In Vivo Therapeutic Gas Delivery for Neuroprotection With Echogenic Liposomes

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Background—Ischemia-related neurological injury is a primary cause of stroke disability. Studies have demonstrated that xenon (Xe) may have potential as an effective and nontoxic neuroprotectant. Xe delivery is, however, hampered by lack of suitable administration methods. We have developed a pressurization-freeze method to encapsulate Xe into echogenic liposomes (Xe-ELIP) and have modulated local gas release with transvascular ultrasound exposure.

Methods and Results—Fifteen microliters of Xe were encapsulated into each 1 mg of liposomes (70% Xe and 30% argon). Xe delivery from Xe-ELIP into cells and consequent neuroprotective effects were evaluated with oxygen/glucose-deprived and control neuronal cells in vitro. Xe-ELIP were administered into Sprague-Dawley rats intravenously or intra-arterially after right middle cerebral artery occlusion. One-megahertz low-amplitude (0.18 MPa) continuous wave ultrasound directed onto the internal carotid artery triggered Xe release from circulating Xe-ELIP. Effects of Xe delivery on ischemia-induced neurological injury and disability were evaluated. Xe-ELIP delivery to oxygen/glucose-deprived neuronal cells improved cell viability in vitro and resulted in a 48% infarct volume decrease in vivo. Intravenous Xe-ELIP administration in combination with the ultrasound directed onto the carotid artery enhanced local Xe release from circulating Xe-ELIP and demonstrated 75% infarct volume reduction. This was comparable to the effect after intra-arterial administration. Behavioral tests on limb placement and grid and beam walking correlated with infarct reduction.

Conclusions—This novel methodology may provide a noninvasive strategy for ultrasound-enhanced local therapeutic gas delivery for cerebral ischemia–related injury while minimizing systemic side effects. (Circulation. 2010;122:1578-1587.)

Key Words: cerebral ischemia ■ contrast media ■ liposomes ■ stroke ■ xenon

Stroke is the third leading cause of death in the United States and the most common cause of adult disabilities. Ischemic stroke follows occlusion of a cerebral artery, resulting in obstructed blood flow to a portion of the brain.1 Emergent treatments of acute ischemic stroke have 2 objectives: rapid restoration of cerebral blood flow (reperfusion) and restriction of neuronal injury (neuroprotection). Currently, the only Food and Drug Administration–approved thrombolytic agent for the treatment of ischemic stroke is tissue plasminogen activator in selected patients who present early with no evidence of intracerebral hemorrhage.2,3 Other approaches using neuroprotective agents have generated great interest as an adjunct to thrombolytic therapy,4 but no agent has been approved for clinical application.

Clinical Perspective on p 1587

Several neuroprotective agents have been developed to interrupt ischemic injury by targeting the N-methyl-D-aspartate (NMDA) receptor.5–8 Many of the NMDA receptor antagonists have not been approved for clinical use because of neurotoxicity9–11 or failure to cross the blood-brain barrier to the site of injury.12 Bioactive gases such as xenon (Xe), isoflurane, sevoflurane, and nitrous oxide are promising neuroprotective agents with minimal adverse effects because of their low blood/gas solubility, with resultant rapid inflow and washout.13 Inhalation of 70% Xe provides a pharmacological profile similar to that of low-affinity NMDA receptor antagonists with few neurotoxic side effects.14–20 This neuroprotective effect is dose dependent, requiring a high concentration of inhaled Xe (50% to 70%) for a noticeable effect. Such a concentration would not provide sufficient inspired oxygen essential for cell survival, and therefore this would be difficult to translate clinically.

Liposomes are artificial submicron vesicles consisting of a phospholipid bilayer and a hydrophilic core. The phospholipid bilayer is ideal for incorporating a variety of hydrophobic drugs, including gases, while maintaining the physiological inertness of its contents. We have developed echogenic...
liposomes (ELIP) that can encapsulate a variety of bioactive gases such as nitric oxide, allowing both echogenicity and gas delivery. Xe may be encapsulated into ELIP with the use of the same methodology, allowing efficient Xe delivery with therapeutic benefit.

