Xenon Neuroprotection in Experimental Stroke

Interactions with Hypothermia and Intracerebral Hemorrhage

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ABSTRACT

Background: Xenon has been proven to be neuroprotective in experimental brain injury. The authors hypothesized that xenon would improve outcome from focal cerebral ischemia with a delayed treatment onset and prolonged recovery interval.
Methods: Rats were subjected to 70 min temporary focal ischemia. Ninety minutes later, rats were treated with 0, 15, 30, or 45% Xe for 20 h or 0 or 30% Xe for 8, 20, or 44 h. Outcome was measured after 7 days. In another experiment, after ischemia, rats were maintained at 37.5° or 36.0°C for 20 h with or without 30% Xe. Outcome was assessed 28 days later. Finally, mice were subjected to intracerebral hemorrhage with or without 30% Xe for 20 h. Brain water content, hematoma volume, rotarod function, and microglial activation were measured.

Results: Cerebral infarct sizes (mean ± SD) for 0, 15, 30, and 45% Xe were 212 ± 27, 176 ± 55, 160 ± 32, and 198 ± 54 mm^3, respectively (P = 0.023). Neurologic scores (median ± interquartile range) followed a similar pattern (P = 0.002). Infarct size did not vary with treatment duration, but neurologic score improved (P = 0.002) at all xenon exposure durations (8, 20, and 44 h). Postischemic treatment with either 30% Xe or subtherapeutic hypothermia (36°C) had no effect on 28-day outcome. Combination of these interventions provided long-term benefit. Xenon improved intracerebral hemorrhage outcome measures.
Conclusion: Xenon improved focal ischemic outcome at 7, but not 28 days postischemia. Xenon combined with subtherapeutic hypothermia produced sustained recovery benefit. Xenon improved intracerebral hemorrhage outcome. Xenon may have potential for clinical stroke therapy under carefully defined conditions.

What We Already Know about This Topic

- Xenon is neuroprotective in several animal models of injury, but previous studies have focused on short-term outcomes or have not controlled physiologic variables that can affect recovery

What This Article Tells Us That Is New

- In rats, exposure to xenon after transient focal ischemia resulted in a concentration- and time-dependent reduction in infarct volume and an improvement in neurologic function 7 days after injury
- Although xenon did not improve function 28 days after injury, its combination with mild hypothermia did
- Xenon also improved outcome after intracerebral hemorrhage

XENON (Xe) interacts with neural elements purported to play important roles in outcome from acute brain injury, including the adenosine triphosphate potassium channel[1,2] and the glutamatergic *N*-methyl-D-aspartate receptor (NMDA).[3] In contrast to competitive or noncompetitive NMDA receptor antagonists (*e.g.*, ketamine, nitrous oxide), xenon acts at the NMDA receptor glycine recognition site.[3] Drugs acting at the glycine recognition site are typically devoid of psychotomimetic properties.[4] Consistent with this and in contrast to ketamine or nitrous oxide, xenon

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does not induce c-fos immunoreactivity (a marker of psychotomimetic properties) in the rat retrosplenial cortex[5] or apoptosis in the developing rat brain.[6] Psychotomimetic and other toxic properties of NMDA receptor antagonism have prevented clinical application of glutamate antagonists for stroke.[7] Xenon may obviate these constraints.

Xenon has other properties that could be favorable to injured brain. Xenon readily penetrates the blood–brain barrier, offering rapid onset of action.[8] Because of a low blood–gas partition coefficient,[9] titration of dose and response is rapid. Xenon has little effect on experimental intracranial hypertension, cerebral autoregulation, or carbon dioxide reactivity.[10,11] Effects on myocardial contractility, mean arterial pressure, and cerebral blood flow are absent or modest in humans.[12,13] Administration of 28% Xe for brief durations, to measure cerebral blood flow, has been found to be generally safe in a large population of patients with intracranial pathology.[14]

Xenon has been reported to provide neuroprotection in numerous experimental models of central nervous system injury, including neonatal asphyxia,[15,16] focal ischemic stroke,[17–19] cardiopulmonary bypass,[20] and cardiac arrest[21] (but see Fries *et al.*[22]). Although promising, the preponderance of this work has not controlled physiologic factors known to alter ischemic outcome and only brief survival intervals were studied. The goal of the following series of experiments was to rigorously assess xenon efficacy in experimental stroke as a prelude to potential clinical application.

Materials and Methods

The following studies were approved by the Duke University Animal Care and Use Committee (Durham, North Carolina).

Xenon Exposure System

A novel exposure system was created to allow continuous servo-regulation of inhaled gas concentrations and pericranial temperature, intravenous infusion of maintenance fluids, and xenon conservation.

The system included a 5-l acrylic box (animal chamber), air pump, inline carbon dioxide soda lime/humidity scrubber cartridge, injection ports for introducing fresh oxygen and xenon into the circulating air within the loop, xenon (inline) and oxygen (in chamber) sensors, and a pressure release vent (to prevent overpressurization within the chamber). These components were connected in series in a closed loop using Tygon® tubing (OD = ½″, ID = ¼″, Saint-Gobain Performance Plastics, Co., Paris, France). Gas was circulated within the closed exposure system at a rate of approximately 10 l/min by an air pump (model 7010Z AC; Garner Denver Thomas, Sheboygan, WI) specifically constructed to not entrain room air. Proximal to the injection ports, gas was continuously monitored with sensors for measuring xenon (Model 439; Nyquist Ltd., Congleton Cheshire, United Kingdom) and oxygen (model 5120k; Datex Ohmeda, Louisville, CO) concentrations. It was assumed that the remaining inhaled gas was nitrogen. Analog output from each sensor was continuously digitized (Model DT9812 universal serial bus analog to digital converter; Data Translation, Marlboro, MA) under the control of a computer-based LabVIEW-implemented controlling system (National Instruments, Austin, TX). The LabVIEW program compared measured and target oxygen and xenon concentration in the circulating gas. This information was used to independently open or close mass flow controllers (Model GFC17; Aalborg Inc., Orangeburg, NY) that regulated the flow of oxygen and xenon into the system through the injector ports. Oxygen injection was required to compensate for respiration. Because fresh gas was intermittently injected into the circulating gas stream, the system was connected to a reservoir and pressure release mechanism (a water-filled cylinder) so as to maintain near-atmospheric pressure within the animal chamber. Although xenon was lost from the system through the vent, the target xenon concentration was maintained by injecting fresh xenon through the xenon mass flow controller. Circulating gas within the exposure system was passed through a canister containing segregated columns of soda lime and anhydrous calcium sulfate (Drierite, W.A. Hammond Co. Ltd., Xenia, OH) desiccant to eliminate carbon dioxide and water vapor from the gas stream. Two identical systems were constructed with animal assignment to the systems being alternated. Air Products and Chemicals, Inc. (Allentown, PA) provided xenon. Arterial blood gas values measured in pilot studies demonstrated that this system provided normocapnic conditions (arterial carbon dioxide partial pressure values = 40 ± 3 mmHg) over the study interval.

