Evaluation of Ultrasound-Assisted Thrombolysis Using Custom Liposomes in a Model of Retinal Vein Occlusion

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Purpose. To study the potential efficacy of ultrasound (US) assisted by custom liposome (CLP) destruction as an innovative thrombolytic tool for the treatment of retinal vein occlusion (RVO).

Methods. Experimental RVO was induced in the right eyes of 40 rabbits using laser photothrombosis; the US experiment took place 48 hours later. Rabbits were randomly divided into four equal groups: US+CLP group, US+saline group, CLP+sham US group, and no treatment group. The latter three groups acted as controls. Fundus fluorescein angiography and Doppler US were used to evaluate retinal blood flow.

Results. CLP-assisted US thrombolysis resulted in restoration of flow in seven rabbits (70%). None of the control groups showed significant restoration of retinal venous blood flow.

Conclusions. US-assisted thrombolysis using liposomes resulted in a statistically significant reperfusion of retinal vessels in the rabbit experimental model of RVO. This approach might be promising in the treatment of RVO in humans. Further studies are needed to evaluate this approach in patients with RVO. Ultrasound assisted thrombolysis can be an innovative tool in management of retinal vein occlusion. (*Invest Ophthalmol Vis Sci.* 2012;53:6920–6927) DOI:10.1167/iovs.12-10389

Retinal vein occlusion (RVO) is the second leading cause of retinal vascular disease after diabetic retinopathy,[1] with a prevalence of 0.7% to 1.6%.[1,2] In a population-based study, the overall incidence of symptomatic RVO in a 4-year period was found to be 0.21% in patients aged 40 or older.[3]

The clinical characteristics, prognosis, and response to treatment are influenced by the location of the occlusion in the retinal venous vasculature and by the extent of retinal nonperfusion.[4–6] RVO is classified, according to where the obstruction is located, into central retinal vein (CRVO), branch retinal vein (BRVO), and hemiretinal vein occlusion (HRVO). In CRVO, the location of the occlusion may occur at the level of or posterior to the lamina cribrosa. BRVO is an occlusion of either a major branch retinal vein draining one quadrant of the retina, a macular branch vein draining a portion of the macula, or a peripheral branch vein draining a portion of the retinal periphery.[7–9] In eyes with a HRVO, venous outflow from the superior or inferior retina is impaired.[10] In 20% of eyes, the merger of the superior and inferior venous trunks occurs posterior to the lamina cribrosa.[11] If one of these trunks becomes occluded, an HRVO occurs.[10–12]

Current treatment options for RVO include laser photocoagulation,[13–15] intravitreal steroid or anti-VEGF drug injection,[16–25] and less frequently, vitrectomy with and without sheathotomy.[26–32] Most of these approaches do not address the thrombus: the inciting underlying pathology. Sheathotomy does surgically try to dislodge the thrombus, but the procedure is complex. Systemic or local thrombolytic therapy using tissue plasminogen activator (tPA) or urokinase once seemed to be an interesting therapeutic modality; but these therapies have been associated with serious complications.[33–39]

The development of liposomes is a translational research endeavor. Its earliest impact has been in areas of biopharmaceuticals (e.g., drug delivery, drug discovery),[40–44] gene therapy,[45] and imaging.[46–50] Liposomes are microspheres with unique acoustic properties that make them useful as ultrasound (US) contrast agents for sonographic imaging, especially when their cores contain air or gas.[47–50] Experimental studies have shown that US-accelerated thrombolysis may be further enhanced by administration of liposome microspheres.[51–62] High power US has been shown to induce cavitation of liposomes and fluid motion into the thrombus.[52,53] Specifically, application of high acoustic pressure US has been shown to induce nonlinear oscillations of liposomes, leading to a continuous absorption of energy until the microspheres explode, releasing the absorbed energy.[55] The synergic effect of US and liposomes on sonothrombolysis has been demonstrated in clinical studies in patients with arteriovenous dialysis graft thrombosis.[56]

The objective of the present study was to test the innovative application of US-mediated liposome destruction in the treatment of RVO in an experimental animal model induced by laser photothrombosis (LPT), which depends on the use of rose bengal; a dye that, when exposed to light at its peak absorption wavelength (550 nm), generates oxygen singlet. This method seems to fairly well reflect the situation in the human disease. Peroxidation of endothelial cell lipids by singlet oxygen is the mechanism of membrane damage and, in blood vessels, may serve as the initial stimulus for platelet adhesion and aggregation.[63–66] Animal studies proved that laser-treated retinal vessels had a platelet-rich clot associated with injured endothelium, but there is no electron microscopic evidence of

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fibrin or damage to cells adjacent to the endothelial cells (e.g., smooth-muscle cells in the walls of arterioles).[67–69]

Materials and Methods

Animal Preparation

Forty age-matched, Dutch pigmented normal rabbits, each weighing 2 to 3 kg, were used. All animal experiments adhered to the regulations of the Institutional Animal Care and Use Committee of University of Southern California and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. For all animal procedures, the rabbits were anesthetized by intramuscular injection of a mixture of ketamine hydrochloride (25 mg/kg) and xylazine hydrochloride (6 mg/kg). The pupils were dilated with a topical application of phenylephrine hydrochloride 2.5% and tropicamide 0.5% eye drops.

Fundus Photography

All rabbits were scheduled for baseline imaging before LPT; imaging was repeated once, 48 hours after laser, and again, immediately after the US experiment. A digital fundus camera system (FF 450^{Plus}; Carl Zeiss Meditec AG, Jena, Germany) was used to obtain color fundus photographs and for fundus fluorescein angiography (FFA). The latter procedure was used to document the status of blood flow in retinal vessels.

RVO Model

Laser photothrombosis of a main branch retinal vein in the right eye of each rabbit was performed as described previously.[70] Briefly, the 532-nm laser (Iris Medical Oculight Glx; IRIDEX Corporation, Mountain View, CA) was applied to the retinal veins (guided by the baseline FFA photos) at or close to the disc margin, 10 seconds after intravenous injection of a dose of 40 mg/kg rose bengal dye (Sigma-Aldrich, Inc., St. Louis, MO). Laser shots were applied to either the main nasal or temporal retinal veins or to both. Care was taken to avoid damaging the adjacent arteries. Laser parameters were as follows: spot size, 125 μm; duration, 500 ms; power starting at 150 mW and rising to 300 mW after a few shots. A total of 10 to 30 shots were applied to the treatment spot (average 20). Whitening of the treated segment of the vein, distal engorgement, and proximal blanching were considered the treatment endpoints. Complete RVO was confirmed by FFA on the day of the US experiment. Rabbits with good occlusion on at least one side (i.e., nasal or temporal) were included in the study, while those with poor or no vein occlusion were excluded from the study.