In this study, we have (1) developed Xe-containing ELIP (Xe-ELIP) using a pressurization-freeze method and evaluated their physical and sonographic characteristics; (2) investigated the effects of ultrasound exposure for triggering Xe release from Xe-ELIP; (3) determined the therapeutic effects of Xe-ELIP on cultured hypoxic PC12 cells in vitro; and (4) evaluated the pathological and behavioral changes in a rodent model of cerebral ischemia with ultrasound-enhanced Xe delivery using Xe-ELIP.

Methods
Preparation of Xe-Containing Liposomes
Liposomes were composed of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC; Avanti Polar Lipids, Alabaster, Ala); 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC; Avanti Polar Lipids); and cholesterol (Sigma, St Louis, Mo) at a molar ratio of 60:30:10. Five milligrams of lipids was mixed in chloroform, and the solvent was evaporated with argon in a 50°C water bath to form a thin film on the glass vial. The lipid film was placed under vacuum (<100 μm Hg) for 4 to 6 hours for complete solvent removal. The dried lipid film was hydrated with 0.32 mol/L mannitol to a concentration of 10 mg lipid per milliliter, followed by sonication for 5 minutes. The sonicated liposomes were transferred to a 2-mL glass vial with a cap sealed with a Teflon-rubber septum. Ten milliliters of a Xe (70%) (Concorde Specialty Gas Inc, Eatontown, NJ) and argon (30%) mixture was injected into the glass vial through the Teflon-rubber septum with a 12-mL syringe attached to a 27-gauge (30%) mixture was injected into the glass vial through the Teflon-rubber septum with a 12-mL syringe attached to a 27-gauge needle. A ratio of 70% Xe and 30% argon was chosen because preliminary data showed that 70% Xe produced the maximal neuroprotective effect. The pressurized liposomal dispersion was frozen at −70°C with dry ice for at least half an hour. The liposomal dispersion was allowed to thaw after the vial was unpressurized by removing the cap.

Xe Release Profile
The spontaneous Xe release profiles from Xe-ELIP were determined by a syringe method as described previously. A dispersion of 200 μL of Xe-ELIP was added into 1.8 mL of phosphate-buffered saline (PBS) (GIBCO; Invitrogen Co, Carlsbad, Calif) or human serum plasma (HSP) (Innovative Research Inc, Novi, Mich) and incubated at 37°C. The release of Xe was measured at different time points (0, 5, 10, 30, 60 minutes and 18 hours) after incubation. PBS and HSP solutions devoid of Xe-ELIP were used as controls to correct Xe release from Xe-ELIP with respect to the residual Xe in the solution. Each Xe-ELIP sample was prepared separately at each time point, and Xe release was compared between the PBS and HSP groups (ie, comparison of Xe release was performed independently at each time point).

Ultrasound-Triggered Xe Release From Xe-ELIP
Ultrasound-triggered Xe release from Xe-ELIP was evaluated with the use of a flow system that mimics physiological flow conditions (Figure 1). Xe-ELIP (250 μL, 10 mg lipid per milliliter) was diluted in 1.75 mL PBS, and 2 mL of the diluted Xe-ELIP was injected into 2 cm of latex tubing (1/8-inch inner diameter, 1/32-inch wall thickness; McMaster-Carr, Atlanta, Ga), submerged in degassed water at 22°C, and placed directly on acoustically absorptive rubber (Precision Acoustics Ltd, Dorchester, Dorset, UK) to minimize acoustic reflections. A calibrated 1-MHz continuous wave ultrasound transducer that has a 6-mm aperture (Sonitron 2000; Rich-Marc Corp, Inola, Okla) was mounted 5 mm above the latex tubing, and the flow was exposed to 0.18-MPa peak-to-peak pressure amplitude. A constant flow rate of 10 mL/min was used with a syringe pump. The ultrasound-exposed Xe-ELIP solution was collected to measure residual Xe.

