Published in final edited form as:

Invest Radiol. 2011 March ; 46(3): . doi:10.1097/RLI.0b013e318200757a.

Microbubbles Improve Sonothrombolysis In Vitro and Decrease Hemorrhage In Vivo in a Rabbit Stroke Model

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Abstract

Introduction—Tissue plasminogen activator (tPA) is the thrombolytic standard of care for acute ischemic stroke, but intracerebral hemorrhage (ICH) remains a common and devastating complication. We investigated using ultrasound (US) and microbubble (MB) techniques to reduce required tPA doses and to decrease ICH.

Materials and Methods—Fresh blood clots (3–5 hours) were exposed in vitro to tPA (0.02 or 0.1 mg/mL) plus pulsed 1 MHz US (0.1 W/cm²), with or without 1.12×10^8 /mL MBs (Definity or albumin/dextrose MBs [adMB]). Clot mass loss was measured to quantify thrombolysis. New Zealand white rabbits (n = 120) received one 3- to 5-hour clot angiographically delivered into the internal carotid artery. All had transcutaneous pulsed 1 MHz US (0.8 W/cm²) for 60 minutes and intravenous tPA (0.1–0.9 mg/kg) with or without Definity MBs (0.16 mL/mg/kg). After killing the animals, the brains were removed for histology 24 hours later.

Results—In vitro, MBs (Definity or adMB) increased US-induced clot loss significantly, with or without tPA ($P < 0.0001$). At 0 and 0.02 mg/mL, tPA clot loss was greater with adMBs compared with Definity ($P = 0.05$). With MB, the tPA dose was reduced 5-fold with good efficacy. In vivo, both Definity MB and tPA groups had less infarct volume compared with controls at $P < 0.0183$ and $P < 0.0003$, respectively. Definity MB+tPA reduces infarct volume compared with controls ($P < 0.0001$), and ICH incidence outside of strokes was significantly lower ($P = 0.005$) compared with no MB. However, infarct volume in Definity MB versus tPA was not different at $P = 0.19$.

Conclusion—Combining tPA and MB yielded effective loss of clot with very low dose or even no dose tPA, and infarct volumes and ICH were reduced in acute strokes in rabbits. The ability of MBs to reduce tPA requirements may lead to lower rates of hemorrhage in human stroke treatment.

Keywords

microbubble; rabbit; tissue plasminogen activator; embolic; stroke

Ischemic stroke affects approximately 700,000 Americans annually, and stroke is the third leading cause of death in the United States.¹ Since initial human studies in 1996, intravenous (IV) tissue plasminogen activator (tPA) has remained the primary Food and Drug Administration-approved treatment for ischemic stroke.^{2–5} It acts by lysis of embolic clots occluding the cerebral arteries. The tPA must be given within 4.5 hours of stroke onset for

positive therapeutic effect, but it can be administered up to 6 hours after onset, if delivered intra-arterially. Although overall effect of tPA is positive, it also causes increased symptomatic intracranial bleeding, a major and often deadly complication.^{6–10} Delayed tPA treatment increases hemorrhage rates, thus limiting its use to a small minority of ischemic stroke cases.

The “most feared complication” of tPA therapy is intracerebral hemorrhage (ICH).^{11–14} This concern is emphasized in several scientific articles and might lead physicians to forego tPA treatment, even when appropriate. Although logistical and diagnostic delays are important roadblocks to care, physician fear of hemorrhage remains a major obstacle to tPA treatment for ischemic stroke. Fewer than 5% of Americans with ischemic stroke are treated with tPA thrombolysis. Continued research has been focused on reducing the incidence and deleterious consequences of tPA-related ICH.¹³ This research includes ultrasound (US) augmentation of tPA, variation of the intensity and frequency of US,^{15,16} and use of microbubbles (MBs)¹⁷ in combination with tPA or without tPA^{18–20} to enhance sonothrombolysis. More rapid restoration of blood flow with US appears to be beneficial in reducing hemorrhage,^{15,16} but excessive US can also cause bleeding.¹⁵

The objective of this study was to determine whether combining MBs with tPA and US could markedly decrease or eliminate the tPA dose required for effective sonothrombolysis and thereby reduce hemorrhage rates. US was tested in vitro with 2 types of MBs and differing tPA doses to quantify effects on clot loss. The in vitro results were then translated to a rabbit arterial embolic stroke model using commercially available Definity MBs, and the effects on stroke volumes and hemorrhage rates were measured.