A separate system was used to regulate animal temperature. The rats were placed in a torso harness (Covance Infusion Harness; CIH95; Instech Laboratories, Inc., Plymouth Meeting, PA) connected to a swivel commutator. During surgery (see Middle Cerebral Artery Occlusion Preparation and Ischemic Insult), a blunt thermistor was implanted percutaneously adjacent to the right temporal bone. The thermistor wire was passed through the swivel commutator and interfaced with a thermoregulation system (YSI Model 73ATA; YSI Inc., Yellow Springs, OH) that was used to servo-regulate pericranial temperature by controlling a cooling fan and heating lamp. Pericranial temperature was continuously recorded to ensure that temperature regulation was successfully maintained (MacLab/4eAD Instruments Pty Ltd., Castle Hill, Australia). Target temperature was defined by experimental protocol. Pilot studies indicated that this system was effective at maintaining temperature at intended target values.

We also performed pilot studies involving subtherapeutic hypothermia. Placement of the exposure chamber within a portable refrigeration chamber covered with a transparent acrylic cover, to allow visual observation of the animal, was

sufficient to achieve and maintain target pericranial temperature in conjunction with the heat lamp/fan system.

Middle Cerebral Artery Occlusion Preparation and Ischemic Insult

Surgical procedures to obtain middle cerebral artery occlusion (MCAO) were performed as previously described.[23] Male Wistar rats 250–300 g (10–12 weeks of age; Harlan Laboratories, Indianapolis, IN) were fasted from food, with free access to water on the night before surgery. Rats were anesthetized in a chamber with 5% isoflurane in 100% O_2. After loss of righting reflex, the trachea was intubated and the lungs were mechanically ventilated (40% O_2/balance nitrogen). The inspired isoflurane concentration was decreased to 1.5–2%. Surgery was performed with aseptic technique, and all surgical fields were infiltrated with 0.25% bupivacaine.

The tail artery was cannulated and used to monitor mean arterial pressure and sample blood. Pericranial temperature was continuously recorded and servo-controlled by surface heating and cooling (heating lamp or fan, respectively) to maintain pericranial temperature at 37.5 ± 0.2°C from surgery onset until indicated by experimental protocol.

A midline ventral cervical skin incision was made, and the right common carotid artery identified. The external carotid artery was isolated, ligated, and divided. The internal carotid artery was dissected distally until the origin of the pterygopalatine artery was visualized. A catheter was placed in the jugular vein and tunneled subcutaneously to the nape of the neck and exteriorized. After surgical preparation, animals were fitted in the torso harness. A 30-min interval was allowed for physiologic stabilization. Heparin (50 U) was then given intravenously. A 0.25-mm diameter nylon monofilament (distal 1-mm tip coated with 0.38-mm diameter silicone) was inserted into the external carotid artery stump and advanced 19–20 mm from the carotid artery bifurcation into the internal carotid artery until a slight resistance was felt, so as to achieve MCAO.

Isoflurane was abruptly discontinued at MCAO onset. The wound was closed, and the trachea was extubated after recovery of the righting reflex. The rat, along with the tethered thermistor assembly, was placed in a 3-l transparent plastic animal enclosure not associated with the xenon exposure system, with the fraction of inspired oxygen maintained at 30%. Rats typically recovered the righting reflex within 5–8 min. This system allowed continuous observation, thermoregulation, and mean arterial pressure monitoring. Seven minutes before the end of MCAO, isoflurane was introduced into the chamber. The rat was removed, and anesthesia maintained by snout mask. The filament was withdrawn to allow reperfusion at 70 min after MCAO onset. The arterial catheter was removed, all wounds were closed with interrupted suture and infiltrated with 0.25% bupivacaine, and isoflurane was discontinued.

Animals were then placed in the xenon exposure system for 90 min in 30% O_2/balance nitrogen. NaCl (0.9%) was infused intravenously at 1 ml/h with thermoregulation continued. At 90 min after onset of reperfusion from MCAO, animals were randomly assigned to experimental groups and inspired gas concentration servo-regulation systems were activated.

Measurement of Neurologic Outcome

After recovery intervals defined by experimental protocol, we used a previously described scoring system that has repeatedly been shown to closely correlate with cerebral infarct size.[23–25] General status, simple motor deficit, complex motor deficit, and sensory deficit were scored.[24] The score given to each animal at the completion of testing was the sum of the four individual scores with 0 being the minimum (best) score and 48 the maximum possible (worst) score. The same experienced observer, who was blinded to group assignment, assigned all scores.