The amount of Xe released (Vrelease) from Xe-ELIP with ultrasound exposure was determined by subtracting the residual Xe (Vpost) from the initial Xe amount (Vpre) in the Xe-ELIP with the use of the same syringe method. The percentage of Xe release, Vrelease%, was calculated as follows:

\[ V_{\text{release}}% = \frac{V_{\text{post}} - V_{\text{pre}}}{V_{\text{pre}}} \times 100 (\%) \]

Neuroprotective Effects of Xe on Cultured PC12 Cells
PC12 cells (ATCC, Manassas, Va) were placed in 24-multiwell plates (Costar, Cambridge, Mass) and incubated in medium consisting of Eagle’s minimum essential medium (ATCC) supplemented with 20 mmol/L glucose, 26 mmol/L NaHCO3, 10% fetal bovine serum (GIBCO), 10% heat-inactivated horse serum, penicillin-streptomycin solution (GIBCO), 2 mmol/L glutamine (Sigma, Poole, UK), and 10 ng/mL murine epidermal growth factor (GIBCO). After confluence was reached, oxygen/glucose deprivation was induced by incubating PC12 cells with deoxygenated PBS solution in the absence of glucose in an anoxic chamber for 3 hours at 37°C. After hypoxic treatment, deoxygenated PBS was removed. Standard PBS was placed in the control wells, no-treatment wells, air-containing ELIP (air-ELIP) treatment wells (80% O2), and Xe-ELIP treatment wells (80% Xe) from the initial Xe amount (Vpost) in the Xe-ELIP with the use of the same syringe method. The percentage of Xe release, Vrelease%, was calculated as follows:
μg/mL) at 37°C in culture medium for 3 hours, washed, and incubated in 0.08 mol/L HCl/isopropanol to dissolve the blue formazan product. The concentration of blue formazan salt, a surrogate of cell viability, was quantified by measuring absorbance at 570 nm with the use of a Tecan Safire® plate reader (Tecan Group Ltd, Männedorf, Switzerland). Data were reported as percentage of the released blue formazan compared with controls.

**Rat Model of Middle Cerebral Artery Ischemia/Reperfusion**

All animal experiments were approved by the Animal Welfare Committee at the University of Texas Health Science Center at Houston. A total of 40 Sprague-Dawley male rats (weight, 260 to 280 g; Harlan Laboratories Inc, Indianapolis, Ind) were fasted for 24 hours with free access to water before surgery. Before surgery, anesthesia was induced by intraperitoneal injection of a cocktail solution of ketamine (25 mg/mL), diazepam (2 mg/mL), and atropine (0.1 mg/mL) at a dose of 2.5 mL/Kg. Marcaine (2 mg/kg) was injected subcutaneously at the surgical site to provide topical analgesia. Cerebral ischemia was induced by occluding the right middle cerebral artery (MCA) for 2 hours by the intraluminal suture method.25–27

With the use of an operating microscope, the right common carotid artery was exposed through a midline neck incision and carefully dissected free from surrounding nerves and fascia. The external carotid artery was isolated and dissected distally to the bifurcation of the lingual and maxillary artery branches. The external carotid artery was ligated close to its distal end. The internal carotid artery (ICA) was isolated and separated from adjacent tissues. A 25-cm 4-0 monofilament nylon suture (Ethicon, Somerville, NJ) was blunted by heating the tip with the use of a low-temperature cautzerizer (World Precision Instruments, Sarasota, Fla). The suture was coated with poly-L-lysine (0.1% [wt/vol]) and heparin solution (1000 U/mL) in deionized water and dried with the cautzerizer tip downward at room temperature for 12 hours. The suture was inserted into the MCA lumen located 18 to 20 mm from the external carotid artery/common carotid artery bifurcation for 2 hours to provoke ischemia. As soon as the suture was removed, Xe-saturated saline (200 μL) or Xe-ELIP (200 μL, 10 mg/mL) was administered via the tail vein or through a catheter into the ICA in the antegrade direction. In all experiments, body temperature was monitored and maintained at 37°C during ischemia and for the first hour of reperfusion with the use of a feed-forward temperature controller that has a heating lamp and heating pad (Harvard Apparatus, Holliston, Mass). A polyethylene catheter was introduced into the right femoral artery for pressure recording.