MATERIALS AND METHODS

Preparation of In Vitro Clot

A 270- μ L aliquot of fresh rabbit blood was placed into an 18-mm well on a Boerner glass slide and then covered with an 18-mm circular glass cover slip to exclude air-blood contact. The Boerner slide was placed inside a humidified 37°C incubator for 3 to 5 hours to promote clot formation. Afterward, clotted blood was removed and placed in a 35-mm plastic culture dish containing 3 to 4 mL of rabbit serum (room temperature) and was cut into square pieces weighing 7.5 to 10 mg.

Microbubbles

Two different MB preparations were used. One was the commercially available perflutren lipid MB (Definity, Lantheus, Medical Imaging, Billerica, MA) with a MB size range of 1 to 4.5 μ m. The other was a more uniformly sized 3.0 ± 0.4 μ m albumin/ dextrose microbubble (adMB), produced in our laboratory. The adMBs were prepared by sonicating a decafluorobutane gas-saturated solution of 5% (wt/vol) bovine serum albumin (Sigma-Aldrich Co., St. Louis, MO: cat A7906) and 10% (wt/vol) of dextrose (Sigma-Aldrich Co, St. Louis, MO: cat G7528), using a Fisher 500 Sonic Dismembrator (Thermo Fisher Scientific, Waltham, MA). Both MB agents were used at a concentration of 1.124×10^8 microbubbles/mL. Sonication was performed in the following 2 steps: the first for 30 seconds at 250 W and the second for 20 seconds at 450 W. This procedure produced a range of MB sizes and the 3- μ m MBs were isolated on the basis of differential buoyancy using multiple fractionation steps.

In Vitro Sonothrombolysis

A piece of clot was weighed and then suspended in the middle of a vertical, acoustically transparent mylar tube (5.2-mm inner diameter, 0.2-mm thick wall) that was prefilled with

Hy-Clone Bovine Calf Serum (APG26112; Thermo Fisher Scientific) (Fig. 1). The calf serum contained either MBs ($1.12 \times 10^8/\text{mL}$), tPA (Activase, Genentech, Inc, San Francisco, CA), or a combination of both; depending on the experimental conditions. Additional serum was introduced into the mylar tube from below at a constant rate of 0.5 mL/min (2.4 cm/min linear velocity) to provide a continuous fresh supply of MBs and/or tPA. An acoustic absorber was used to shield the serum before entering the mylar tube to prevent premature MB lysis.

Sonication was performed in a tank filled with degassed water at 37.0°C. The serum reservoir, mylar sonication chamber, and the ultrasonic transducer were all pre-equilibrated to 37.0°C. US was delivered horizontally using a 10-cm² diameter (730 transducer, Mettler Electronics, Anaheim, CA) piezoelectric transducer located 6.5 cm from the mylar tube's center. Sinusoidal 1 MHz US with a temporal average spatial average intensity of 0.1 W/cm² was delivered using repeated 2 milliseconds pulses with an 8-milliseconds interpulse delay (Sonicator 716; Mettler Electronics, Anaheim, CA). Each clot was exposed to US for 15 minutes. Subsequently, the clot was removed from the chamber, serum was wicked off the clot using absorbent paper, the clot was weighed, and the fractional change in weight was calculated.

Preparation of In Vivo Embolus

Emboli were prepared by obtaining an arterial blood sample (2 mL) from a donor rabbit. The blood was transferred immediately into a 30.5 cm length of butterfly pediatric infusion set (No. 4506; Abbot Hospitals, Inc, North Chicago, IL) with an internal diameter of 1.0 mm and allowed to clot at 37°C for 3 to 5 hours. The cylindrical clot was expelled from the tubing into a dish containing physiological saline and cut into several pieces of length 1.0 mm with an approximate diameter of 0.6 mm after shrinkage. A single clot piece was drawn into a 3.0-mL syringe containing physiological saline for injection into the ICA with 0.7 to 2.0 mL of saline flush.

In Vivo Description of Animals and Surgical Procedures

All animal procedures were approved by the Institutional Animal Care and Use Committee. New Zealand white rabbits (n = 120, average weight = 5.2 kg) were used in this study.

