Effect of 40-kHz Ultrasound on Acute Thrombotic Ischemia in a Rabbit Femoral Artery Thrombosis Model
Enhancement of Thrombolysis and Improvement in Capillary Muscle Perfusion

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Background—We have shown previously that 40-kHz ultrasound (US) at low intensity accelerates fibrinolysis in vitro with little heating and good tissue penetration. These studies have now been extended to examine the effects of 40-kHz US on thrombolysis and tissue perfusion in a rabbit model.

Methods and Results—Treatment was administered with either US alone at 0.75 W/cm², streptokinase alone, or the combination of US and streptokinase. US or streptokinase resulted in minimal thrombolysis, but reperfusion was nearly complete with the combination after 120 minutes. US also reversed the ischemia in nonperfused muscle in the absence of arterial flow. Tissue perfusion decreased after thrombosis from 13.7±0.2 to 6.6±0.8 U and then declined further to 4.5±0.4 U after 240 minutes. US improved perfusion to 10.6±0.5 and 12.1±0.5 U after 30 and 60 minutes, respectively. This effect was reversible and declined to pretreatment values after US was discontinued. Similarly, tissue pH declined from normal to 7.05±0.02 after thrombosis, but US improved pH to 7.34±0.03 after 60 minutes. US-induced improvement in tissue perfusion and pH also occurred after femoral artery ligation, indicating that thrombolysis did not cause these effects.

Conclusions—40-kHz US at low intensity markedly accelerates fibrinolysis and also improves tissue perfusion and reverses acidosis, effects that would be beneficial in treatment of acute thrombosis. (Circulation. 2000;101:2296-2301.)

Key Words: ultrasonics ■ thrombolysis ■ tissue ■ perfusion ■ streptokinase ■ thrombosis

The expanding use of fibrinolytic therapy has resulted in improved outcomes in patients with myocardial infarction and peripheral vascular disease and the promise of reduced disability after stroke. These advances have also focused attention on the limitations of therapy and have stimulated efforts to improve effectiveness and decrease adverse effects. Thus, in patients with acute myocardial infarction, up to 20% do not achieve reperfusion, and the benefit decreases with longer periods of ischemia, emphasizing the need for rapidly acting therapy. For stroke, the need for very early treatment and the serious consequences of intracranial bleeding limit application. Problems with treatment of peripheral artery occlusion include the need for proper catheter replacement, a longer duration of treatment, and a requirement for subsequent endovascular or surgical reconstruction in most patients. Its limited use for venous thromboembolic disease reflects the high incidence of therapeutic failure and lower benefit-risk ratio. Efforts to overcome these obstacles have focused on development of new plasminogen activators, more effective dosing regimens, and adjunctive antiplatelet and anticoagulant therapy.

The use of ultrasound (US) represents a completely different, nonpharmacological approach to improving fibrinolytic therapy and offers unique potential to increase reperfusion and limit bleeding complications. Several reports have shown marked acceleration of fibrinolysis with low-intensity US in vitro1–5 and in animal models.6–10 Miniaturized transducers have also been attached to catheters for endovascular use,11–13 and this offers the potential to deliver localized US at the site of thrombosis while limiting insonification of normal tissue. The choice of US frequency is critical for successful clinical application, because it influences both efficacy and safety. Early studies used frequencies of ≥500 kHz, but poor tissue penetration and unacceptable heating were limiting. These problems are smaller at lower frequencies, and we recently showed that the enhancement of thrombolysis in vitro is greater at 40 kHz than at 1 MHz.5 In the present study, therefore, we have extended these observations to a model of rabbit femoral artery thrombosis. The findings indicate that 40-kHz US accelerates thrombolysis, with little evidence of tissue injury and minimal heating, and also improves tissue perfusion and metabolism independent of clot lysis.
Methods

Animal Preparation
Rabbits were anesthetized with ketamine 60 mg/kg, xylazine 6 mg/kg, and chlorpromazine 25 mg/kg, and sedation was maintained with sodium pentobarbital as needed. The femoral arteries were dissected 5 cm distal to the origin of the superficial branch, and the profunda femoris and superficial arteries were ligated close to their origin. A Doppler flow probe was placed distally around the isolated segment, and 2 parallel ligatures were placed around the femoral artery 1 cm distal to the profunda branch. These reduced flow by \( \approx 50\% \) and remained in place for the duration of the experiment. After this, filter paper saturated with 20% ferric chloride was placed on the femoral artery, and thrombosis was assessed by monitoring of flow, which approached 0 after occlusion. In some animals, a completely occlusive suture was placed around the artery.

