 25 mW/cm2. In the current series of experiments, we used a power density setting of 10 mW/cm2 and treatment duration of 2 minutes to parallel the settings used in the NEST-2 clinical trial. TLT was administered 90 minutes after embolization, which is consistent with a time of administration when TLT effectively improves clinical rating scores in rabbits after small clot embolic strokes.8,9 In our previous studies, we showed that TLT effectively improved behavior when given up to 6 hours after embolization8,9. Moreover, to simulate the clinical setting, TLT would most likely be given to patients with acute ischemia after they have received infusions of tPA.11

Thrombolytic Therapy

For tPA administration, the thrombolytic was infused 60 minutes after embolization using the marginal ear vein as described previously.19,22,26,28 Briefly, tPA was reconstituted with sterile water for injection and the treatment regimen used in this study is as follows: 3.3 mg/kg tPA, 20% as a bolus injection given over 1 minute followed by the remainder infused over 30 minutes.17,19,22,26,28 The 3.3-mg/kg dose of tPA used in this study is consistent with that used in previous rabbit embolic stroke studies and has been shown to improve behavioral function after small clot embolic strokes and also significantly increase hemorrhage rate after large clot embolic strokes.9,26,29 Clinical-grade tPA was either purchased or graciously supplied by Genentech, Inc (South San Francisco, Calif) as a lyophilized cake in 50-mg (or 100-mg) configurations. For 50-mg configurations, vials contained 50 mg tPA (29 million IU), 1.7 mg L-arginine, 0.5 g phosphoric acid, and less than 4 mg Polysorbate 80.

Power Analysis

Power calculations for ICH rate were based on a 2-sided sided χ2 test for detecting a difference between 2 proportions assuming a Type 1 error of 0.05. With a sample size of 20 rabbits in each group (40 overall) and assuming a baseline ICH rate of approximately 20% in the untreated embolized group, we have 80% power to detect a hemorrhage rate of 70% in the treated group. Power analysis for ICH volume indicates that assuming α=0.05 and β=0.90, a coefficient of variation of 15%, and a difference between means of 20% for ICH volume, we would require a sample size of 14 animals per group.

Treatment Groups

In the present study, a full control group was run in parallel to the 3 different treatment groups and all groups were rated simultaneously to preclude any experimental or investigator bias. For all experiments in this study, rabbits were randomly allocated into treatment groups before the embolization procedure with concealment of the randomization guaranteed by using an independent third party (P.A.L.). The randomization sequence was not revealed until all postmortem analyses were complete. The 4 treatment groups were: (1) control embolization group; (2) TLT group (90 minutes postembolization); (3) intravenous Thrombolytic group (60 minutes postembolization); and (4) TLT + intravenous thrombolytic group (90 minutes and 60 minutes postembolization, respectively).

Statistical Analysis

Data were analyzed using MedCalc Version 9.4.1.0 (www.Medcalc.be) for comparison of proportions for hemorrhage and ischemia incidence rates. Behavioral reaction and survival data were analyzed using comparison of proportions. Analysis of variance followed by the t test was used for the analysis of differences among radioactive counts and hemorrhage volume using SigmaStat 3.5.

Results

Stroke Behavioral Manifestation and Radioactivity Recovery

As shown in Table 1, 86.9% to 100% of the rabbits included in the 4 treatment groups had a visible behavioral reaction to blood clot administration. Table 1 also provides the recovery and presence of radioactive counts in the brain after embolization with the blood clots containing tracer quantities of Co57 microspheres. In all groups, 32.85% to 36.74% of administered radioactive counts, which were present in the administered blood clots, were recovered in brain tissue. The remaining isotope and clot did not enter the brain and could be located in the vasculature outside of the brain. There were no statistical differences between counts recovered in the control group compared with the other 3 groups that were run in parallel.

Stroke Ischemia Incidence and Survival Rate

The survival rate of rabbits included in each of the 4 treatment groups was measured 24 hours after embolization. The survival rate was similar in all 4 groups and there were no statistically significant differences between the rates observed for the treatment groups compared with the control group. Table 2 also presents the ischemia incidence resulting from quantitation of brain sections after embolization. The ischemia incidence was similar in all 4 treatment group, and there was no statistically significant difference among the groups.

Effect of Transcranial Near-Infrared Laser Therapy on Hemorrhage Rate and Volume: Combination Studies

In these experiments, TLT was administered for 2 minutes starting 90 minutes postembolization. After removal and

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formalin fixation of the brains, hemorrhage incidence and volume were quantified (Figure; Table 3). We found that in TLT-treated embolized rabbits, the hemorrhage rate was similar to that measured in the control group (Figure). In TLT-treated rabbits, 4 of 23 (17.4%) of the group had hemorrhages compared with 5 of 23 (21.7%) in the control group.

In the additional 2 groups included in this study, we determined the effects of TLT in the presence of tPA administration on hemorrhage and infarct measures. As we have previously found,18–25 tPA administration significantly increased hemorrhage rate compared with the control group. For these studies, TLT was applied for 2 minutes coincident with the end of tPA infusion (ie, 90 minutes after embolization), a time when thrombolysis was occurring or had already occurred. We found that combining TLT and thrombolytic therapy did not result in any significant alteration of hemorrhage incidence (Figure). Moreover, there was no change in hemorrhage volume in the combination-treated group.