The surgical and angiographic procedures were as previously described.²¹ Briefly, rabbits were sedated with an intramuscular injection of ketamine (30 mg/kg, Ketaset, Fort Dodge, IA) and xylazine (3 mg/kg, AnaSed, Lloyd Laboratories, Shenandoah, IA) and anesthetized with isoflurane (Novaplus, Hospira Inc, Lake Forrest, IL). The right femoral artery was surgically exposed, and using a 3F vascular sheath, a modified 65-cm angled-tip 3F catheter (SlipCath, Cook Inc, Bloomington, IN) was placed into the artery. Common carotid arteriography and selective internal carotid artery catheterization were performed.

Embolization was performed by injecting a single cylindrical blood clot with 0.7 to 2.0 mL of saline into the internal carotid artery. Flow usually carried the clot to the middle cerebral artery. One minute following embolization, repeat angiography was performed and the degree and location of the arterial occlusion was documented and recorded.

Immediately after the embolization and repeat angiography, each respective treatment was initiated. An IV catheter (Instyle-W, Becton Dickinson, Sandy, UT) placed into the left ear vein was used for administration of tPA and Definity MB. Following the procedure, the 3F arterial catheter was removed and the incision was sutured. Each rabbit was allowed to recover from surgery and monitored for adverse effects.

US and Treatments

The clot embolus was treated transcutaneously for 1 hour with 1 MHz US at 0.8 W/cm² using the same instrument and pulse mode used for the in vitro experiments. Positioning of the US probe was confirmed fluoroscopically and centered in a dehaired area just behind the rabbit's eye on the lateral side of the head. US was administered in combination with tPA and/or Definity MB.

Rabbits were randomly assigned to 1 of 5 tPA treatment groups. The tPA groups were as follows: (1) control 0 mg/kg (n = 45), (2) 0.1 mg/kg (n = 12), (3) 0.3 mg/kg (n = 16), (4) 0.8 mg/kg (n = 11), and (5) 0.9 mg/kg (n = 36). These rabbits were then randomly selected for Definity MB at 0.16 mg/kg at (1) (n = 27), (2) (n = 5), (3) (n = 9), (4) (n = 5), and (5) (n = 14). Rabbits-administered tPA received IV tPA with first dose given at 10% as a single bolus and the remaining 90% administered for 60 minutes in groups 2 to 5. Rabbits-administered MB received IV perflutren lipid MB, Definity, at a dose rate of 0.16 mg/kg for 30 minutes in bolus doses every 5 minutes.

Measurement of Infarct Volume

After killing the rabbits, the brains were harvested, immediately placed in ice-cold physiological saline for 60 minutes, and then sliced coronally at 0.4 cm intervals. Sections were placed in 1% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich, St. Louis, MO) for 45 minutes at 37°C, fixed in 10% formalin, and then digitally photographed. Pale areas of infarction were measured by reviewers blinded as to treatment groups, using an image analyzer (NIH ImageJ). Each brain section volume was calculated by multiplying the section area by the slice thickness (0.4 cm). Similarly, the infarct volume for each section was calculated by multiplying the area(s) of infarction for each section by the slice thickness. Percent infarct volume was calculated by dividing the sum of the infarct volumes into the sum of the section volumes and multiplying by 100%.

Hemorrhage Determination

Intracranial hemorrhage was defined as extravasation of erythrocytes into the extravascular space. Each brain section was fixed in 10% formalin, embedded in paraffin, sectioned at 4 µm, stained with hematoxylin and eosin, and examined microscopically. The evaluation was performed by a veterinary pathologist without knowledge of treatment group. The incidence of ICH (presence or absence) and the location (within the infarct or clearly outside of the infarct, presumably in penumbra) were recorded (Figs. 2A, B).

Statistical Analysis

Statistical analyses were conducted using SAS, version 9.2 (SAS Institute, Inc, Cary, NC). In vitro data were analyzed by analysis of variance (ANOVA) as a 3 × 3 factorial arrangement of treatments (tPA dose rate [0, 0.02, or 0.1 mg/mL] and MB type [no MBs, Definity or adMB]) within a completely randomized design. Five replicates were performed per treatment. Percent clot loss was analyzed by ANOVA using the generalized linear model procedure of SAS. The model included tPA dose rate, MB type, and the interaction. Standard errors for group means are reported as the square root of mean square for error from the ANOVA model divided by 5 (as there were 5 clot observations per treatment group). Pairwise comparisons of least squares means of the various tPA dose—MB type treatment groups were carried out using the PDIF function of SAS with Bonferroni adjustments for multiple comparisons.