Experimental Protocol
Rabbits received (1) US alone, (2) streptokinase alone, (3) both US and streptokinase, or (4) no treatment. There were 7, 6, 6, and 9 rabbits in groups 1, 2, 3, and 4, respectively. The source of US was a 3.5-cm-diameter probe with a 1-cm-diameter transducer driven in continuous mode at 0.75 W/cm\(^2\), and acoustic pressures were measured before and after each experiment with a hydrophone. A balloon filled with water at 37°C was placed over the thrombosed segment for temperature control and US transduction. The interface between the balloon and the artery was covered by a layer of US gel. Streptokinase was administered as an intravenous bolus of 15,000 U/kg, followed by an infusion of 15,000 U kg\(^{-1}\) h\(^{-1}\) for 2 hours. This dose was selected because our previous experience with this model indicated that it was relatively ineffective alone and that 1-MHz US enhanced its effects,\(^8\) and data in vitro indicated that 40-kHz US had a greater effect on thrombolysis than 1 MHz.\(^2\) The pH of the muscle was also monitored with a pH meter (model HI 9023C, Hanna Instruments). Perfusion in the gracilis muscle was measured with a laser-Doppler flowmeter (BFL 21, Transonic Systems) with an output in units (TPU) that is linearly related to the number of red cells times their velocity in the hemispheric measuring volume.\(^14\) The measuring surface was 1 mm\(^2\), and the light penetration depth was \( \approx 1 \) mm.

Temperature monitoring in 4 rabbits was performed with a copper-constantan fine-wire thermocouple placed under the femoral artery or on the exposed surface of the femur and connected to a temperature gauge. To assess the effects of heating on tissue perfusion, a balloon containing water at 32°C to 42°C was laid over the gracilis muscle. At the completion of each experiment, animals were euthanized, and samples for histology were excised and fixed in 10% buffered formalin. Specimens were processed in paraffin, sectioned at 4 \( \mu m \), and stained with hematoxylin and eosin. The stained sections were encoded to obscure treatment and examined by an observer (R.B.B.) blinded as to code.

Statistical Methods
The 3 primary outcome measures that were used to assess the effect of US were flow intensity, TPU, and pH. The mixed linear model was used for statistical analysis of each of the primary measures. The 3 primary outcome measures that were used to assess the effect of US were flow intensity, TPU, and pH. The mixed linear model was used for statistical analysis of each of the primary measures. The least-squares means, their standard errors, and covariances were calculated, and the adjusted differences between treatment means at different time points were obtained. They were used for testing for treatment effect as well as for effect sizes for each level of grouping variables.

Results
Occlusive thrombi formed in all femoral arteries within 20 to 30 minutes of placement of the constriction and application of 20% ferric chloride. Arterial flow was 12.0\( \pm \)0.7 mL/min at baseline, declined to 5.8\( \pm \)0.4 mL/min after placement of the constriction, and was 0.1\( \pm \)0.1 mL/min after thrombosis. Three different treatment regimens were administered after thrombosis: US alone, streptokinase alone, or the combination of streptokinase and US. Flow in control vessels receiving no treatment remained at near 0 after 30, 60, and 120 minutes (Figure 1). Treatment with US alone resulted in no significant increase in flow, whereas treatment with streptokinase alone resulted in a small but significantly increased flow at 120 minutes to 0.4\( \pm \)0.1 mL/min (P<0.001). The combination of streptokinase and US resulted in greater reperfusion, with flow of 2.6\( \pm \)0.7 mL/min at 30 minutes, 4.6\( \pm \)0.4 mL/min at 60 minutes, and 4.8\( \pm \)0.6 mL/min at 120 minutes. The 120-minute flow represented 83% of the flow of 5.8\( \pm \)0.4 mL/min after placement of the external constrictor but before thrombosis. These results indicate that the application of US markedly accelerates arterial reperfusion compared with streptokinase alone and that US by itself had no appreciable effect.