Measurement of Cerebral Infarct Size

Methods for cerebral infarct measurement have previously been reported.[23] After neurologic evaluation, animals were weighed, anesthetized with isoflurane, and decapitated. The brains were removed, snap frozen at −20°C in 2-methylbutane, and stored at −80°C for later analysis. Infarct volume was measured by using the method of Swanson *et al.*[26] Serial quadruplicate 20-μm thick coronal sections were taken by using a cryotome at 800-μm intervals over the rostral–caudal extent of the infarct. The sections were dried and stained with hematoxylin and eosin. A representative section from each 800-μm interval was digitized with a video camera controlled by an image analyzer (MCID Elite, Interfocus Imaging, Linton, England). The image of each section was stored as a 1280 × 960-pixel matrix and displayed on a video monitor. With the observer blinded to experimental conditions, the following regions of interest were cursor-outlined: noninfarcted ipsilateral cerebral cortex, noninfarcted ipsilateral subcortex, contralateral cerebral cortex, and contralateral subcortex. The area within each region of interest (mm^2) was determined by automated counting of the calibrated pixels contained within the region of interest. Ipsilateral noninfarcted cortex and subcortex areas were subtracted from the corresponding contralateral region of interest values. Infarct volumes (mm^3) were computed as running sums of subtracted infarct area multiplied by the known interval (*e.g.*, 720 μm) between sections over the rostral–caudal extent of the infarct calculated as an orthogonal projection.[27]

Experiment 1: Xenon Dose–Response Analysis

This experiment was designed to determine the optimally efficacious xenon concentration for postischemic intervention. Ninety minutes post-MCAO, rats were randomly assigned to one of four groups defined by the inspiratory gas mixture (30% O_2/0% Xe/ balance nitrogen, n = 13; 30% O_2/15% Xe/ balance nitrogen, n = 13; 30% O_2/30% Xe/balance nitrogen,

n = 13; 30% O_2/45% Xe/balance nitrogen, n = 13). Exposure was continued for 20 h with assigned xenon and oxygen concentrations and pericranial temperature continuously recorded. Food was provided within the chamber, and 0.9% NaCl was infused intravenously at 1 ml/h. After exposure, rats were removed from the chamber, briefly anesthetized with isoflurane, and the pericranial thermistor and jugular venous catheters were removed and wounds closed after 0.25% bupivacaine infiltration. Rats were allowed to recover from anesthesia and then returned to their home cages for 7 days followed by outcome assessment (see Measurement of Neurologic Outcome and Measurement of Cerebral Infarct Size).

Experiment 2: Xenon Treatment Duration Analysis

On the basis of the results of Experiment 1, we accepted 30% Xe as the maximally efficacious dose. This experiment was designed to determine the xenon treatment duration necessary to optimize functional and histologic outcome. The protocol was otherwise identical to that described for Experiment 1.

Ninety minutes post-MCAO, rats were randomly assigned to one of four groups based on xenon treatment duration: 0% Xe in 30% O_2/balance nitrogen (n = 18) or 30% Xe in 30% O_2/balance nitrogen for 8, 20, or 44 h (n = 17 per group). All rats remained within the exposure chamber with thermoregulation for 44 h. For the 8- and 20-h xenon exposure groups, the inspiratory gas was changed to 30% O_2/balance nitrogen for the remainder of the confinement interval within the exposure chamber. As in Experiment 1, xenon exposure commenced 90 min after removal of the occlusive filament.

After these experimental treatments, rats were removed from the exposure chamber and recovered as described in Experiment 1. Animals were allowed to survive 7 days, after which outcome was assessed (see Measurement of Neurologic Outcome and Measurement of Cerebral Infarct Size).

Experiment 3: Xenon as an Adjunct to Subtherapeutic Hypothermia

We then asked whether postischemic 35% Xe could provide a sustained neuroresuscitative benefit and whether this benefit could also serve as an adjunct to subtherapeutic hypothermia. We used a magnitude of induced hypothermia previously reported to have no effect on MCAO outcome.[28] Ninety minutes after removal of the occlusive filament, rats were randomly assigned to one of four groups:

37.5°C/0% Xe (n = 18): Pericranial temperature = 37.5 ± 0.2°C while rats breathed 30% O_2/balance nitrogen for 20 h.

37.5°C/30% Xe (n = 17): Pericranial temperature = 37.5 ± 0.2°C while rats breathed 30% O_2/30% Xe/balance nitrogen for 20 h.

36.0°C/0% Xe (n = 17): Pericranial temperature = 36.0 ± 0.2°C while rats breathed 30% O_2/balance nitrogen for 20 h.

36.0°C/30% Xe (n = 17): Pericranial temperature = 36.0 ± 0.2°C while rats breathed 30% O_2/30% Xe/balance nitrogen for 20 h.

After exposure, rats remained in the chamber an additional 1 h to allow recovery of pericranial temperature to 37.5°C in the hypothermic groups. On completion of the exposure protocol, rats were returned to their home cages and allowed to survive for 28 days, after which outcome was assessed (see Measurement of Neurologic Outcome and Measurement of Cerebral Infarct Size).

Experiment 4: Xenon in Intracerebral Hemorrhage

To determine whether xenon could worsen intracerebral hemorrhage (ICH) preventing potential use before the determination of stroke subtype (ischemic *vs.* hemorrhagic), we assessed the effects of xenon in our previously described ICH model.[29,30] Male C57BL/6J mice (10–12 week of age; Jackson Laboratory, Bar Harbor, ME) were used in these experiments. Baseline rotarod performance was obtained, as previously described.[31,32] Mice were placed on an accelerated rotating rod (4–40 rpm, ENV-577M; Med Associates Inc., Georgia, VT). The latency to fall from the rod was automatically recorded by the action of the mouse dropping from the rotating rod. Three trials were performed at each session with an intertrial interval of 15 min. The best value from the three trials was used for statistical analysis.

The following day, anesthesia was induced with 4.6% isoflurane and the trachea was intubated. The lungs were mechanically ventilated with 1.6% isoflurane in 30% O_2/70% N_2. Rectal temperature was maintained at 37.0 ± 0.2°C by an underbody circulating waterbed. The animal's head was secured in a stereotactic frame. The scalp was incised, and a burr hole was created 2.2 mm left lateral at bregma. A 1.0-μl syringe needle (Hamilton, Reno, NV) was advanced to a depth of 3 mm from the cortical surface by micromanipulator. Type IV-S clostridial collagenase (Sigma, St. Louis, MO) was injected over 5 min (0.075 U in 400 μl 0.9% NaCl). The incision was then closed with suture, infiltrated with 0.25% bupivacaine, and isoflurane was discontinued. Animals were allowed to recover spontaneous ventilation, and the trachea was extubated.