Randomization and Animal Subgroups

Rabbits that were included in the study were divided into four equal groups: Group 1 (US+custom liposome [CLP]) received CLP-assisted US thrombolysis 48 hours after LPT. Group 2 (US+saline) received normal saline infusion during therapeutic US application. Group 3 (CLP+sham US) received CLP infusion, while the therapeutic US was turned off. Group 4 (no treatment) received 100 mL normal saline infusion, while the therapeutic US was turned off.

To reduce confounding by independent variables that have not been accounted for in the experimental design, animals that were included in the study were assigned randomly to the four animal subgroups using computer generated randomization tables.

CLP Preparation

The liposomes used in our study were custom built and composed of a core of sulfur hexafluoride gas and an outer shell of phospholipids (macrogol, distearoylphosphatidylcholine, dipalmitoylphosphatidylglycerol sodium, and palmitic acid). For the saline+US and the no treatment groups, 100-mL normal saline was infused intravenously (IV).

Probe Positioning

Each rabbit was placed on its left side with the speculum in place, and US gel (Aquasonic; Parker Laboratories, Inc., Fairfield, NJ) was applied. The probe unit was then attached to a stereotactic stand, allowing proper positioning of the probe, and avoiding untoward pressure on the globe that could give rise to erroneous measurements. The probe was applied to the inferior sclera with the long axis of the probe parallel to the limbus.

Spectral Doppler US Imaging

Measurement of the retinal vein blood velocity from both the nasal and temporal sides was obtained (as previously described),[71] both before and after therapeutic or sham US application.

For this purpose, we used a dedicated, small animal, high resolution imaging unit (Vevo 770; Visual Sonics, Toronto, Canada) and a 20-MHz, high frequency, linear transducer (lateral resolution, 140 μm at the focal depth; axial resolution, 140 μm; focal point, 15 mm with a depth of focus of 2.7 mm).

Therapeutic US

For therapeutic US, we applied the contrast imaging mode; we then applied 10 cycles of low-power destruction pulses (frequency, 10 MHz) every 5 minutes. For the US+saline group, 10 cycles of destruction pulses were run. For the CLP+sham US and the no treatment groups, the ultrasound probe was applied to the eye, but therapeutic US was turned off during the infusion.

Statistical Analysis

Data are shown as mean ± SD. Comparison between means was conducted by running the one-way ANOVA test. Statistical significance was defined as P less than 0.05. All analyses were conducted using the SPSS 17.0 statistical package (SPSS Inc., Chicago, IL).

Results

Analysis and interpretation of data obtained from FFA and Doppler flow studies were performed by an experienced co-author who was blinded to the animal subgroup when presented with the raw data.

Fluorescein Angiography

The Doppler results were confirmed by FFA in all cases. Evaluation of the status of the retinal vein blood flow as seen by the Doppler US correlated well with the FFA findings. All animals that were included in the study had complete RVO (except four animals), which were allocated randomly to the four groups of the study, thus, each group included nine complete RVO and one partial RVO.

Two patterns of branch retinal arterial occlusion (BRAO) developed in our study; immediate and delayed patterns. In the immediate (direct) pattern, the laser burns touched the arterial wall resulting in immediate spasm of the artery and subsequent arterial occlusion. We had four animals that developed this pattern of BRAO and these animals were excluded from the study.

On the other hand, the delayed (indirect) pattern is the pattern that developed within 48 hours as a part of the natural course of this model of RVO particularly with the complete venous occlusion pattern. This developed in four animals and these animals were randomly allocated to the four animal

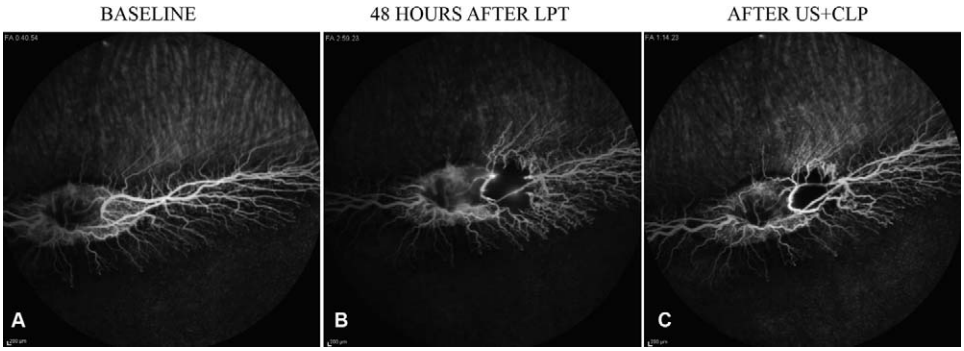

Figure 1. Fundus fluorescein angiographic images of a rabbit in the US+CLP group. Baseline imaging (**A**) shows normal retinal venous filling. Post laser imaging (**B**) shows no flow in a segment of the temporal retinal vein but retrograde filling from venous tributaries (indicates partial venous occlusion). Final imaging after CLP-enhanced US thrombolysis (**C**) shows return of flow in the occluded retinal vein and complete retinal venous filling.

subgroups. Thus, each animal subgroup included complete RVO ($n = 8$), complete RVO+arterial occlusion ($n = 1$), and partial RVO ($n = 1$).

In the US+CLP group, seven rabbits (70%) showed reperfusion of the occluded retinal veins; however, the reperfused vessels were smaller in diameter than they were before the laser occlusion (Figs. 1, 2, 3). None of the control groups showed any significant restoration of blood flow in the occluded veins after the experiment.

The Table shows the number of animals used in our study and the fate of each animal according to the pattern of vascular occlusion.

Retinal Venous Blood Velocity Measurement

Averaged pulse Doppler measurements of blood flow velocity in the experimentally occluded retinal veins at the end of the experiment were compared with those taken at the start of therapeutic or sham US experiments (Figs. 4, 5).

The mean venous blood velocity for the US+CLP group improved from 0.05 ± 0.1 cm/s before the experiment to 1.28 ± 0.9 cm/s at the end of the experiment, with a statistically significant difference (P value = 0.002). On the other hand, the final measurements of retinal venous blood velocity for the three control groups were not statistically different from the post laser measurements.