US application can cause heating, and temperature was monitored with a thermocouple placed adjacent to the thrombosed vessel or at the surface of the femur. With application of US, the average initial temperature increase at the femoral artery was 0.02°C/min, and it was 0.04°C/min at the surface of the femur. The maximum temperature increase after 60 minutes was 1.6\( \pm \)1.3°C at the femoral artery and 1.1\( \pm \)0.7°C at the femur. Histologically, examination showed that vessels exposed to US, regardless of other treatment components (streptokinase, ligation, clot), had a pronounced tendency toward endothelial cell vacuolization, and some cells lifted up off the underlying basement membrane (Figure 2). Occasionally, erythrocytes were seen in direct contact with the basement membrane.

During these experiments, we observed that muscle adjacent to the femoral artery lost its normal pink, vital color soon after thrombosis and became a brownish-purple. Application of US restored the normal pink color even when no
thrombolysis occurred and femoral artery flow remained near zero. Therefore, we characterized perfusion in the gracilis muscle using a probe that is sensitive to capillary blood flow (Figure 3). In control experiments, tissue perfusion was stable for periods up to 60 minutes, indicating that application of the unactivated probe by itself did not affect tissue perfusion. At baseline, before vessel constriction or thrombosis, capillary perfusion was 13.7±0.4 U (Figure 4A). This declined to 6.8±0.4 U immediately after thrombosis and then declined progressively to 4.5±0.4 U after 240 minutes in animals receiving no treatment. The application of US resulted in a significant increase in perfusion to 10.0±0.5 U at 30 minutes and a further increase to 12.1±0.5 U at 60 minutes ($P<0.001$ for both). To determine whether the effect of US was reversible, the transducer was switched off at 60 minutes, and perfusion then declined progressively to 9.7±0.2 U at 90 minutes and to 8.5±0.2 U at 120 minutes ($P<0.001$ for both in comparison with 60 minutes). At 120 minutes, the transducer was reactivated, and this resulted again in improved perfusion to 11.8±0.8 U at 150 minutes and 12.7±0.4 U at 180 minutes ($P<0.001$ for both compared with 120 minutes). When the transducer was switched off at 180 minutes, perfusion again declined and reached 8.2±0.8 U at 240 minutes. The Doppler flow probe placed distally on the artery showed no flow for the duration of the experiment. The same changes were observed when the vessel was ligated completely with sutures to preclude any change in femoral artery flow. Because tissue perfusion is sensitive to temperature, we investigated whether US-induced heating could explain the changes. Muscle was heated from 32°C to 42°C by use of warm water in a balloon, and TPU increased from 6.2 to 7.2 U over this temperature range. Because the maximum temperature increase with US was <2°C, heating alone could not account for the US-induced increase in perfusion in the ischemic area.

We hypothesized that the increased tissue perfusion after application of US would improve the metabolism of ischemic muscle and ameliorate acidosis, and this was investigated with a similar experimental design with a pH probe (Figure 4B). After surgical exposure but before thrombosis, the baseline muscle pHi was 7.41±0.02, but this declined to 7.05±0.02 after thrombotic occlusion. In the absence of treatment, pHi declined slowly but progressively to reach 6.86±0.02 at 240 minutes. The application of US reversed the acidosis, and muscle pHi increased significantly to 7.31±0.02 after 30 minutes and to 7.34±0.03 after 60 minutes.

Figure 2. Histological changes in vessels exposed to US. A, Vessel walls with endothelial cells showing occasional cytoplasmic vacuoles (arrowheads) and lifting of a single endothelial cell (arrow). B, Segment of treated artery with all endothelial cells showing cytoplasmic vacuoles (arrowheads) and some rounding up with lifting (arrow). C, Segment of artery with vacuolization (arrowheads) and complete lifting of an endothelial cell (arrow) with erythrocytes under lifted endothelial cell in contact with basement membrane. Bar=20 μm.
minutes (P<0.001 for both). At 60 minutes, the US was turned off, and muscle pH declined to 7.17±0.01 at 90 minutes and then showed little change to 7.16±0.02 at 120 minutes. The transducer was then turned on, and pH again improved to 7.32±0.02 at 150 minutes and 7.30±0.03 at 180 minutes. At this time, the transducer was again turned off, and pH declined to 7.13±0.04 at 210 minutes. The differences between means were all significant (P<0.001).