Cerebral blood flow was measured before and after MCA occlusion and after reperfusion, just before Xe delivery, and after Xe delivery with the use of a PR407–1 straight-needle laser Doppler flowmeter probe (Perimed, Järfälla, Stockholm, Sweden) connected to a standard laser Doppler monitor (PF5010 LDPM unit and PF5001 main unit; Perimed, Järfälla). Interruption of blood flow was recorded in the region of ischemic penumbra (2 mm lateral and 2 mm anterior to the ICA).24

Animals were randomly divided into 7 groups (n=8 in each group): (1) normal group: skin incision without MCA occlusion; (2) no-treatment group: MCA occlusion only; (3) treatment group A: intra-arterial Xe-ELIP administration into common carotid artery after MCA occlusion; (4) treatment group B: intravenous Xe-saturated saline administration after MCA occlusion; (5) treatment group C: intravenous Xe-saturated saline administration with ultrasound activation on ICA after MCA occlusion; (6) treatment group D: intravenous Xe-ELIP injection after MCA occlusion; and (7) treatment group E: intravenous Xe-ELIP injection with ultrasound activation on ICA after MCA occlusion.

**Transvascular Ultrasound Application for Enhanced Xe Release**

The Soniton ultrasound probe was placed 5 mm above the ICA to trigger Xe release from Xe-ELIP during the 4-minute period of liposomal injection. A PBS solution was filled between the artery and probe to ensure adequate acoustic coupling. The ICA was exposed to 1-MHz continuous wave ultrasound at a peak-to-peak pressure amplitude of 0.18 MPa (1-W/cm² dial setting) during tail vein injection of the Xe-ELIP.

**Neurological Assessment**

All behavioral tests in mice were conducted in a quiet and low-lit room by an observer blinded with respect to the treatment groups. At days 1, 2, and 3 after surgery, each animal was tested for motor function and neurological outcomes by recording limb-placing, beam-walking, and grid-walking abilities.24 Limb placement was assessed by observing the animal’s ability to lift its head and extend its forelimbs toward a table while the animal was suspended over the table by its tail (zero score, no response; score of 1, when response was sluggish or delayed; score of 2, when response was rapid and fully executed). The ability to walk across a beam (2.5×2.5×80 cm) was assessed by observing the ability to maintain balance while navigating across the beam. The response scores were assigned as follows: score 0, traversed the beam without foot slip; score 1, traversed with grasping of the lateral side of the beam; score 2, showed difficulty crossing the beam but able to traverse; score 3, required >10 seconds to traverse the beam because of difficulty in walking; score 4, unable to traverse the beam; score 5, unable to move the body or any limb on the beam; and score 6, unable to stay on the beam for >10 seconds. Grid-walking ability was assessed by placing the animal on a stainless steel grid floor (20×40 cm with a mesh size of 2×2 cm). The total number of steps was counted up to a maximum of 50 steps. The number of foot fault errors as defined by the misplacement of a forelimb or hindlimb that fell through the grid was recorded.

**Infarct Volume Measurement**

Animals were euthanized on the third day after neurological assessment. Brains were harvested. With the use of a Jacobowitz brain slicer, 2-mm-thick coronal sections were cut before staining with 2% 2,3,5-triphenyltetrazolium chloride (TTC) in PBS for 20 minutes at 37°C for infarct volume determination. Stained sections were transferred to 10% phosphate-buffered formalin for storage. Sections were photographed with a Canon G7 10.0-megapixel camera fitted on a Polaroid land tripod at an object distance of 8.5 cm. Images were transferred and analyzed with Image Pro-Plus to calculate infarct volumes. Infarct volume was calculated by measuring infarct areas on evenly sliced (1 mm) brain sections and adding them together (Simpson’s rule). Normalized infarct volume with respect to whole brain volume was calculated by dividing the volume of TTC-unstained (infarcted) tissue by that of the whole brain.

**Statistical Analysis**

In this preclinical animal study, because of the small sample sizes within the experimental groups, nonparametric statistical methods were used to assess differences between the groups. Pairwise comparisons of spontaneous Xe release from Xe-ELIP in PBS or HSP and release with or without ultrasound were performed by the Wilcoxon rank sum test for 2 groups. For comparisons of multiple groups, the Kruskal-Wallis ANOVA of ranks and median test was used to assess whether there were global differences between the groups. This was followed by post hoc multiple comparisons of mean ranks for all groups by computation of normal z-values for each comparison followed by probabilities adjusted for the number of comparisons for 2-sided tests of significance.28 Data were plotted as means and SDs for most experiments. Neurological outcomes between the treatment groups were also reported as medians and quartiles. Statistica (version 9, StatSoft Inc, Tulsa, Okla) software was utilized for the statistical analyses. P<0.05 was considered significant.