In the first experiment, collagenase injection was performed. Two hours later, these mice were randomized to begin treatment with 30% Xe (n = 8) or 0% Xe (n = 8) for 20 h in the xenon exposure system. Rotarod testing was repeated 1–2 h after removal from the exposure chamber by an observer blinded to group assignment. The mice were then euthanized, the brains removed intact, flash frozen in 2-methyl butane (−20°C), and stored at −80°C. The brains were serially sectioned and stained using techniques identical to those described for the MCAO model.

Computerized planimetric analysis was performed to measure hemorrhage volume by an observer blinded to treatment group.

In the second experiment, 32 mice were subjected to collagenase injection using the same protocol. Ten of these mice were reanesthetized with isoflurane (maintenance = 0.7–1.0% delivered *via* snout cone) to undergo magnetic resonance (MR) imaging of the brain at 90 min postcollagenase injection. Respiratory rate was continuously monitored. An MR compatible cradle with circulating water was used to maintain rectal temperature within 36.0°–37.5°C. MR imaging was performed on a 7T Bruker Biospec 70/30 horizontal bore system (Billerica, MA). Sequential axial 2D T2-weighted fast spin echo data sets were obtained for anatomical assessment and volumetry (TURBO-RARE, echo time/repetition time = 11/4200 ms with 1-mm slice thick, matrix = 256 × 256 and field of view of 2.4 × 2.4 cm, five averages, 0.0 mm interslice gap). The scanning sequence required approximately 45 min.

Volumetric analysis of MR data sets was performed using Osirix software (Pixmeo, Geneva, Switzerland). Hemorrhage and ventricular volumes were manually segmented in contiguous axial slices in each animal by an investigator blinded to experimental group. Selected areas were reviewed for consistency on coronal and sagittal representations. Two separate hemorrhage and ventricular segmentations were obtained for each animal, with the average volume then taken. Intrarater reliability (κ value) was 0.97.

After MR imaging, isoflurane was discontinued and the mice awakened. The mice were then randomly assigned to either 30% Xe (n = 5) or 30% O_2/balance nitrogen (n = 5), beginning at approximately 2.25 h postcollagenase injection using the xenon exposure system. Because of their size, the mice were not placed in the harness system, nor were they thermoregulated. The exposure system was maintained at room temperature (25°C). Food and water were provided within the chamber. Exposure duration was 20 h. Mice were subjected to repeat MR imaging. These mice were then decapitated under isoflurane anesthesia, and brain water content was measured as described previously.[32] In brief, the brains were harvested and the hindbrain discarded. Each hemisphere was immediately weighed ("wet" weight). Hemispheres were allowed to dehydrate over 24 h at 105°C and then reweighed ("dry" weight). Brain water content was calculated as ([wet weight − dry weight]/[wet weight] × 100).

The remaining 22 mice were randomly assigned to be exposed to either 30% Xe (n = 11) or 30% O_2/balance nitrogen (n = 11) for 20 h, beginning at approximately 2.25 h postcollagenase injection (no MR imaging was performed in these mice). On postinjection days 1 (4–5 h after removal from the exposure chamber), 3, 5, and 7, these mice were assessed for rotarod performance by an observer blinded to group assignment. Body weights were measured before collagenase injection and after 7 days recovery.

The 7-day recovery mice were then anesthetized with isoflurane and subjected to *in situ* perfusion with 30 ml phosphate-buffered saline. The brains were rapidly removed and immersion fixed in 4% formaldehyde for 24 h, transferred into 30% sucrose/1× phosphate-buffered saline, and stored at 4°C for 48 h. Frozen coronal sections (40 μm) were collected on a freezing sliding microtome. Floating brain sections were incubated in 1% hydrogen peroxide, permeabilized with 0.1% saponin, and blocked with 10% goat serum. Monoclonal rat antimouse F4/80 antibody (1:20,000; Serotec, Raleigh, NC) was incubated overnight, and biotinylated goat antirat IgG secondary antibody (1:3000; Vector Laboratories, Inc., Burlingame, CA) was applied for 1h, followed by avidin–biotin–peroxidase complex treatment for 1 h (ABC kit; Vector Laboratories, Inc.). Staining was visualized with diaminobenzidine (Vector Laboratories, Inc.). After being mounted onto slides, all sections were counterstained with hematoxylin (Fisher Scientific, Fair Lawn, NJ). Cell counting was conducted using a light microscope interfaced with the Stereo Investigator software package (MicroBrightField, Williston, VT) by an observer blinded to treatment group. The number of stained cells per volume of hippocampus was estimated by using an optical fractionator method as described before.[30]

Statistical Analysis

Statistical analysis was performed using StatView 5.0.1 and JMP Pro 9.0.0 (SAS Institute, Inc., Cary, NC). Samples sizes were selected based on previous experience in the respective MCAO and ICH models. Long-term effects of xenon on postischemic recovery have not been evaluated. Therefore, data were treated as a two-tailed hypothesis for statistical analysis. Mortality was less than 8% in all MCAO groups. These rats were not replaced. Seven mice died in the ICH experiments and were not replaced. No other data points were missing. Parametric values (physiologic values, cerebral infarct sizes, neurologic scores, brain water content, F4/80 cell counts, and hemorrhage and ventricular volumes) were compared by one-way ANOVA. In the presence of a significant F ratio, *post hoc* testing was performed using Scheffe test for experiments containing three or more groups. For Experiment 4, a two-way repeated-measures ANOVA was conducted (with a between-subjects factor for group and a repeated-measures factor for time). The relationship between neurologic score and total cerebral infarct volume was tested with linear regression analysis. A *P* value less than 0.05 was considered statistically significant. Values are reported as mean ± SD.