Data for the infarct volumes from the animal experiment did not meet the normality assumption required for ANOVA, so nonparametric procedures on the basis of ranks were used for statistical analysis. Within MB levels (present/absent) the infarct volumes for all

nonzero doses of tPA did not differ significantly from each other, so rabbits were recategorized into 4 groups: controls (no tPA or MB), tPA only (all nonzero doses combined), MB only, tPA+MB. The generalized linear model procedure of SAS was used to compare the ranks of the infarct volumes among these 4 groups. This is equivalent to a Kruskal-Wallis test of infarct volume among the 4 groups, but using this procedure facilitated conduct of the between group comparisons. Because of the limited power owing to small sample sizes in some of the groups and the conservative nature of the Bonferroni adjustment, we did not adjust the *P* values in this exploratory in vivo study for multiple comparisons. To assess rates of ICH, tPA doses of 0.1 and 0.3 mg/kg were combined as were doses of 0.8 and 0.9 mg/kg to create low and high dose groups. ICH rates were then compared across the combinations of 3 tPA dose groups (none, low, high) and 2 MB categories (none, 0.16 mg/kg) using exact *P* value calculations for contingency table analysis with the software package StatXact because of the small frequencies of ICH in many groups.

RESULTS

In vitro results showed that clot loss was influenced by MB type and tPA dose interactions ($P < 0.0001$). US combined with adMB or Definity yielded more effective clot loss than US alone ($P < 0.0001$) (Fig. 3). In a control group without US, simple tPA therapy alone produced $1.3\% \pm 0.4\%$ clot loss. Exposing the clot to US alone yielded a clot loss of $4.25\% \pm 0.6\%$. Without tPA administration, the adMBs were more effective at potentiating ultrasonic thrombolysis than Definity, with clot loss levels of $18.1\% \pm 0.6\%$ and $13.7\% \pm 0.6\%$ ($P = 0.001$), respectively. The use of 0.02 mg/mL tPA and US (without MB) increased clot loss from $4.25\% \pm 0.6\%$ to $15.9\% \pm 0.6\%$ ($P < 0.0001$), and increase of tPA to 0.1 mg/mL improved clot loss significantly to $23.5\% \pm 0.6\%$ ($P < 0.0001$). The addition of 0.02 mg/mL tPA improved sonothrombolysis with adMBs to $28.3\% \pm 0.6\%$ and with Definity MBs to $25.1\% \pm 0.6\%$. The different results with adMB and Definity were significant at these low doses of tPA ($P = 0.05$). However, at a dose of 0.1 mg/mL tPA, there were no differences between clot loss enhancement by the 2 MB types (adMB, $31.0\% \pm 0.6\%$ vs. Definity, $30.0\% \pm 0.6\%$ [$P = 0.27$]). Using either MB type at this level of tPA remained superior to tPA+US ($23.5\% \pm 0.6\%$), ($P < 0.0001$).

In vivo results indicate a significant difference in infarct volume within tPA dose rate groups at $P = 0.006$ (Table 1). Infarct volumes differ between controls and those with tPA+Definity MB ($P < 0.0001$) or Definity MB ($P = 0.018$). Infarct volumes with tPA alone differed from controls ($P = 0.003$), but not the other 2 groups. However, the 0.8 to 0.9 mg/kg tPA group (high dose) also had the greatest magnitude of penumbra ICH at 61% comparable with the control group at 56% ($P = 0.77$). ICH incidence in penumbra was significantly lower with MB only (19%) when compared with controls (56%) ($P = 0.02$) (Table 1). Hemorrhage within the stroke occurred with an incidence of 5% and outside the stroke incidence was 43% of cases. Compared with controls, the incidence of ICH within strokes was not different within tPA treatment groups or within MB treatment groups, (both at $P = 0.65$).

DISCUSSION

A safe and effective ischemic stroke therapy that provides improved thrombolysis without the added risk of ICH remains elusive. With careful selection of patient and exclusion of those with added risk of hemorrhage, such as patients with severe hypertension or hyperglycemia, hemorrhage rates and deaths have decreased moderately.¹¹ However, very demanding inclusion criteria and physician's great fear of ICH have led to surprisingly low proportions of eligible stroke patients who actually receive tPA. Consequently, the insurance risk for not receiving therapy, failure to treat, is higher than the complication risk.²² These

factors make a compelling case to investigate safer thrombolytic stroke therapies, including combining therapeutics with reduced tPA doses to achieve increased clot lysis and lower ICH rates.