**Results**

**Encapsulation and Release of Xe**

With the use of the pressurization-freeze method at 6 atm, a total of 15±2.3 μL of Xe gas was encapsulated into 1 mg of
liposomes. The spontaneous Xe release profiles from Xe-ELIP demonstrated a biphasic pattern with the rapid phase (initial 10 minutes) characterized by 30% release of the encapsulated gas (Figure 2). Although Xe release from Xe-ELIP in HSP was somewhat slower than in PBS, there was no difference between the 2 groups for the first hour.

**Ultrasound-Triggered Xe Release From Xe-ELIP**

The average of initial Xe volume encapsulated in 2 mg Xe-ELIP (10 mg/mL) was 33±5 μL. Under physiological steady flow conditions (10 mL/min; Figure 1) mimicking blood flow in the ICA, it took 12 seconds to have 2 mL of Xe-ELIP pass through the latex tubing. Ultrasound exposure triggered enhanced Xe release (44±4%) compared with 5±1% Xe release without ultrasound exposure (Figure 3).

**Effects of Xe-ELIP on Cell Viability During Oxygen/Glucose Deprivation**

The effects of Xe-ELIP on cell viability during oxygen/glucose deprivation were evaluated by measuring the release of blue formazan salt. Live cells maintain their metabolic capability to convert MTT to blue formazan salt. Figure 4 demonstrates a 54% decrease in viability of the cultured PC12 cells at 4 hours after oxygen/glucose deprivation in the no-treatment group ($P=0.004$ versus no hypoxia). Air-ELIP did not protect the cells from oxygen/glucose deprivation damage. However, with exposure to 40 μg/mL of Xe-ELIP, 90% of the cells remained viable ($P=1.00$ versus no hypoxia).

**Neurological Assessment**

The effects of Xe-ELIP treatment on the neurological disability of animals with ischemia after transient MCA occlusion were investigated (Table 1 and Figure 5). Animals without treatment showed major deficits in locomotor performance on day 1 with little improvement on day 3. Both Xe-saturated saline and Xe-ELIP treatment groups demonstrated improved performance in all behavioral tests from day 1 and close to normal performance in the limb placement test on day 3.
Anchored with Xe-ELIP treatment combined with ultrasound exposure demonstrated an enhanced neurological improvement on all tests compared with those without ultrasound exposure. Animals treated with Xe-ELIP showed earlier recovery from anesthesia and earlier restoration of daily activities including grooming, exploratory behavior, and feeding compared with the no-treatment group.

### Effects of Xe-ELIP on Cerebral Infarct Volume After MCA Occlusion

Local delivery of Xe-ELIP to moderate cerebral ischemia was achieved by tail vein injection in combination with or without ultrasound application over the ICA. Representative images of the TTC-stained brain section of rats subjected to MCA occlusion with and without treatment are shown in Figure 6A. In the no-treatment group, a large infarction developed and predominantly involved the cerebral cortex and striatum. Figure 6B quantitatively demonstrates the effects of Xe delivery by Xe-saturated saline solution or Xe-ELIP. The normalized infarct volume in the no-treatment group was $16\pm5.2\%$ ($228\pm74\,\text{mm}^3$). The normalized infarct volumes with intravenous Xe-saturated saline treatment with and without ultrasound activation were $7.5\pm1.4\%$ ($P=0.23$ versus no treatment) and $7.8\pm2.0\%$ ($P=0.40$ versus no treatment), respectively. The normalized infarct volume with intravenous delivery of Xe-ELIP without ultrasound treatment was $8.3\pm1.4\%$ ($108\pm18\,\text{mm}^3$; $P=1.00$ versus no treatment). However, intravenous administration of Xe-ELIP combined with ultrasound treatment over the carotid artery further decreased the normalized infarct size to $4.0\pm1.4\%$ ($56\pm19\,\text{mm}^3$), a 75% reduction compared with the no-treatment group ($P<0.001$). The effect of intra-arterial delivery of Xe-ELIP was compared with the treatment groups with intravenous injection (Table 2). Intra-arterial injection of Xe-ELIP decreased the normalized infarct volume to $4.9\pm1.3\%$ ($82\pm18\,\text{mm}^3$; 64% reduction; $P<0.001$), which was comparable to that in the treatment group with intravenous Xe-ELIP injection with ultrasound activation ($P=1.00$). No deaths or seizures were observed in all groups.