Results

Experiment 1: Dose–Response Analysis

Physiologic data collected in the peri-ischemic interval are presented in table 1. There were no differences among groups. Body weight values were similar among groups

Table 1. Periischemic Physiologic Values for Experiment 1 (Dose–Response Analysis)

	0% Xe	15% Xe	30% Xe	45% Xe
n	13	12	12	13
Preischemia				
Body weight, g	272 ± 19	276 ± 16	271 ± 20	277 ± 14
MAP, mmHg	93 ± 5	89 ± 13	88 ± 6	86 ± 4
Hematocrit, %	42 ± 2	43 ± 1	44 ± 2	42 ± 2
Blood glucose, g/dl	82 ± 15	75 ± 10	82 ± 9	77 ± 12
Arterial, pH	7.42 ± 0.05	7.42 ± 0.04	7.42 ± 0.06	7.43 ± 0.05
$Paco_2$, mmHg	38 ± 5	39 ± 5	38 ± 8	36 ± 6
Pao_2, mmHg	152 ± 25	138 ± 19	151 ± 24	153 ± 16
Intraischemia (45 min)				
MAP, mmHg	103 ± 19	102 ± 10	104 ± 14	97 ± 14
Arterial pH, mmHg	7.48 ± 0.05	7.48 ± 0.01	7.47 ± 0.03	7.48 ± 0.04
$Paco_2$, mmHg	32 ± 4	32 ± 2	33 ± 4	30 ± 3
Pao_2, mmHg	135 ± 27	136 ± 22	134 ± 32	134 ± 29
Pericranial temperature, °C	37.5 ± 0.2	37.5 ± 0.2	37.5 ± 0.2	37.5 ± 0.2
Postischemia (20 min)				
MAP, mmHg	80 ± 6	80 ± 5	80 ± 6	82 ± 5
Arterial pH, mmHg	7.42 ± 0.02	7.43 ± 0.03	7.42 ± 0.02	7.43 ± 0.01
$Paco_2$, mmHg	37 ± 4	36 ± 2	36 ± 4	36 ± 3
Pao_2, mmHg	172 ± 17	162 ± 16	163 ± 14	161 ± 22
Pericranial temperature, °C	37.5 ± 0.2	37.5 ± 0.2	37.5 ± 0.2	37.5 ± 0.2
Postischemia day 7				
Body weight, g	241 ± 45	289 ± 37	295 ± 26*	248 ± 57

Values = mean ± SD.

* Difference *vs.* 0% xenon group ($P = 0.04$).

MAP = mean arterial pressure; $PaCO_2$ = arterial blood carbon dioxide partial pressure; PaO_2 = arterial blood oxygen partial pressure; Xe = xenon.

before ischemia. On day 7, there was a main effect for group ($P = 0.005$). *Post hoc* analysis revealed greater body weights in the 30% Xe *versus* 0% Xe groups ($P = 0.04$).

Figure 1 depicts neurologic outcome and cerebral infarct size measured 7 days postischemia. Main effects were present for neurologic score ($P = 0.0009$), total infarct volume ($P = 0.02$), and subcortical infarct volume ($P = 0.03$). A main effect neocortical infarct size could not be proven ($P = 0.06$). *Post hoc* analysis identified significant improvement in rats exposed to 30% Xe for both neurologic score and total infarct size *versus* 0% Xe. This finding led us to use 30% Xe for subsequent studies. There was an association between total infarct size and neurologic score (neurologic score = 4.976 + 0.053 × total infarct volume, $R^2 = 0.47$, 49 total degrees of freedom, $P < 0.0001$).

Although not quantitatively assessed, rats treated with 45% Xe were visibly sedated, but the righting reflex was retained. Neither sedation nor agitation was evident with either 15% or 30% Xe.

Experiment 2: Treatment Duration Analysis

Physiologic values were similar to those reported for Experiment 1 and thus are not reported. Figure 2 shows the effect of 30% Xe treatment duration on 7-day MCAO outcome. A main effect for treatment condition was observed for neurologic score ($P = 0.001$). All treatment durations were different from 0% Xe (8 h, $P = 0.004$; 20 h, $P = 0.05$; 44 h, $P = 0.02$) with no difference detected among treatment durations (8 h *vs.* 20 h, $P = 0.99$; 8 h *vs.* 44 h, $P = 0.93$; 20 h *vs.* 44 h, $P = 0.87$). Xenon did not have a main effect on total ($P = 0.06$) or cortical ($P = 0.11$) infarct volumes. Subcortical infarct volume was decreased ($P = 0.006$). A *post hoc* difference was present between 44 h 30% Xe and 0% Xe ($P = 0.01$). There was a positive association between total infarct size and neurologic score (neurologic score = 4.845 + 0.051 × total infarct volume, $R^2 = 0.51$, 68 total degrees of freedom, $P < 0.0001$).

Because of the lack of differences among treatment groups for infarct size, we postulated that this reflected insufficient statistical power to detect an effect of xenon inhalation on infarct size by using a four-group study design. To explore this, a *post hoc* analysis was performed in which all three xenon treatment durations were collapsed into a single xenon exposure group. Comparison of this collapsed group to 0% xenon identified differences for neurologic score ($P = 0.0001$) and total ($P = 0.007$), cortical ($P = 0.02$), and subcortical ($P = 0.0006$) infarct sizes. Because of negligible differences in treatment duration on 7-day recovery, and because 20-h exposure allowed the most convenient experimental protocol, the following study used 30% Xe for a duration of 20 h.