To determine whether the effect of US on tissue perfusion and pH was limited to the insonified tissue, measurements were made at multiple locations laterally and distally during insonification (Figure 5A). Perfusion measured from the center of the transducer to 4 cm distally at baseline was between 12.4 and 13.6 U, and after thrombosis it decreased to between 6.3 and 6.5 U. Perfusion measurements were then made after application of US for 30 and 60 minutes. At 0 cm (Figure 5A), perfusion increased to 11.1±0.5 U at 30 minutes and further to 14.0±0.6 U at 60 minutes. The effect declined at sites distal from the center of the transducer. This was most evident at 60 minutes, with values of 12.1±0.4, 8.3±0.3, 6.0±0.3, and 5.3±0.3 at 1, 2, 3, and 4 cm, respectively. The readings at 3 and 4 cm were the same as those in control animals not exposed to US. Because the diameter of the transducer was 1 cm, these findings suggest that the US effect is limited to the insonified tissue. In other experiments, the transducer was applied at sites 1, 2, 3, and 4 cm distally. Insonification at these sites resulted in normalization of TPU, indicating that the effect could be induced at these sites but required direct US exposure.

Similar experiments were performed measuring muscle pH (Figure 5B). At baseline and before thrombosis, muscle pH was between 7.36 and 7.42 within the 4-cm area. This declined to between 7.03 and 7.08 after thrombus formation. US application improved tissue pH at the center of the transducer to 7.34±0.04 at 30 minutes and to 7.39±0.07 U at 60 minutes. As with perfusion (Figure 5A), the effect was limited to the insonified area, and muscle pH at 3 and 4 cm was not improved during insonification.

Discussion

The results demonstrate that application of 40-kHz US at 0.75 W/cm² greatly accelerates thrombolysis in a rabbit femoral artery thrombosis model. Streptokinase was chosen as the plasminogen activator for the study because the rabbit is relatively resistant to this activator, primarily as a result of slower activation of plasminogen to plasmin. This was confirmed by our findings showing <0.5 mL/min flow after 120 minutes of therapy with streptokinase alone (Figure 1). US by itself resulted in no significant reperfusion, but the combination of both US and streptokinase had a marked effect by 30 minutes, with restoration of flow to 83% of baseline by 120 minutes. The femoral artery thrombosis model was modified for these experiments to avoid any endovascular instrumentation or injury to the endothelium, and no injection of procoagulant was used. Instead, thrombosis was induced by application of ferric chloride externally. This avoided the possibility of injecting air into the vessel, which would increase the potential for cavitation and possibly increase the US effect artfactually. Therefore, clot lysis resulted only from the interaction of plasminogen activator and US with the thrombus. The magnitude of the US effect appears to be greater than that previously demonstrated with a similar model at 1 MHz, and this is consistent with the demonstration of greater acceleration of fibrinolysis in vitro with 40 kHz than with 1 MHz.

The mechanism(s) by which US accelerates thrombolysis is complex. Transport of plasminogen activator into a thrombus is rate-limiting, because flow is obstructed. Drug access by diffusion is very slow, but previous studies in a static system have shown that US increases clot uptake of activator and results in greater depth of penetration. US also increases pressure-mediated perfusion through a fibrin matrix, and both of these effects would increase drug delivery and accelerate fibrinolysis. In addition, US reversibly alters fibrin fiber structure, generating a larger number of thinner fibers, and this may contribute to changes in equilibrium binding of activator to fibrin. The physical mechanism by which US accelerates fibrinolysis is nonthermal, and the ability of stabilized microbubbles to augment the effect suggests that...
cavitation plays a role, possibly by increasing fluid motion and thereby drug transport.