### Table 1. Changes in Neurological Disability of Animals With Ischemia on Day 1 and Day 3 After Transient MCA Occlusion

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 1</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (no hypoxia)</td>
<td>2.0*</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>1.0*</td>
<td>1.0</td>
</tr>
<tr>
<td>No treatment</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>5.0</td>
<td>4.8</td>
</tr>
<tr>
<td>Xe-solution treatment</td>
<td>1.0*</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0*</td>
<td>2.5*</td>
<td>2.0</td>
</tr>
<tr>
<td>Xe-ELIP treatment</td>
<td>1.0*</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0*</td>
<td>2.5*</td>
<td>2.0</td>
</tr>
<tr>
<td>Xe-ELIP treatment + ultrasound exposure</td>
<td>1.5*</td>
<td>1.0</td>
<td>2.0</td>
<td>2.0*</td>
<td>3.5‡</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Data are presented as median and quartiles. Scores for limb placement are as follows: 0, no response; 1, response was sluggish or delayed; 2, response was rapid and fully executed. Scores for beam walking are as follows: 0, traversed the beam with no foot slip; 1, traversed with grasping of the lateral side of the beam; 2, showed difficulty crawling across the beam but able to traverse; 3, required >10 seconds to traverse the beam because of difficulty in walking; 4, unable to traverse the beam; 5, unable to move the body or any limb on the beam; 6, unable to stay on the beam for >10 seconds. Scores for grid walking are as follows: total number of foot fault errors.

* $P<0.001$, † $P=0.002$, ‡ $P=0.01$, § $P=0.03$ vs no-treatment group (n=8).
was no difference in core body temperature between the groups during MCA occlusion and the initial hours of reperfusion. Figure 7 demonstrates an estimate of blood velocity profile that supports blood restoration after removal of MCA occlusion. Xe-ELIP treatment showed no additional vasodilatory effect.

Discussion

Over the last 2 decades, neuroprotective agents designed to block the NMDA receptor have demonstrated promising results in animal models of cerebral ischemia but have failed to produce clinical benefits. This lack of efficacy may be related to factors such as delay in administration of the neuroprotective agents beyond 6 hours after stroke onset, use of a single agent for complicated neuroprotective therapy, and inability to achieve sufficiently high local doses in the ischemic area. The present study is the first to demonstrate the therapeutic effect of Xe delivery intravenously with ultrasound-enhanced local Xe release after cerebral ischemic injury. This novel strategy with Xe-ELIP may represent a major step in overcoming these obstacles in the management of ischemic and reperfusion brain injury.

ELIP as Theranostic Agents

The encapsulation of therapeutic and diagnostic gases into ELIP provides enhanced echogenicity as an echo contrast agent, as well as a vehicle to deliver a bioactive gas for therapeutic effect. We have previously demonstrated a novel methodology to encapsulate nitric oxide into ELIP with directed local delivery to balloon-injured carotid arteries to attenuate neointimal hyperplasia. In the present study, we employed the same gas encapsulation technique and investigated the Xe release profiles. Spontaneous Xe release from Xe-ELIP was observed in 2 phases: an initial rapid release in the first 10 minutes and a slow release over 18 hours (Figure 2).

ELIP are effective vehicles for delivering bioactive gases to target tissues with the potential to overcome obstacles in delivering therapeutic gases by inhalation, such as the reduction of partial pressure of inspired oxygen and toxicity associated with inhaling high concentrations of bioactive gases. The efficiency of Xe delivery to infarct areas by Xe-saturated saline is limited by the solubility of Xe in solution, whereas Xe delivery by Xe-containing ELIP can be conducted via both soluble and insoluble formats. Xe solubility in solution is 89 μL/mL, whereas in lipid it is 1853 μL/mL. The total amount of Xe loaded in ELIP consists of 3 components: Xe dissolved in solution (89 μL), Xe in vapor phase (150 μL), and Xe dissolved in lipid bilayer (19 μL) per 1 mL of lipid dispersion (10 mg lipid per milliliter). Xe-containing ELIP thus provide a more effective strategy for Xe delivery. Gas-containing liposomes therefore represent a new class of “theranostic” agents with unique characteristics that allow their visualization in the circulation and delivery of the payload in a site-specific fashion.