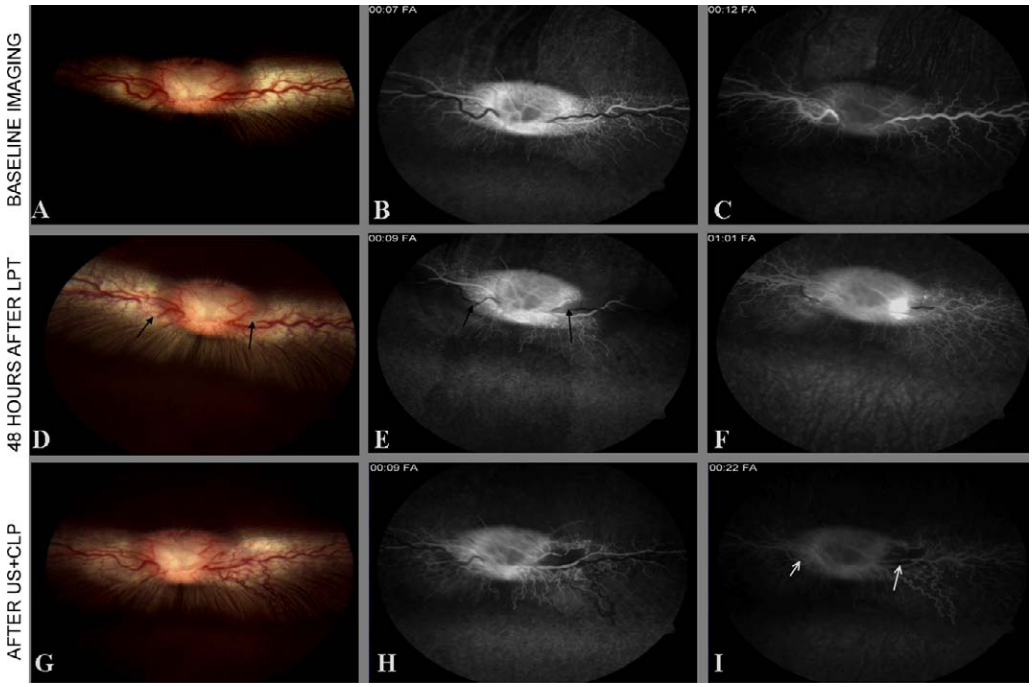

Figure 2. Fundus photography and fundus fluorescein angiogram of a rabbit in the US+CLP group with partial venous occlusion pattern. Baseline imaging shows normal retinal vessels in color photograph (**A**), early arterial flow shows up at 8 seconds (**B**), and when retinal venous filling is complete (**C**). Post laser imaging shows no flow in the nasal and temporal retinal veins in a small segment at the edge of the optic disc (**D**, **E**), while the remaining retinal veins fill late by collaterals (**F**). *Black arrows* point to the site of laser application. Final imaging after US experiment shows modest return of flow in the partially occluded retinal veins with complete filling by 22 seconds (**G**, **H**, **I**). *White arrows* point to filling of the previously occluded venous segment.

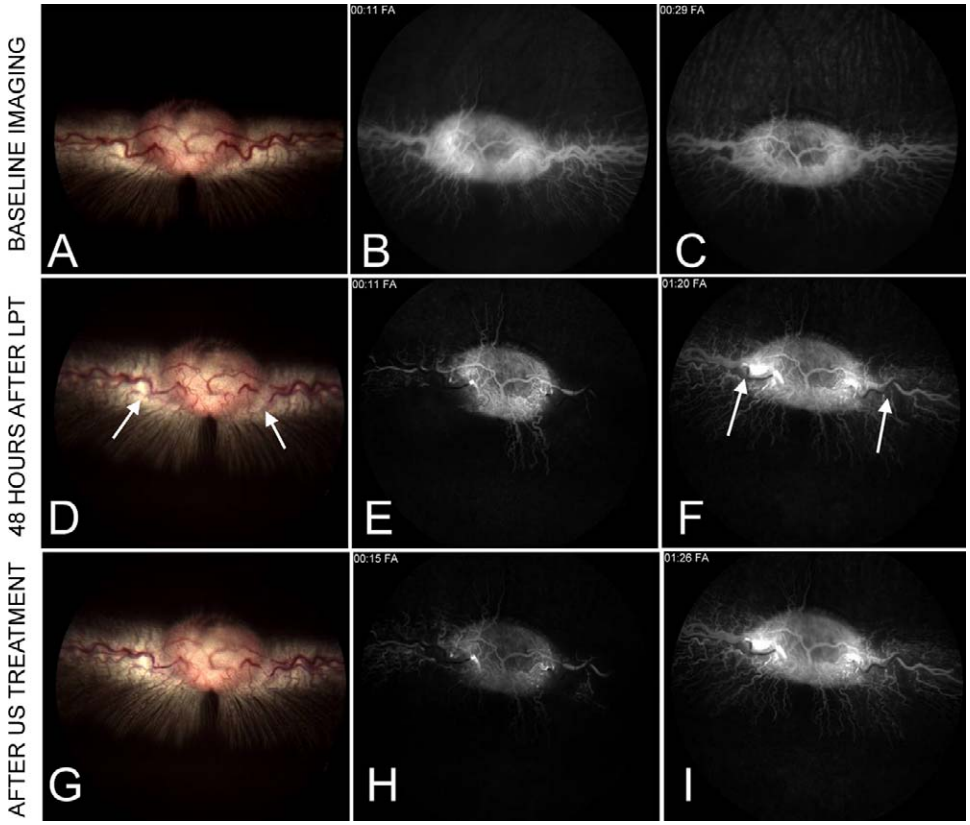

Figure 3. Fundus photographs and fundus fluorescein angiogram of a rabbit in the US+saline group. Images (**A**, **B**, **C**) represent those taken before laser photothrombosis, while (**D**, **E**, **F**) represent the images taken 48 hours after retinal vein occlusion, and (**G**, **H**, **I**) represent images taken at the end of therapeutic US application. *Arrows* in (**D**) and (**F**) point to site of laser application. Note the non filling of the proximal segment of the laser-treated retinal veins and the absence of filling after US application. The distal segments of these veins fill from collaterals.

Discussion

Although RVO represents a common, potentially blinding disease, most of the available treatment modalities are neither safe nor efficacious. This issue has led to a search for a safer and more effective treatment approach. A number of studies point to the possible value of US-assisted thrombolysis as an effective and potentially safe procedure, with several studies on experimental US thrombolysis in animals.