Previous reports have shown that US can accelerate fibrinolysis in vivo, but they differ from our findings in several respects. In some reports, thrombi were mechanically disrupted in vitro\textsuperscript{22,23} or in animal models\textsuperscript{23,24} with wires vibrating at US frequencies in the absence of plasminogen activator, and this approach has been tested in small studies in patients with coronary\textsuperscript{25} or peripheral\textsuperscript{26,27} arterial occlusion and with occluded coronary bypass grafts.\textsuperscript{28} This treatment requires endovascular positioning of the wire, and it can result in vessel wall damage, excessive heating, and distal embolization of clot fragments. Externally applied high-intensity US alone without plasminogen activator at 20 kHz has been used to recanalize femoral artery thrombi in a rabbit model, but excessive heating also occurred.\textsuperscript{21}

In considering therapeutic application, the choice of US frequency is of particular importance, because tissue penetration declines and heating increases at higher frequencies. We have previously demonstrated excellent transmission of US through bone at 40 kHz,\textsuperscript{5} and others have demonstrated that 40-kHz\textsuperscript{29} and 211.5-kHz\textsuperscript{30} US transmitted through the skull can accelerate fibrinolysis in vitro. Toxicity from US can result from heating or from the effects of cavitation, but we found little evidence of adverse effects in our study. Heating of $<2^\circ$C at the bone surface would be of little clinical consequence, and the endothelial changes observed histologically (Figure 2) are similar to those observed previously at 1 MHz\textsuperscript{8} and are likely to be reversible. In those experiments, there was also evidence of US-induced accumulation of platelets on the thrombus,\textsuperscript{8} a change that was not seen in the present experiments.

Femoral artery thrombosis results in distal muscle ischemia and metabolic changes, including acidosis. In the experiments reported, muscle perfusion was measured with a probe sensitive to movement of red blood cells to a depth of $\approx 1$ mm in the regional microcirculation.\textsuperscript{31} Unexpectedly, the application of US improved tissue perfusion, and this resulted in reversal of acidosis. Although the laser-Doppler measurement is limited to 1 mm in depth, the prolonged duration of the US effect accompanied by normalization of tissue pH and muscle color suggests that it was more general. This occurred without clot lysis and was observed even when the vessel was completely ligated, indicating that reperfusion with flow through the femoral artery was not the explanation. The improved perfusion was reversible, and the effect was limited
to the insonified area, with no discernible increase in perfusion either distally or laterally. The proximal leg muscles receive their primary blood supply from the femoral artery, and occlusion reduced perfusion to \(\approx 50\%\) of baseline, but the residual perfusion after occlusion indicates that an alternative arterial supply provided collateral flow. The US-induced increase in perfusion in the absence of femoral artery flow suggests that arterial supply through these collateral vessels increased.

The mechanism of improved perfusion is unclear, but redistribution of collateral flow into the ischemic area may be modulated by neural or hormonal influences. Humoral mediators of vasomotor tone include endothelin, prostacyclin, and nitric oxide. The local secretion of nitric oxide is regulated by nitric oxide synthase, an enzyme that can be induced by mechanical stress on endothelial cells\(^\text{32,33}\) that could result from US. Thus, we hypothesize that nitric oxide–induced redistribution of flow may account for the effects of US, but further studies will be necessary to elucidate the physiological mechanisms. Therapeutic application will require careful attention to limiting application of US only to ischemic tissue, because inappropriate insonification to adjacent nonischemic tissues could result in redistribution of flow away from ischemic tissue, thereby extending ischemia.

Both the accelerated clot lysis and the increased tissue perfusion resulting from US have the potential to improve clinical outcomes with fibrinolytic therapy. Rapid reversal of tissue ischemia is essential in preventing myocardial necrosis and, in particular, neuronal loss with stroke. This could be achieved either through removal of the arterial obstruction or by an increased flow to the ischemic area through collateral vessels. The accelerated clot lysis with US offers the potential to accelerate clot dissolution and speed reperfusion, thereby preserving ischemic tissue. Alternatively, US could be used to achieve an equivalent rate of clot lysis with a lower concentration of plasminogen activator to reduce bleeding complications. The augmentation of collateral flow offers a new approach to increasing perfusion of ischemic tissue and limiting dysfunction and necrosis.

Acknowledgments

This work was supported in part by grant HL-30616 from the NHLBI, NIH, Bethesda, Md, and by a Grant-in-Aid from the American Heart Association. We appreciate the statistical assistance provided by Vladimir Dragalin and the secretarial assistance of Carol Weed.

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_Circulation_. 2000;101:2296-2301
doi: 10.1161/01.CIR.101.19.2296

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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