In addition, liposomes can be conjugated to ligands for targeted delivery of payload to specific tissues. We have incorporated tissue plasminogen activator into our ELIP and demonstrated a formulation that is suitable for fibrin targeting and clot lysis. We have also demonstrated that the combination of an ultrasound contrast agent and ultrasound treatment can facilitate thrombolysis. Coencapsulation of both Xe and tissue plasminogen activator into ELIP may provide another approach to targeted treatment of ischemic stroke when both reperfusion and neuroprotection are vital to reduce neuronal damage.

Ultrasound for Therapeutic Gas Release

Drug delivery by intravenous administration has low efficiency because of the large dilation in the blood volume. An alternative delivery method to increase local drug concentration is intra-arterial administration. Intra-arterial delivery of tissue plasminogen activator has been used clinically for selected cases of embolic cerebral infarction and intra-arterial delivery of papaverine has been used for relief of vasospasm after subarachnoid hemorrhage. Although intra-arterial drug administration is efficient, it involves invasive arterial catheterization procedures with potential complications and risk to patients. Intravenous administration of ELIP carrying a neuroprotective gas in combination with ultrasound application over the carotid artery can overcome this problem. In this study, we have demonstrated intravenous Xe-ELIP injection with good effects.

Xe release from circulating Xe-ELIP can be triggered by low-power ultrasound exposure. There are 2 types of ultrasound-induced gas release from ELIP: ultrasound-driven diffusion or rapid fragmentation. We have previously demonstrated these ELIP destruction thresholds. Rapid fragmentation can be accompanied by cavitation, which has the potential to cause harmful target tissue bioeffects. In this study, low-power ultrasound (ultrasound-driven diffusion) was utilized with the ultrasound focused over the ICA but not directly over the brain tissue. This strategy avoids possible...
Ultrasound bioeffects on the ischemia-compromised microvasculature and neurons.

Xe-ELIP are stable under physiological conditions, allowing Xe release for hours. This ensures that Xe-ELIP administered into the tail vein can circulate systemically and release Xe locally to the cerebral infarction sites on ultrasound activation. One-megahertz continuous wave ultrasound with 0.18-MPa peak-to-peak pressure amplification...

Figure 6. Effects of intravenous Xe delivery on cerebral ischemia in an animal model. A, Representative TTC-stained coronal brain sections demonstrating brain infarction in rats at 3 days after MCA occlusion with no treatment, Xe-saturated saline treatment, and Xe-ELIP treatment. B, Infarct size comparison between treatment groups demonstrating a 48% infarct volume reduction with Xe-ELIP alone and 75% reduction with Xe-ELIP combined with ultrasound activation. IA indicates intra-arterial; IV, intravenous; US, ultrasound; w/o, without; and w/, with.

Table 2. Changes in Normalized Infarct Size With Intra-Arterial or Intravenous Xe Delivery

<table>
<thead>
<tr>
<th></th>
<th>No Treatment</th>
<th>Xe-ELIP Without Ultrasound (IA)</th>
<th>Xe-Saturated Saline Without Ultrasound (IV)</th>
<th>Xe-Saturated Saline With Ultrasound (IV)</th>
<th>Xe-ELIP Without Ultrasound (IV)</th>
<th>Xe-ELIP With Ultrasound (IV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normalized infarct volume, %</td>
<td>15.9±5.2</td>
<td>4.9±1.3*</td>
<td>7.5±1.4</td>
<td>7.8±2.0†‡</td>
<td>8.3±1.4</td>
<td>4.0±1.4§</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD; n=8. IA indicates intra-arterial; IV, intravenous.