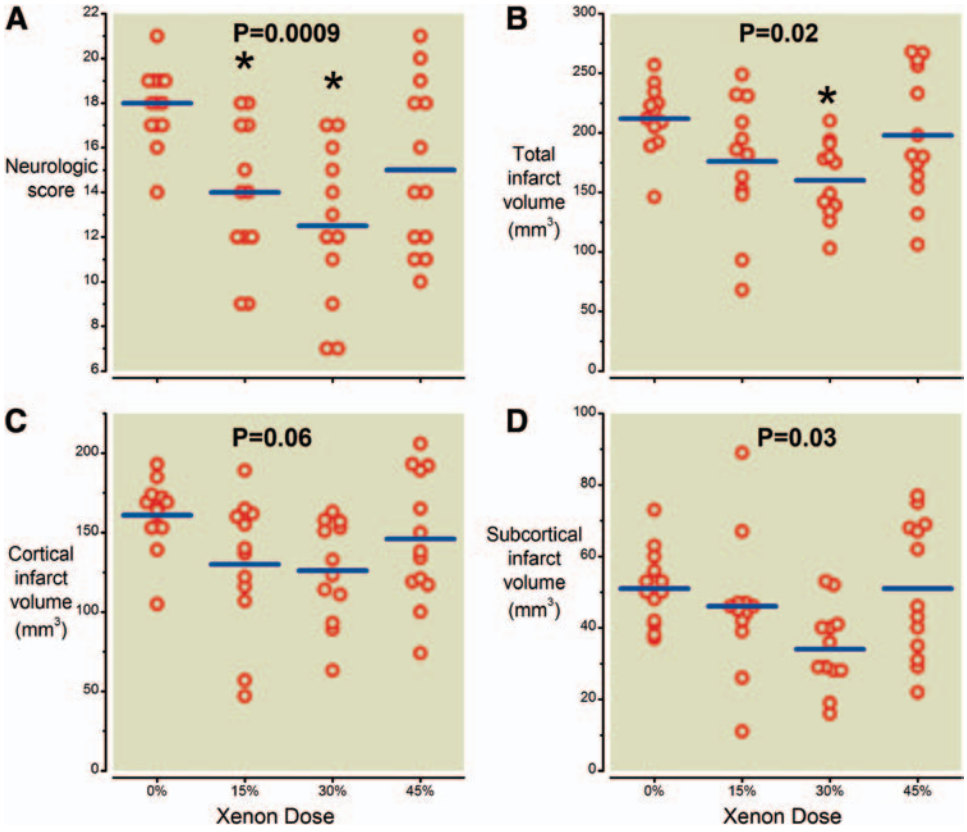

Fig. 1. Xenon (Xe) dose–response analysis. Rats were exposed to 0, 15, 30, or 45% Xe (in 30% O_2/balance nitrogen) by random assignment for 20 h, beginning 90 min after reperfusion from 70-min awake middle cerebral artery occlusion. A 7-day recovery interval was allowed. *Open circles* indicate individual animal values. *Horizontal lines* indicate mean values for (*A*) neurologic score and (*B*) total, (*C*) cortical, and (*D*) subcortical infarct sizes. For neurologic scores, 0 = no neurologic deficit (potential range 0–48). *P* values indicate ANOVA main effect. * $P < 0.05$ *versus* 0% Xe (*post hoc* analysis).

Experiment 3: Xenon as a Subtherapeutic Hypothermia Adjunct

The thermoregulation system was effective in achieving and maintaining target temperatures in the normothermia and hypothermia groups (fig. 3). Physiologic values were otherwise similar to those reported in table 1 for Experiment 1 and thus are not reported. As intended, body weight was similar among groups before the ischemic insult ($P = 0.78$). After 4 weeks recovery, a main effect was present for body weight ($P = 0.04$). Between-group differences were absent except for greater body weight in the 36.0°C/30% Xe *versus* the 37.5°C/0% Xe group (404 ± 37 g *vs.* 340 ± 103 g, respectively, $P = 0.04$). This pattern persisted when we compared body weight change from preischemic baseline values ($P = 0.02$).

Figure 4 shows neurologic and histologic findings. A main effect for treatment condition was observed for neurologic score ($P = 0.0003$). *Post hoc* analysis for between-group differences indicated improved neurologic score only in the 36.0°C /30% Xe *versus* 37.5°C/0% Xe groups ($P = 0.0005$). Main effects were present for total ($P = 0.015$) and cortical ($P = 0.03$) infarct volumes. A main effect was absent for subcortical infarct volume ($P = 0.06$). There were no between-group differences for cortical infarct volumes. For total infarct volume, only the combination of 36.0°C/30% Xe was statistically different from 37.5°C/0% Xe ($P = 0.04$).

Experiment 4: Xenon in ICH

In the experiment designed to evaluate 24 h ICH functional and histologic outcome, there was no difference between groups for preinjury rotarod latency to fall (0% Xe = 258 ± 58 s, 30% Xe = 250 ± 40 s, $P = 0.76$). Twenty-four hours postcollagenase injection, rotarod latency to fall was better in mice treated with xenon (0% Xe = 55 ± 34 s, 30% Xe = 122 ± 59 s, $P = 0.015$). Xenon decreased planimetrically defined hemorrhage volume (0% Xe = 48 ± 17 mm^3, 30% Xe = 32 ± 7 mm^3, $P = 0.027$; figure 5).

In the MR imaging/brain water content experiment, one mouse in the 0% Xe group died before the final MR imaging sequence and wet–dry weight analysis. Any difference between groups for hemorrhage volume at 2 or 24 h (0% Xe = 5.2 ± 1.8 mm^3, 30% Xe = 4.1 ± 1.9 mm^3, $P = 0.42$) or at 24 h (0% Xe = 14.9 ± 1.2 mm^3, 30% Xe = 13.8 ± 2.0 mm^3; $P = 0.37$) postcollagenase injection was not statistically significant. The increase in hemorrhage volume was nearly identical between groups over the 22-h observation interval (0% Xe = 9.7 ± 2.7 mm^3, 30% Xe = 9.7 ± 2.3 mm^3, $P = 0.98$; fig. 6). Three-dimensional volume measurements of the ventricular