The simplicity and controllability of in vitro testing can be used to rapidly test hypotheses and evaluate potential combined therapy solutions for achieving effective clot lysis with reduced tPA doses. Greater precision of in vitro data allows clearer identification of procedural changes that improve thrombolysis efficacy significantly. These results must then be confirmed in more complex in vivo stroke models to determine whether the modifications that improved thrombolysis efficacy in vitro do so in vivo while reducing, rather than increasing, the risk of tPA associated complications including hemorrhage. The complex clinical phenomena including embolic ischemic stroke, autolysis of clot, reperfusion, and complications of therapies including hemorrhage often produce unexpected results. In addition, the variables particular to a small animal stroke model can obscure important findings specific to species and scale. Animal and human testing involves so many uncontrolled factors ranging from anatomic arterial variation to spontaneous recanalization and variable collateral formation that large volumes of data are required to obtain reliable information. Yet, early work of Zivin and coworkers in rabbits did translate successfully to human beings, and further use of a similar model is promising.⁶

Our in vitro testing demonstrated convincingly that when tPA was combined with MBs and US, tPA dose could be reduced markedly without reducing thrombolysis efficacy (Fig. 3). Using a more uniformly sized 3- μ m diameter adMB at the same concentration as Definity, improved in vitro clot lysis possibly by enhancing the US-MB interactions responsible for sonothrombolysis, eg, cavitation and microstreaming (Fig. 3). These results suggest that further development and testing on MBs with size and other properties optimized for sonothrombolysis might hold promise for improved clot lysis in the clinical setting.

In the rabbit embolic stroke model, Definity MBs decreased rates of hemorrhage versus controls at $P = 0.02$ (Table 1). The addition of the MBs plus US to our embolic stroke model showed therapeutic results similar to or better than those with standard tPA therapy levels, even with greatly reduced tPA or no exogenous tPA given to the animal. Certainly, autolysis with no exogenous tPA is often a factor in humans, as in transient ischemic attacks, and small amounts of endogenous tPA are thought to be produced locally in ischemic endothelium.²³ The combination of MBs and endogenous tPA might be entirely adequate for thrombolysis in some cases, and this possibility deserves further study. The ability to use MBs plus US to reduce the exogenous tPA dose required for thrombolytic therapy may well translate into a reduction in symptomatic ICH in human beings, but this has yet to be proven clinically.²⁴

Although several studies with 2-MHz small-beam US transcranial Dopplers are associated with improved tPA stroke therapy and limited bleeding using MBs,^{25–29} other studies have shown excessive bleeding with wide beam lower frequency (300 KHz) US and tPA without MBs.^{15,16} Alexandrov et al also reported increased bleeding at 2 MHz with very high doses of MBs in a brief dose escalation study in human strokes.²⁷ Molina et al used only 3 IV doses of MBs in the course of 1 hour.²⁶ Depending on the half life of those MBs, those results might have been further improved with more frequent dosing. Our unpublished work suggests that the half-life for commercial bubbles ranges from 4 to 10 minutes in a 20 kg dog and these times also depended on US conditions. It is slightly longer in the rabbit model. In our study, use of 1 MHz with a broad US beam, we chose frequent doses of IV MBs to maintain therapeutic levels. Additional work will be required to optimize all of the thrombolytic factors involved.

Although the lowered incidence of microscopic bleeding observed in rabbits treated with MB therapy was promising, the mechanism and the clinical significance of this finding are not clear. It has been speculated that US-induced bleeding in the brain might be because of the formation of standing waves or possibly the focusing of US into pockets of higher intensity by the cranium. MBs circulating throughout the brain might provide enough additional ultrasonic absorption and scattering to diminish standing waves and/or cranial focusing sufficiently to reduce penumbral ICH. Although the reduction of any treatment-associated trauma should be viewed as a positive factor, any clinical relevance for this reduction in penumbral ICH is unknown. Small bleeds may or may not progress to symptomatic ICH. In human beings, such cases have been described,³⁰ but other series have suggested that microbleeds are not likely to worsen.^{31,32} Because the significance of the small bleeds remains uncertain, its full characterization is of utmost importance for future studies.