*P<0.001 vs no-treatment group; †P=1.00 vs Xe-saturated saline without ultrasound (IV) group; ‡P=0.03 vs Xe-ELIP with ultrasound (IV) group; §P=0.004 vs Xe-ELIP without ultrasound (IV) group; ††P=1.00 vs Xe-ELIP without ultrasound (IA) group.
tude was able to trigger 44% of Xe release from Xe-ELIP under a flow rate of 10 mL/min (Figure 3). Xe release from intravenously injected Xe-ELIP resulted in 48% reduction of infarct size and further increased to 75% reduction after ultrasound-enhanced Xe release. This was comparable to the therapeutic effect by intra-arterially injected Xe-ELIP (Figure 6 and Table 2). Ultrasound treatment demonstrated further infarct volume reduction only in the Xe-ELIP group, whereas there was no ultrasound effect in the Xe-saturated solution group. This indicates that, with strategic ultrasound activation over the ICA, Xe can be continuously released from circulating Xe-ELIP into the cerebral circulation, allowing a therapeutic amount of Xe delivery to ischemic areas (Figure 8).

Limitations
A limited number of methods are available to determine Xe concentration in vivo. Although computed tomography or magnetic resonance imaging has potential to investigate Xe distribution in vivo, no modality can accurately determine Xe concentration. We injected 200 μL (10 mg/mL) of Xe-ELIP via the tail vein, which contained a total of 20 μL Xe for our desired neuroprotective effect in vivo. Our in vitro testing demonstrated 40% of Xe release from Xe-ELIP on ultrasound exposure (Figure 1). We thus speculate that, with ultrasound application, 40% of Xe release from the circulating Xe-ELIP that reached to the ICA via intravenous injection was achieved in our in vivo studies. The mechanisms of increased infarct size reduction after Xe release from our Xe-ELIP have not been fully investigated. Previous studies suggest that Xe may share characteristics similar to those of some NMDA antagonists. The activation of NMDA receptors is a very early event during the cascade of ischemic injury, and viable neurons in the ischemic penumbra may only be salvageable with early administration of an NMDA antagonist or other neuroprotective agents.

Figure 7. Velocity profile of cerebral blood flow after MCA occlusion, reperfusion, and Xe-ELIP delivery estimated with the use of a laser Doppler flow meter.

Figure 8. Diagram of a strategy to apply ultrasound treatment over the common carotid artery, a site that is distant from the cerebral infarct site, for both imaging of Xe-ELIP and ultrasound-enhanced therapeutic gas delivery.
agent. However, in a rat myocardial infarct model, reduction of infarct size after Xe administration was greater than that produced by an NMDA antagonist (MK801), suggesting that other mechanisms play a role in Xe protection.

Conclusions
This study demonstrates a methodology to encapsulate therapeutic gases such as Xe into ELIP with the use of a pressurization-freeze method. Xe-ELIP protected PC12 cells from hypoxic cell death. In an animal model of cerebral ischemia/reperfusion injury, intravenous administration of Xe-ELIP reduced infarct size. On ultrasound activation over the carotid artery, Xe release from circulating Xe-ELIP was enhanced, with further reduction of infarct size and restoration of sensorimotor function. Intravenous administration of ELIP carrying a neuroprotective gas in combination with ultrasound application over the carotid artery is a novel noninvasive strategy for local therapeutic delivery to modulate cerebral ischemia while minimizing systemic side effects.

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Disclosures
None.

References


**CLINICAL PERSPECTIVE**

Stroke is the third leading cause of death and the most common cause of adult disabilities in the United States. The emergent treatment of acute ischemic stroke has two primary goals: rapid restoration of cerebral blood flow (reperfusion) and limitation of neural injury (neuroprotection). Although many therapeutic strategies have demonstrated pharmacological effect for neuroprotection including their ability to decrease stroke size, these strategies often fail when translated to the clinical setting. Inability to deliver high local doses sufficiently to the ischemic area and inadequate penetration of therapeutic agents across the blood-brain barrier are major contributory factors. The present study suggests that intravenous delivery of a noble gas, xenon, which readily diffuses across the blood-brain barrier, with the use of a delivery vehicle (liposomes) and a release mechanism (ultrasound), enhances local xenon delivery and decreases stroke size. Site-specific payload release from liposomes can be controlled by low-power ultrasound over the common carotid artery with enhanced neuroprotective delivery to the ischemic circulation. This study demonstrates that both intra-arterial and intravenous carrier deliveries with low-power ultrasound activation over the common carotid artery have the same therapeutic effect for neuroprotectant payload delivery to the cerebral ischemic area. Together, this novel delivery vehicle and delivery methodology have the potential to provide a clinically effective and efficient means for therapeutic gas/pharmaceutical delivery for improved stroke treatment.
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