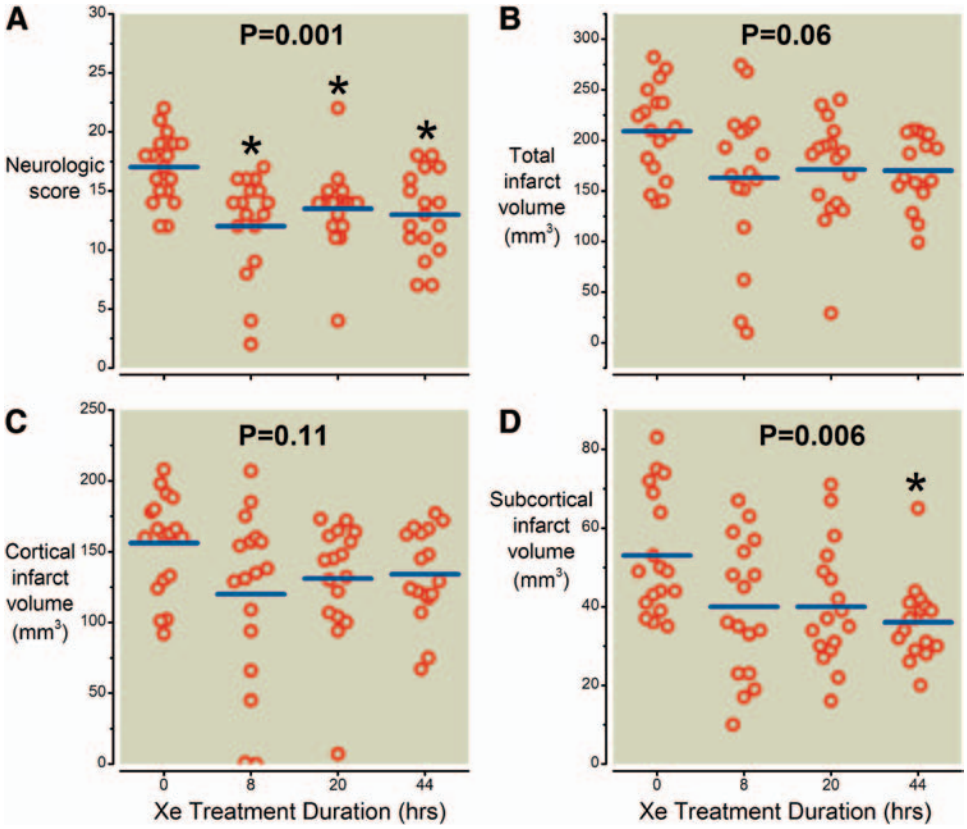

Fig. 2. Xe treatment duration analysis. Rats were randomly exposed to 30% Xe (in 30% O_2/balance nitrogen) for 0, 8, 20, or 44 h, beginning 90 min after reperfusion from 70-min awake middle cerebral artery occlusion. A 7-day recovery interval was allowed. *Open circles* indicate individual animal values. *Horizontal lines* indicate mean values for (*A*) neurologic score and (*B*) total, (*C*) cortical, and (*D*) subcortical infarct sizes. For neurologic scores, 0 = no neurologic deficit (potential range 0–48). *P* values indicate main effect. *P* values = one-way ANOVA main effect for each analysis. * $P < 0.05$ *versus* 0 h Xe. Xe = Xenon.

systems identified no difference between groups at 2 h post-collagenase injection ($P = 0.6$). However, at 24 h postcollagenase, hydrocephalus was less severe in the 30% Xe *versus* 0% Xe group ($P = 0.02$, fig. 7). Xenon treatment resulted in lower brain water content at 24 h post-ICH ($P = 0.02$, fig. 8A).

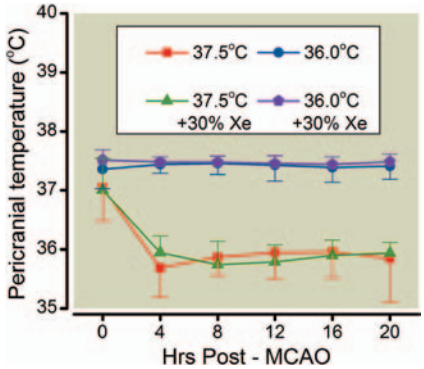

Fig. 3. Xe as an adjunct to subtherapeutic hypothermia (temperature values). Pericranial temperature in rats subjected to 70-min awake normothermic middle cerebral artery occlusion followed by 20 h normothermia (target = 37.5°C) or subtherapeutic hypothermia (target values = 36.0°C) with or without 30% Xe inhalation. Values = mean ± SD. MCAO = middle cerebral artery occlusion; Xe = Xenon.

In the 7-day functional outcome groups, four mice in the 0% Xe and two mice in the 30% Xe treatment groups died over the 7-day recovery interval. There was no effect of xenon detected on % body weight change *versus* baseline ($P = 0.38$). Rotarod performance in survivors was improved by xenon exposure (fig. 8B).

Because a neuroinflammatory response involving microglial activation has been implicated after ICH injury,[33,34] we examined whether 30% Xe was associated with suppressed inflammatory responses to ICH. Immunohistochemistry with F4/80 staining was performed to identify ramified microglia in the hippocampi of mice treated with or without 30% Xe at 7 days after collagenase (n = 6/group). Xenon treatment decreased the number of F4/80+ cells in the hippocampus of the left (ipsilateral) hemisphere (fig. 9).

Discussion

These experiments were designed to examine preclinical efficacy of xenon in focal ischemic stroke. Postischemic xenon produced a dose-dependent benefit when outcome was measured at 7 days postischemia, with 30% Xe offering maximal benefit. However, when examined in a 28-day recovery model, we could not detect benefit. In