Table. This Table Describes the Distribution of Animals according to the Pattern of Occlusion and the Fate of Each.

Pattern of Vascular Occlusion	*n*	Fate of the Animal
Complete venous occlusion	36	Completed the study Randomly assigned to the 4 groups of the study (with each group having 9 animals of this pattern)
Partial venous occlusion	4	Completed the study Randomly assigned to the 4 groups of the study (with each group having 1 animal of this pattern)
Combined arterial+venous occlusion	5	Excluded from the study
None	4	Excluded from the study

The first approach examined the ability of high intensity US delivered via catheter to disrupt vascular thrombi without administration of plasminogen activator. The US frequency range was 19.5 to 26.5 kHz, and time of US application was 4 to 5 minutes.[59,61,72–76] The second approach used low intensity US in the range of 0.75 to 2 W/cm^2 to accelerate plasminogen-induced enzymatic thrombolysis. The US frequency range used was 20 kHz to 1MHz.[77–84]

Since their introduction in the market, liposomes have been extensively studied for use in drug delivery and molecular imaging.[85–87] One of the most important advantages of liposomes is the utilization of phospholipids that are natural components of cell membranes. Therefore, they can be eliminated from the body by simple degradation pathways without causing any toxic effect.[88] Similarly, numerous publications since 1995 demonstrate the safety of sulfur hexafluoride (SF_6) with the major route of elimination through the lungs.

Our approach in the present study was to use US-assisted destruction of liposomes to restore blood flow in cases with RVO. The time to apply the US pulse destruction cycles was governed by the appearance of a well-flowing CLP, as seen in the contrast imaging mode. We report success in 70% of cases treated with CLP-assisted US, without the use of any fibrinolytic agents.

To our knowledge, the only study on the effectiveness of US-assisted thrombolysis in RVO in rabbits was performed by Larsson et al.[79] In their study, instead of liposomes they used streptokinase with US. Moreover, the only tool they used to document reperfusion was FFA, performed 12 hours after the experiment. This may have compromised their results, as our

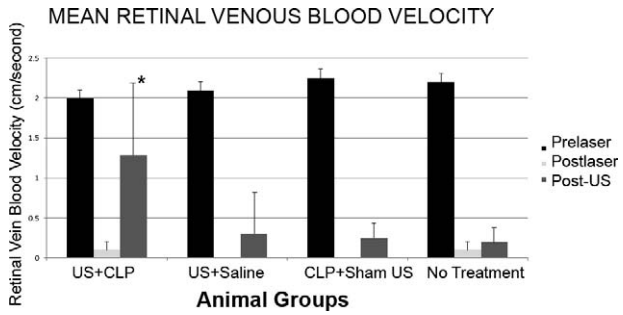

Figure 4. Graph shows the mean retinal venous blood velocity as measured before LPT (*baseline*), post laser, and after the US experiment (*after treatment*). Only the US+CLP group showed statistically significant improvement of blood flow at the end of the US experiment. *Refers to $P < 0.05$ with statistical significance

experience indicates that the laser model of RVO in rabbits can vary in terms of duration of occlusion. Specifically, the natural history of the vein occlusion itself may make the vein patent within hours of conducting the experiment. This reperfusion can be spontaneous and unrelated to the US experiment itself. We chose to perform our experiments 48 hours after LPT because, based on our previous studies,[70] maximum vascular occlusion occurs 2 days after LPT. The occluded vessels start recanalizing afterwards, reaching maximum revascularization several weeks later.[70]

The only way to obviate the possibility of natural opening of the vein occlusion is to perform FFA immediately after the experiment, as was done in our study. To further enforce our proof of assessment of the retinal blood flow, we measured the blood velocity in the retinal veins using pulse Doppler US during the experiment itself in the real time of the US experiment.

Many challenges were encountered in our study. First, the LPT model in rabbits is not fully reliable; we excluded four rabbits that underwent light laser application because of incomplete RVO, as determined by FFA on the third day post laser. Second, measurement of blood velocity in tiny vessels such as the retinal veins is difficult, and a good deal of experience is needed to ensure accuracy. We were able to successfully identify these vessels with a combination of power Doppler with pulse Doppler mode. As soon as power Doppler located a superficial blood flow near the retinal surface, pulse Doppler was used to measure the blood velocity and identify the retinal veins by their characteristic waveforms. Third, artifacts are produced during the measurement of blood velocity by pulse Doppler when a