Lastly, Table 3 presents the types of hemorrhage found in the 4 different treatment groups. Under the hemorrhage column, we have entered the number of each specific hemorrhage type observed in each of the groups. We found that in both the tPA-treated and TLT/tPA-treated rabbit groups, in some rabbits, there was more than one type of hemorrhage present and this is indicated in the table. It does not appear that TLT preferentially affects any particular type of hemorrhage. However, we did find that parenchymal hemorrhages made up 50% of all hemorrhages in the TLT- and tPA-treated groups, and 20% of all hemorrhages in the control and combination-treated groups.

**Discussion**

In the present study, we assessed the effects of TLT on hemorrhage incidence and volume after large clot embolic strokes in rabbits because of the usefulness of the model at predicting neutral or deleterious effects of pharmacological treatments.17,18,21,22,25 We found that TLT could be administered safely either alone or in the presence of tPA because neither the monotherapy nor combination therapy affected hemorrhage rate compared with their respective controls. Treatments also did not alter hemorrhage volume compared with the embolized control group.

There was little concern that TLT alone would adversely affect the end points in this study because both preclinical studies in rodents and rabbits and a Phase II human study in patients with ischemic stroke have shown that transcranial laser therapy is safe8–10,30 and can produce significant behavioral improvement.8–10,30 Moreover, our preclinical studies in rabbits8,9 did not reveal any deleterious effects of TLT. However, because we were interested in combination therapy (ie, TLT plus tPA), we were concerned that TLT may exacerbate the deleterious effects of tPA such as an increased risk of ICH.11,12,16,20,31–34 To date, TLT has not been studied in the presence of the thrombolytic tPA and historically, an increased hemorrhage rate has been found when tPA is administered to rats, rabbits, and patients with stroke.11,12,16,20,31–34

We deemed this study necessary for several reasons. First, the mechanisms of action of TLT are not fully understood and

**Table 2. Survival Rate and Ischemia Incidence**

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>24-Hour Survival Rate</th>
<th>Ischemia Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17/23 (73.9%)</td>
<td>19/23 (82.6%)</td>
</tr>
<tr>
<td>TLT</td>
<td>15/23 (65.2%)</td>
<td>22/23 (95.6%)</td>
</tr>
<tr>
<td>tPA</td>
<td>12/23 (52.2%)</td>
<td>17/23 (73.9%)</td>
</tr>
<tr>
<td>TLT+tPA</td>
<td>12/20 (60.0%)</td>
<td>15/20 (70.0%)</td>
</tr>
</tbody>
</table>

*Statistically significant differences between any treatment group and the control group (P<0.05).

**Table 3. Hemorrhage Types and Volume**

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>n</th>
<th>Hemorrhage Volume</th>
<th>Hemorrhage Types</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23</td>
<td>6.51±2.32 (5)</td>
<td>HIN 1</td>
</tr>
<tr>
<td>TLT</td>
<td>23</td>
<td>2.25±1.12 (4)</td>
<td>PH 1, HIN 2, HPT 0</td>
</tr>
<tr>
<td>tPA</td>
<td>23</td>
<td>4.46±0.62 (13)</td>
<td>PH 7, HIN 2, HPT 5</td>
</tr>
<tr>
<td>TLT+tPA</td>
<td>20</td>
<td>5.00±0.56 (8)</td>
<td>PH 2</td>
</tr>
</tbody>
</table>

*Hemorrhage volumes as mean±SEM for only those rabbits exhibiting an ICH (n) and the types of hemorrhage found in the brain of embolized rabbits in each experimental group. There were no statistically significant differences in hemorrhage volume among the 4 groups (P>0.05).

PH indicates parenchymal hemorrhage; HIN, hemorrhagic infarction; HPT, punctuate hemorrhage.

**Figure. Hemorrhage rate after TLT: Effect of tPA administration.**

Effect of treatments on hemorrhage incidence in brain after embolic strokes. Results are presented as hemorrhage incidence (percent population) for each treatment group included in the study. tPA administration increased hemorrhage incidence (P<0.05). TLT in combination with tPA did not have any effect on hemorrhage rate. *Significance (P<0.05) compared with control. All other treatments did not significantly affect hemorrhage rate compared with control (P>0.05).
it has been suggested that photon energy photobiostimulation or infrared light energy as used in this study may promote angiogenesis and neurogenesis.35–39 It has been reported that angiogenesis can cause transiently leaky microves-sels35,39; therefore, we wanted to determine the effect of TLT alone and in combination with tPA treatment, which is known to cause vascular damage and hemorrhage. We were concerned that TLT might exacerbate thrombolytic-induced ICH. Also, because light treatment can increase protein turnover,37 it is possible that the treatment may affect protein molecules and pathways known to be involved in spontaneous and thrombolytic-induced ICH such as metalloproteinase membrane-associated proteins and tumor necrosis factor-α.19,22,40–43 However, as we have shown in this study, the hemorrhage rate in the combination treatment group was, if anything, somewhat lower than that of the tPA group. Moreover, the hemorrhage volume was not increased when compared with the thrombolytic group, suggesting that TLT when administered after thrombolytic does not expand the volume of a hemorrhage. The results indicate that TLT may not increase the level or activity of proteins known to be involved in ICH in rabbits after embolization. In conclusion, we have demonstrated that both TLT alone and TLT administered after thrombolytic therapy can be administered after a thromboembolic stroke without adversely affecting the hemorrhage rate, hemorrhage volume, or survival. Thus, based on this study, we recommend a clinical trial of Alteplase plus TLT when Alteplase is administered to patients before TLT.

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Disclosures
J.A.Z. is the Principal Investigator for the NEST-2 clinical trial. J.A.Z. and P.A.L. are consultants for Photothera Inc and are on the Scientific Advisory Board of Photothera Inc. J.S. is Founder and Executive Chairman of Photothera, Inc, and has a financial interest in the company.

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