The limitations of this study include the short survival time, which may have not been long enough to include symptomatic ICH that often occurs after 24 hours. A long-term survival study is required to better characterize the natural history of these small bleeds and the less common larger bleeds. Human studies¹¹ suggest that almost all infarcts will eventually show some hemorrhage within the infarct during the healing phase. The hemorrhages reported here are outside the infarct in what appears to be penumbra and are thought to be different.

An efficacious and safer form of ischemic stroke therapy will dramatically improve delivery of care to the stroke patient population that is so badly underserved at this time. If symptomatic human ICH can be decreased from about 6% with standard therapy to about 2% with MBs as suggested here, physician fears will subside, the clinical decision will become more obvious, and treatment of strokes will dramatically increase. If what now is an improved outcome in less than 14% of treated patients⁸ could improve to about 18% of treated patients by removing the threat of increased ICH due to therapy, utilization should increase dramatically. With increased delivery of a safer therapy a many fold improvement in eventual outcomes for the stroke population is ensured. Considering the almost 700,000 ischemic strokes annually in the United States, the potential effect is striking.

In summary, the addition of MB+US to fresh clot provides significant levels of clot lysis and the addition of low dose tPA provides an even greater reduction in clot size to levels similar to full tPA dose levels. The combination of low-dose or no-dose tPA and MB+US improved thrombolytic treatment of acute embolic strokes in rabbits with a strong trend to decrease infarct volumes and significantly decreased ICH outside the area of infarct. These therapeutic combinations may provide needed improvement in human stroke treatment and lead to lower rates of symptomatic hemorrhage, currently the major treatment complication of IV tPA in humans. With lowered risks of therapeutic thrombolytics, improvements in utilization and in patient outcomes should dramatically increase.

Acknowledgments

The authors acknowledge and thank Sean Woods and Janath Mckee for their technical assistance, Leah J. Hennings, DVM, for pathology analysis and John D. Lowery, DVM, and Jeff Hatton, for their assistance with surgical and histologic applications.

Supported from the National Institutes of Health, NIH R01HL82481 (to W.C.C.); and R01CA99178 (to M.J.B.).

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FIGURE 1.
Schematic of in vitro sonothrombolysis flow chamber.

FIGURE 2.

ICH. A, Hematoxylin and eosin-stained microscopic brain section shows noninfarcted brain (penumbra) hemorrhage. B, Coronal gross of brain slice with TTC staining illustrating an area of hemorrhage (small red spots) in larger pale area of infarct.

FIGURE 3.

Percentages of in vitro clot loss between MB types. Rabbit blood clots (3 hours clots at 37°C) ($n = 5/\text{treatment combination}$) were exposed to pulsed (20% duty cycle) US at 1 MHz, 0.1 W/cm² for 15 minutes at 37°C. MBs were either 0 (None), Definity, or designed albumin bubbles (adMB) at $1.128 \times 10^8/\text{mL}$. Tissue plasminogen activator (tPA) doses were 0, 0.02, or 0.1 mg/mL. Percent clot loss was influenced ($P < 0.0001$) by MB type and tPA dose interactions. All bars with different letters (a–e) are significant at $P = 0.05$ after Bonferroni adjustment.

TABLE 1

Therapy Effects on Infarct Volume and Bleeding

	MB Dose (mg/kg) *					
	0			0.16		
tPA dose (mg/kg)	0	0.1–0.3 (low)	0.8–0.9 (high)	0	0.1–0.3 (low)	0.8–0.9 (high)
Rabbits (n)	18	14	28	27	14	19
Infarct volume (%) †	1.0	0.21	0.13	0.2	0.08	0.09
ICH (%) ‡	56	50	61	19	57	26

* All rabbits were embolized with a single blood clot (3–5 hours) and had transcutaneous therapeutic ultrasound application of 1 MHz at 0.8 W/cm² for 60 minutes. Following embolization rabbits were either treated with or without Definity microbubbles.

† P values for differences in overall comparisons of infarct volume for tPA versus control were $P = 0.0003$ and differences between both microbubble groups (MB vs. tPA+MB) $P = 0.02$.

‡ Among rabbits without MBs, ICH did not differ between tPA treatments at $P = 0.84$; in rabbits receiving MB ICH differed between tPA groups at $P = 0.04$. The usual (0.8–0.9 mg/kg) dose of tPA+MB was significantly lower in ICH incidence compared usual dose tPA alone ($P = 0.04$).

MB indicates microbubble; tPA, tissue plasminogen activator; ICH, intracerebral hemorrhage.