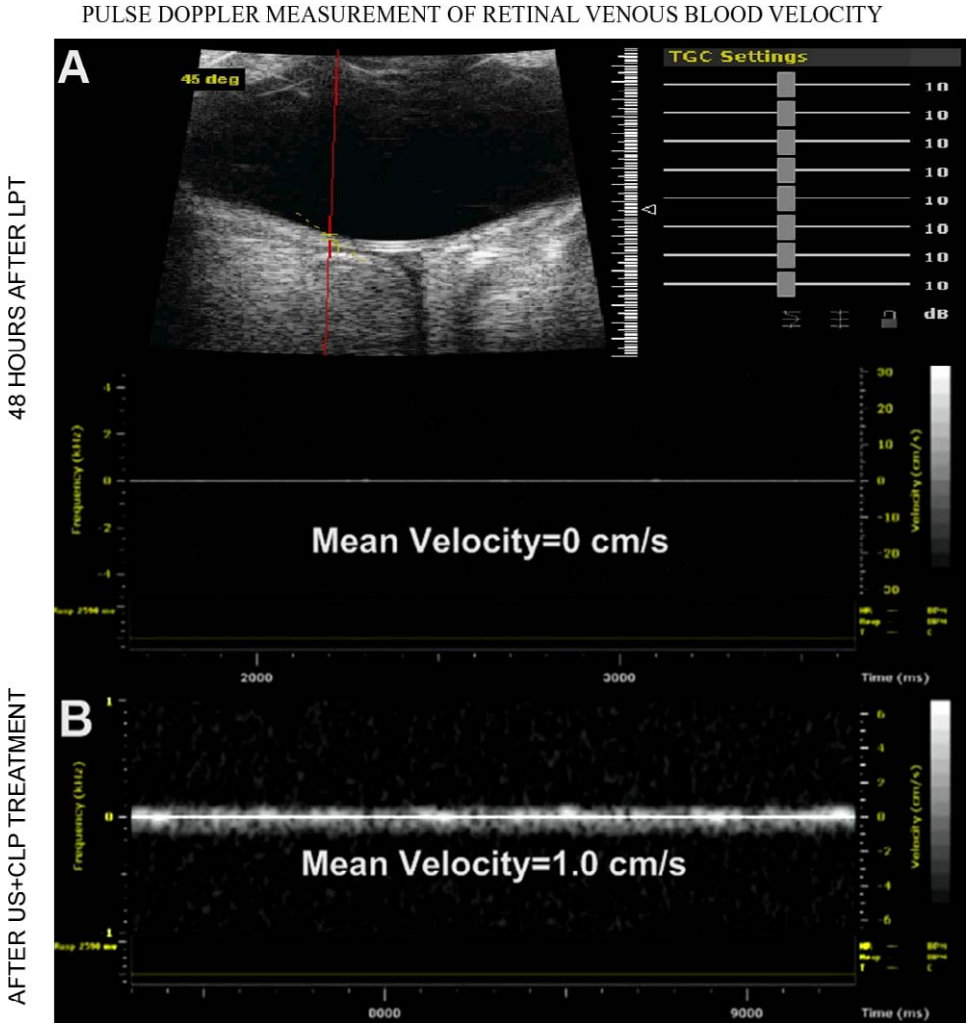

Figure 5. Spectral Doppler imaging of a rabbit in the US+CLP group shows no recordable retinal venous blood flow as measured from the retinal surface (**A**), while after CLP-enhanced US thrombolysis, retinal venous blood flow in the treated vein is restored, and the calculated average blood velocity is 1.0 cm/s (**B**).

high concentration of CLP is still in the circulation. We overcame this problem by avoiding any Doppler measurements while CLP were still in the blood stream. The CLP can be identified by the presence of spikes on the pulse Doppler and the blooming artifact on power Doppler.[83,84]

Our current study had several limitations, namely the relatively small sample of animals used, possible technical errors with Doppler US measurements, and BRAO that developed in some animals. Regarding the animal number, this study was designed as a feasibility study to better evaluate the use of US thrombolysis in RVO without fibrinolytic agents. But even with this limited number of cases, the results clearly demonstrate efficacy with the use of CLP-enhanced US. Also, the translation of this approach to patients with RVO, though exciting, should not be taken as a given and needs to be demonstrated. Patients have RVO due to a different pathophysiologic mechanism than the rabbit model in this study, and the duration of RVO is slightly different, usually longer. The latter results in structural changes to the thrombus and, possibly, in remodeling of the retinal vasculature, not to mention the long-term effects of retinal edema and hemorrhage. The safety of the technique seems good with minimal potential side effects. These do not seem to be directly related to the use of US+liposomes and are being summarized for future publication.

Regarding BRAO, we did not exclude these four cases, as we believe that arterial stagnation or even occlusion from extension of the thrombus is a natural evolution for a perfectly complete venous occlusion and can be unavoidable incident in some cases as reported in previous studies.[70]

We hypothesize that a difference exists between the two patterns of arterial occlusion. In the immediate BRAO, severe arterial wall damage was found on histopathologic studies (unpublished data), which in turn would have complicated our interpretation of the results if we had used these animals. On the other hand, in the delayed BRAO, thrombus formation was detected in the animals of the (CLP+sham US) and the (US+saline) groups, which explains why we decided to keep the animals with this occlusion pattern included in our study.

Regarding possible technical difficulties with Doppler measurements of retinal venous blood velocity, it was difficult to image such small vessels as the retinal veins in rabbits with 100% certainty. Hence, we relied mainly on FFA as the gold standard in order to document and confirm the presence or absence of the blood flow in the laser-treated retinal veins. However, we felt it would be more interesting to assess the flow by Doppler US using a combined technique of pulse Doppler and power Doppler in addition to fundus angiography.

Contrast-enhanced therapeutic ultrasound may carry some risk to patients. First, a fraction of MB is likely to burst under diagnostic acoustic intensities because of bubble wall impurities. Under high acoustic pressures, the stabilizing shells of MB may rupture, freeing gas bubbles. SF_6 is an inert, nontoxic, and safe gas. The route of SF_6 elimination is via the lungs in the exhaled air with approximately 40% to 50% of the dose being eliminated within the first minute and with greater than 75% of the dose eliminated by 11 minutes.[89,90] Second, MB cavitation risks involve chemical hazards (the creation of reactive free radicals) and mechanical hazards to inner vascular wall tissues.[89] Third, vascular bruising or vascular rupture normally heals, but the potential for cavitation harm could be clinically significant for some patients. Patients with hemophilia or on aspirin or other anticlotting therapies may be affected by microvascular ruptures.[91–93] Finally, the FDA ordered a black box warning on the use of perflutren-containing microbubbles in patients with acute coronary syndromes, acute myocardial infarction, and worsening or clinically unstable heart failure, severe emphysema and pulmonary emboli, or other conditions that cause pulmonary hypertension.[94,95]

Overall, however, microbubbles are very safe contrast agents with a minor adverse event rate of 0.13% (dizziness, erythematous rash, itching, nausea, and vomiting) and major adverse event rate of 0.0086% (dyspnea, bronchospasm, slight hypotension, clouding of consciousness, dorsolumbar pain, severe hypotension, and cutaneous rash).[96]

In our study, no major or minor adverse effects have been observed in any of the animal subgroups using FFA and optical coherence tomography (OCT) imaging, electroretinography, immunohistochemistry, and light and electron microscopy (unpublished data).

Conclusions

Liposome-assisted US thrombolysis is an innovative therapeutic tool for RVO. Further studies are needed to study the short- and long-term effects of this therapy in order to try to develop it as a possible treatment for patients with RVO.

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References

1. Klein R, Klein BE, Moss SE, Meuer SM. The epidemiology of retinal vein occlusion: the Beaver Dam Eye Study. *Trans Am Ophthalmol Soc*. 2000;98:133–141; discussion 141–133.
2. Mitchell P, Smith W, Chang A. Prevalence and associations of retinal vein occlusion in Australia. The Blue Mountains Eye Study. *Arch Ophthalmol*. 1996;114:1243–1247.
3. David R, Zangwill L, Badarna M, Yassur Y. Epidemiology of retinal vein occlusion and its association with glaucoma and increased intraocular pressure. *Ophthalmologica*. 1988;197:69–74.
4. Ho JD, Tsai CY, Liou SW, Tsai RJ, Lin HC. Seasonal variations in the occurrence of retinal vein occlusion: a five-year nationwide population-based study from Taiwan. *Am J Ophthalmol*. 2008; 145:722–728.
5. D'Amico DJ, Lit ES, Viola F. Lamina puncture for central retinal vein occlusion: results of a pilot trial. *Arch Ophthalmol*. 2006;124:972–977.
6. Jonas JB, Harder B. Ophthalmodynamometric differences between ischemic vs nonischemic retinal vein occlusion. *Am J Ophthalmol*. 2007;143:112–116.
7. Green WR, Chan CC, Hutchins GM, Terry JM. Central retinal vein occlusion: a prospective histopathologic study of 29 eyes in 28 cases. *Trans Am Soc*. 1981;79:371–422.
8. Hayreh SS. Prevalent misconceptions about acute retinal vascular occlusive disorders. *Prog Retin Eye Res*. 2005;24:493–519.
9. Beaumont PE, Kang HK. Clinical characteristics of retinal venous occlusions occurring at different sites. *Br J Ophthalmol*. 2002;86: 572–580.
10. Hayreh SS, Hayreh MS. Hemi-central retinal vein occlusion: pathogenesis, clinical features, and natural history. *Arch Ophthalmol*. 1980;98:1600–1609.
11. Chopdar A. Dual trunk central retinal vein incidence in clinical practice. *Arch Ophthalmol*. 1984;102:85–87.
12. Hayreh SS, Zimmerman MB, Podhajsky P. Incidence of various types of retinal vein occlusion and their recurrence and demographic characteristics. *Am J Ophthalmol*. 1994;117:429–441.
13. Anonymous. Argon laser scatter photocoagulation for prevention of neovascularization and vitreous hemorrhage in branch vein occlusion. A randomized clinical trial. Branch Vein Occlusion Study Group. *Arch Ophthalmol*. 1986;104:34–41.

14. Arnarsson A, Stefánsson E. Laser treatment and the mechanism of edema reduction in branch retinal vein occlusion. *Invest Ophthalmol Vis Sci*. 2000;41:877–879.
15. Finkelstein D. Argon laser photocoagulation for macular edema in branch vein occlusion. *Ophthalmology*. 1986;93:975–977.
16. Cekiç O, Chang S, Tseng JJ, et al. Intravitreal triamcinolone injection for treatment of macular edema secondary to branch retinal vein occlusion. *Retina*. 2005;25:851–855.
17. Jonas JB, Akkoyun I, Kamppeter B, Kreissig I, Degenring RF. Branch retinal vein occlusion treated by intravitreal triamcinolone acetonide. *Eye*. 2005;19:65–71.
18. Ozkiris A, Evereklioglu C. Erkiliç K, Ilhan O. The efficacy of intravitreal triamcinolone acetonide on macular edema in branch retinal vein occlusion. *Eur J Ophthalmol*. 2005;15:96–101.
19. Gelston CD, Olson JL, Mandava N. Macular oedema in central retinal vein occlusion treated with intravitreal triamcinolone. *Acta Ophthalmol Scand*. 2006;84:314–318.
20. Greenberg PB, Martidis A, Rogers AH, Duker JS, Reichel E. Intravitreal triamcinolone acetonide for macular oedema due to central retinal vein occlusion. *Br J Ophthalmol*. 2002;86:247–248.
21. Ip MS, Kumar KS. Intravitreous triamcinolone acetonide as treatment for macular edema from central retinal vein occlusion. *Arch Ophthalmol*. 2002;120:1217–1219.
22. Ahmadieh H, Moradian S, Malihi M. Rapid regression of extensive retinovitreal neovascularization secondary to branch retinal vein occlusion after a single intravitreal injection of bevacizumab. *Int Ophthalmol*. 2005;26:191–193.
23. Jaissle GB, Ziemssen F, Petermeier K, et al. Bevacizumab for treatment of macular edema secondary to retinal vein occlusion. *Ophthalmologe*. 2006;103:471–475.
24. Rabena MD, Pieramici DJ, Castellarin AA, Nasir MA, Avery RL. Intravitreal bevacizumab (Avastin) in the treatment of macular edema secondary to branch retinal vein occlusion. *Retina*. 2007; 27:419–425.
25. Iturralde D, Spaide RF, Meyerle CB, et al. Intravitreal bevacizumab (Avastin) treatment of macular edema in central retinal vein occlusion: a short-term study. *Retina*. 2006;26:279–284.
26. Charbonnel J, Glacet-Bernard A, Korobelnik JF, et al. Management of branch retinal vein occlusion with vitrectomy and arteriovenous adventitial sheathotomy, the possible role of surgical posterior vitreous detachment. *Graefes Arch Clin Exp Ophthalmol*. 2004; 242:223–228.
27. Kumagai K, Furukawa M, Ogino N, Uemura A, Larson E. Long-term outcomes of vitrectomy with or without arteriovenous sheathotomy in branch retinal vein occlusion. *Retina*. 2007;27:49–54.
28. Saika S, Tanaka T, Miyamoto T, Ohnishi Y. Surgical posterior vitreous detachment combined with gas/air tamponade for treating macular edema associated with branch retinal vein occlusion: retinal tomography and visual outcome. *Graefes Arch Clin Exp Ophthalmol*. 2001;239:729–732.
29. Stefánsson E. The therapeutic effects of retinal laser treatment and vitrectomy. A theory based on oxygen and vascular physiology. *Acta Ophthalmol Scand*. 2001;79:435–440.
30. Hvarfner C, Larsson J. Vitrectomy for non-ischaemic macular oedema in retinal vein occlusion. *Acta Ophthalmol Scand*. 2006; 84:812–814.
31. Liang XL, Chen HY, Huang YS, et al. Pars plana vitrectomy and internal limiting membrane peeling for macular oedema secondary to retinal vein occlusion: a pilot study. *Ann Acad Med Singapore*. 2007;36:293–297.
32. Mandelcorn MS, Nrusimhadevara RK. Internal limiting membrane peeling for decompression of macular edema in retinal vein occlusion: a report of 14 cases. *Retina*. 2004;24:348–355.
33. Elman MJ. Thrombolytic therapy for central retinal vein occlusion: results of a pilot study. *Trans Am Ophthalmol Soc*. 1996;94:471–504.
34. Howden GD. The successful treatment of a case of central retinal vein thrombosis with intravenous fibrinolysin. *Can Med Assoc J*. 1959;81:382–384.
35. Lahey JM, Fong DS, Kearney J. Intravitreal tissue plasminogen activator for acute central retinal vein occlusion. *Ophthalmic Surg Lasers*. 1999;30:427–434.
36. Sharma A, D'Amico DJ. Medical and surgical management of central retinal vein occlusion. *Int Ophthalmol Clin*. 2004;44:1–16.
37. Glacet-Bernard A, Kuhn D, Vine AK, Oubraham H, Coscas G, Soubrane G. Treatment of recent onset central retinal vein occlusion with intravitreal tissue plasminogen activator: a pilot study. *Br J Ophthalmol*. 2000;84:609–613.
38. Weiss JN. Treatment of central retinal vein occlusion by injection of tissue plasminogen activator into a retinal vein. *Am J Ophthalmol*. 1998;126:142–144.
39. Weiss JN, Bynoe LA. Injection of tissue plasminogen activator into a branch retinal vein in eyes with central retinal vein occlusion. *Ophthalmology*. 2001;108:2249–2257.
40. Farshi FS, Ozer AY, Ercan MT, Hincal AA. In-vivo studies in the treatment of oral ulcers with liposomal dexamethasone sodium phosphate. *J Microencapsul*. 1996;13:537–544.
41. Huwyler J, Yang J, Pardridge WM. Receptor mediated delivery of daunomycin using immunoliposomes: pharmacokinetics and tissue distribution in the rat. *J Pharmacol Exp Ther*. 1997;282: 1541–1546.
42. Elbayoumi TA, Pabba S, Roby A, Torchilin VP. Antinucleosome antibody-modified liposomes and lipid-core micelles for tumor targeted delivery of therapeutic and diagnostic agents. *J Liposome Res*. 2007;17:1–14.
43. Ramana LN, Sethuraman S, Ranga U, Krishnan UM. Development of a liposomal nanodelivery system for nevirapine. *J Biomed Sci*. 2010;17:57.
44. Pierre MB. Dos Santos Miranda Costa I. Liposomal systems as drug delivery vehicles for dermal and transdermal applications. *Arch Dermatol Res*. 2011;303:607–621.
45. Yamashita T, Sonoda S, Suzuki R, et al. A novel bubble liposome and ultrasound-mediated gene transfer to ocular surface: RC 1 cells in vitro and conjunctiva in vivo. *Exp Eye Res*. 2007;85:741–748.
46. Sachse A, Leike JU, Rössling GL, Wagner SE, Krause W. Preparation and evaluation of lyophilized iopromide-carrying liposomes for liver tumor detection. *Invest Radiol*. 1993;28:838–844.
47. Demos SM, Alkan-Onyuksel H, Kane BJ, et al. In vivo targeting of acoustically reflective liposomes for intravascular and transvascular ultrasonic enhancement. *J Am Coll Cardiol*. 1999;33:867–875.
48. Erdoğan S, Ozer AY, Volkan B, Caner B, Bilgili H. Thrombus localization by using streptokinase containing vesicular systems. *Drug Deliv*. 2006;13:303–309.
49. Chow TH, Lin YY, Hwang JJ, et al. Therapeutic efficacy evaluation of 111In-labeled PEGylated liposomal vinorelbine in murine colon carcinoma with multimodalities of molecular imaging. *J Nucl Med*. 2009;50:2073–2081.
50. Ferrara KW, Borden MA, Zhang H. Lipid-shelled vehicles: engineering for ultrasound molecular imaging and drug delivery. *Acc Chem Res*. 2009;42:881–892.
51. Delorme S, Krix M. Contrast-enhanced ultrasound for examining tumor biology. *Cancer Imaging*. 2006;27:148–152.
52. Mizushige K, Kondo I, Ohmori K, Hirao K, Matsuo H. Enhancement of ultrasound-accelerated thrombolysis by echo-contrast agents: dependence on microbubble structure. *Ultrasound Med Biol*. 1999;25:1431–1437.
53. Cintas P, Nguyen F, Boneu B, Larrue V. Enhancement of enzymatic fibrinolysis with 2-MHz ultrasound and microbubbles. *J Thromb Haemost*. 2004;2:1163–1166.
54. Culp WC, Porter TR, Lowery J, Xie F, Roberson PK, Marky L. Intracranial clot lysis with intravenous microbubbles and transcranial ultrasound in swine. *Stroke*. 2004;35:2407–2411.

55. Dijkmans PA, Juffermans LJM, Musters RJP, et al. Microbubbles and ultrasound: from diagnosis to therapy. *Eur J Echocardiogr*. 2004;5:245–256.
56. Xie F, Tsutsui JM, Lof J, et al. Effectiveness of lipid microbubbles and ultrasound in declotting thrombosis. *Ultrasound Med Biol*. 2005;31:979–985.
57. Trübestein G, Engel C, Etzel F, Sobbe A, Cremer H, Stumpff U. Thrombolysis by ultrasound. *Clin Sci Mol Med Suppl*. 1976;3:697s–698s.
58. Tachibana K, Tachibana S. Albumin microbubble echo-contrast material as an enhancer for ultrasound accelerated thrombolysis. *Circulation*. 1995;92:1148–1150.
59. Birnbaum Y, Iakobishvili Z, Porter A, et al. Microparticle-containing oncotic solutions augment in-vitro clot disruption by ultrasound. *Thromb Res*. 2000;98:549–557.
60. Behrens S, Daffertshofer M, Spiegel D, Hennerici M. Low-frequency, low-intensity ultrasound accelerates thrombolysis through the skull. *Ultrasound Med Biol*. 1999;25:269–273.
61. Nishioka T, Luo H, Fishbein MC, et al. Dissolution of thrombotic arterial occlusion by high intensity, low frequency ultrasound and dodecafluoropentane emulsion: an in vitro and in vivo study. *J Am Coll Cardiol*. 1997;30:561–568.
62. Danis RP, Yang Y, Massicotte SJ, Boldt HC. Preretinal and optic nerve head neovascularization induced by photodynamic venous thrombosis in domestic pigs. *Arch Ophthalmol*. 1993;111:539–543.
63. Banks JS, Board RG, Carter J, et al. The cytotoxic and photodynamic inactivation of microorganisms by rose bengal. *J Appl Bacteriol*. 1985;58:391–400.
64. Pooler JP, Valenzeno DP. Dye sensitized photodynamic inactivation of cells. *Med Phys*. 1981;8:614–628.
65. Hull DS, Green K, Laughter L. Corneal endothelial rose bengal photosensitization. *Invest Ophthalmol Vis Sci*. 1984;25:455–460.
66. Hull DS, Strickland EC, Green K. Photodynamically induced alteration of corneal cell endothelial function. *Invest Ophthalmol Vis Sci*. 1979;18:1226–1231.
67. Arroyo JG, Dastgheib K, Hatchell DL. Antithrombotic effect of ticlopidine in an experimental model of retinal vein occlusion. *Jpn J Ophthalmol*. 2001;45:359–362.
68. Royster AJ, Nanda SK, Hatchell DL, Tiedeman JS, Dutton JJ, Hatchell MC. Photochemical initiation of thrombosis. Fluorescein angiographic, histologic, and ultrastructural alterations in the choroid, retinal pigment epithelium, and retina. *Arch Ophthalmol*. 1988;106:1608–1614.
69. Nanda SK, Hatchell DL, Tiedeman JS, Dutton JJ, Hatchell MC, McAdoo T. A new method for vascular occlusion. Photochemical inititation of thrombosis. *Arch Ophthalmol*. 1987;105:1121–1124.
70. Ameri H, Ratanapakorn T, Rao NA, Chader GJ, Humayun MS. Natural course of experimental retinal vein occlusion in rabbit; arterial occlusion following venous photothrombosis. *Graefes Arch Clin Exp Ophthalmol*. 2008;246:1429–1439.
71. Abdallah W, Fawzi A, Patel H, et al. Blood velocity measurement in the posterior segment of the rabbit eye using combined spectral Doppler and power Doppler ultrasound. *Graefes Arch Clin Exp Ophthalmol*. 2010;248:93–101.
72. Rosenschein U, Bernstein JJ, DiSegni E, Kaplinsky E, Bernheim J, Rozenzsajn LA. Experimental ultrasonic angioplasty: disruption of atherosclerotic plaques and thrombi in vitro and arterial recanalization in vivo. *J Am Coll Cardiol*. 1990;15:711–717.
73. Ariani M, Fishbein MC, Chae JS, et al. Dissolution of peripheral arterial thrombi by ultrasound. *Circulation*. 1991;84:1680–1688.
74. Steffen W, Fishbein MC, Luo H, et al. High intensity, low frequency catheter-delivered ultrasound dissolution of occlusive coronary artery thrombi: an in vitro and in vivo study. *J Am Coll Cardiol*. 1994;24:1571–1579.
75. Hamano K. Thrombolysis enhanced by transcutaneous ultrasonic irradiation. *Tokyo Jikeikai Med J*. 1991;106:533–542.
76. Luo H, Nishioka T, Fishbein MC, et al. Transcutaneous ultrasound augments lysis of arterial thrombi in vivo. *Circulation*. 1996;94:775–778.
77. Suchkova VN, Baggs RB, Francis CW. 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. *Circulation*. 2000;101:2296–2301.
78. Riggs PN, Francis CW, Bartos SR, Penney DP. Ultrasound enhancement of rabbit femoral artery thrombolysis. *Cardiovasc Surg*. 1997;5:201–207.
79. Larsson J, Carlson J, Olsson SB. Ultrasound enhanced thrombolysis in experimental retinal vein occlusion in the rabbit. *Br J Ophthalmol*. 1998;82:1438–1440.
80. Kashyap A, Blinc A, Marder VJ, Penney DP, Francis CW. Acceleration of fibrinolysis by ultrasound in a rabbit ear model of small vessel injury. *Thromb Res*. 1994;76:475–485.
81. Stone MJ, Frenkel V, Dromi S, et al. Pulsed-high intensity focused ultrasound enhanced tPA mediated thrombolysis in a novel in vivo clot model, a pilot study. *Thromb Res*. 2007;121:193–202.
82. Wu Y, Unger EC, McCreery TP, et al. Binding and lysing of blood clots using MRX-408. *Invest Radiol*. 1998;33:880–885.
83. Forsberg F, Liu JB, Burns PN, Merton DA, Goldberg BB. Artifacts in ultrasonic contrast agent studies. *J Ultrasound Med*. 1994;13:357–365.
84. Correas JM, Bridal L, Lesavre A, Méjean A, Claudon M, Hélénon O. Ultrasound contrast agents: properties, principles of action, tolerance, and artifacts. *Eur Radiol*. 2001;11:1316–1328.
85. Geers B, Lentacker I, Sanders NN, et al. Self-assembled liposome-loaded microbubbles: The missing link for safe and efficient ultrasound triggered drug-delivery. *J Control Release*. 2011;152:249–256.
86. Li T, Zhang M, Han Y, Zhang H, Xu L, Xiang Y. Targeting therapy of choroidal neovascularization by use of polypeptide- and PEDF-loaded immunoliposomes under ultrasound exposure. *J Huazhong Univ Sci Technolog Med Sci*. 2010;30:798–803.
87. Schroeder A, Kost J, Barenholz Y. Ultrasound, liposomes, and drug delivery: principles for using ultrasound to control the release of drugs from liposomes. *Chem Phys Lipids*. 2009;162:1–16.
88. Silindir M, Erdoğan S, Ozer AY, Liposomes Maia S. and their applications in molecular imaging. *J Drug Target*. 2012;20:401–415.
89. Duck FA. Hazards, risks and safety of diagnostic ultrasound. *Med Eng Phys*. 2008;30:1338–1348.
90. Bokor D. Diagnostic efficacy of SonoVue. *Am J Cardiol*. 2000;86:19G–24G.
91. Blomley M, Claudon M, Cosgrove D. WFUMB Safety Symposium on Ultrasound Contrast Agents: clinical applications and safety concerns. *Ultrasound Med Biol*. 2007;33:180.
92. Miller DL, Quddus J. Diagnostic ultrasound activation of contrast agent gas bodies induces capillary rupture in mice. *Proc Natl Acad Sci U S A*. 2000;97:10179–10184.
93. Kusnetzky LL, Khalid A, Khumri TM, et al. Acute mortality in hospitalized patients undergoing echocardiography with and without an ultrasound contrast agent. *J Am Coll Cardiol*. 2008;51:1704–1706.
94. Main ML, Goldman JH, Grayburn PA. Ultrasound contrast agents: balancing safety versus efficacy. *Expert Opin Drug Saf*. 2009;8:49–56.
95. Grayburn PA. Product safety compromises patient safety (an unjustified black box warning on ultrasound contrast agents by the Food and Drug Administration). *Am J Cardiol*. 2008;101:892–893.
96. Piscaglia F, Bolondi L. The safety of Sonovue in abdominal applications: retrospective analysis of 23188 investigations. *Ultrasound Med Biol*. 2006;32:1369–1375.