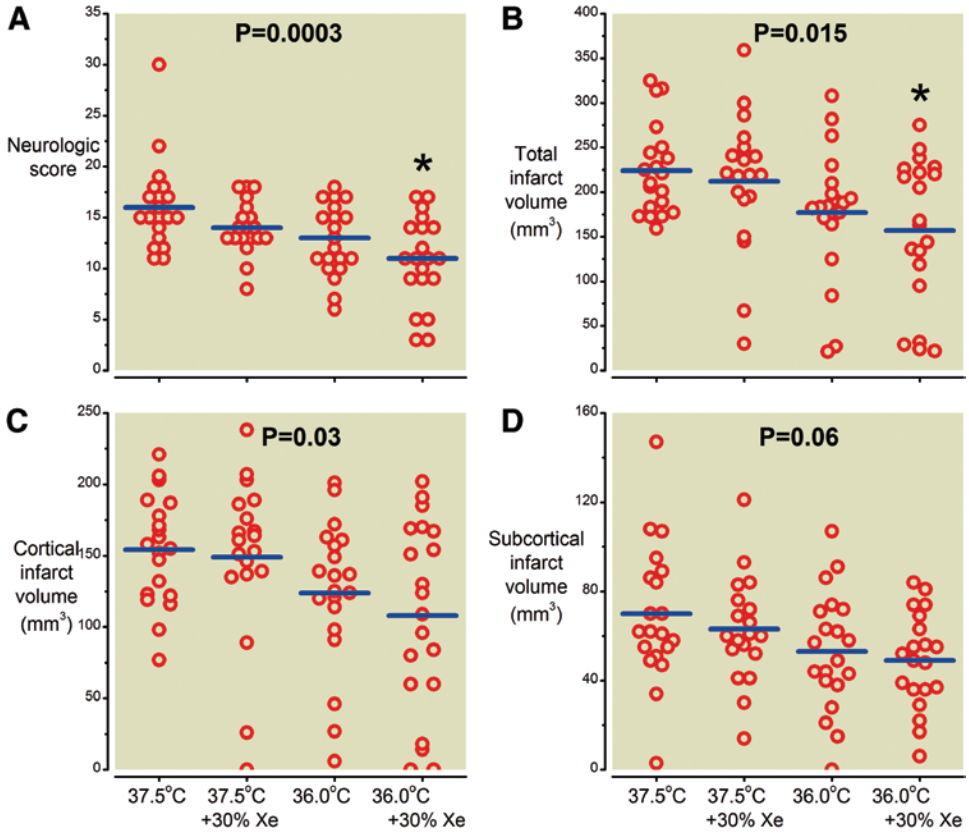

Fig. 4. Xe as an adjunct to subtherapeutic hypothermia (outcome). Rats were subjected to 70-min awake normothermic (37.5°C) middle cerebral artery occlusion and 90-min reperfusion. They were then randomly allocated to control of pericranial temperature at 37.5° or 36.0°C with or without exposure to 30% Xe for 20 h. Neurologic score and cerebral infarct size were measured 4 weeks postischemia. *Open circles* indicate individual animal values. *Horizontal lines* indicate mean values for (*A*) neurologic score and (*B*) total, (*C*) cortical, and (*D*) subcortical infarct sizes. For neurologic scores, 0 = no neurologic deficit (potential range 0–48). *P* values indicate main effect. * $P < 0.05$ *versus* 37.5°C/0% Xe. Xe = Xenon.

contrast, when combined with subtherapeutic hypothermia (36.0°C), 30% Xe improved outcome measured at 28 days postischemia. Thus, under rigorous experimental conditions, we found xenon to offer transient neuroprotective properties when given as a sole therapeutic during recovery from focal cerebral ischemia, but when used as an adjunct to subtherapeutic hypothermia, the net benefit was substantial and sustained. We also examined xenon in a mouse ICH model in which 30% Xe alone was found to improve functional outcome in two independent experiments.

Xenon has been widely studied to define its therapeutic potential. *In vitro* studies indicated that xenon interacts with glutamatergic but not GABAergic receptors.[35] Later, xenon was specifically defined to be a competitive inhibitor of the glycine recognition site on the NMDA receptor.[3] Subanesthetic doses of glycine recognition site antagonists have little effect on cerebral blood flow or metabolic rate,[36] consistent with lack of xenon effect on intracranial hypertension.[10,11] Glycine recognition site antagonists are largely devoid of adverse psychotomimetic effects,[4,37] that have otherwise stymied development of competitive and noncompetitive NMDA glutamate antagonists.[38] However, although glycine recognition site antagonists have previously been found efficacious in preclinical stroke models with short-term recovery (*i.e.*, <7 days),[39] this class has failed to translate into clinical efficacy when postischemic treatment onset was evaluated at 3 months post-ictus.[40]

Several studies have investigated the role of xenon in experimental focal ischemia. The first involved a 3-h postischemic exposure to 50% or 75% Xe, begun 15 min after 90-min MCAO.[18] Although major reduction in cerebral infarct size was reported, the recovery interval after MCAO was brief (24 h), brain temperature was not controlled, and neurologic function was not assessed.

In subsequent work, the therapeutic window for xenon treatment onset after rat MCAO was examined.[41] The window was narrow, with efficacy lost when 50% Xe treatment was started 2 h after 60-min MCAO. Xenon treatment, begun at 1 h after the ischemic insult, improved neurologic function and decreased cortical infarct volume by a remarkable 85% when measured at 48 h postischemia. Brain temperature was not controlled during ischemia or xenon exposure. To assess a potential confound from xenon-induced hypothermia, brain temperature was measured before and after a 3-h exposure to air or 50% Xe in normal

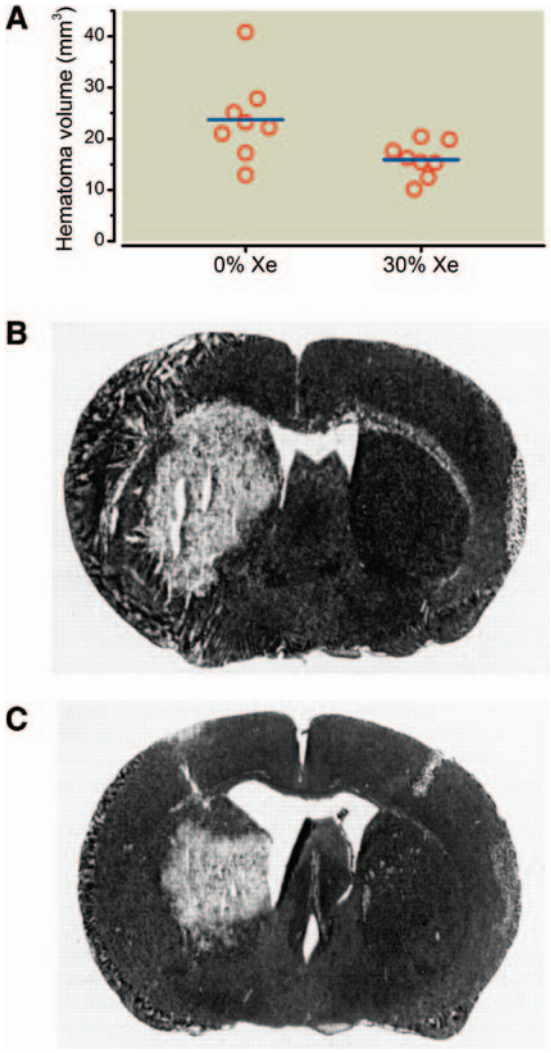

Fig. 5. Effects of Xe on collagenase-induced hematoma volume. Mice were stereotactically injected with intrastriatal collagenase. Two hours later, mice were randomized to receive 0 or 30% Xe for 20 h. Hematoma volume was measured at 24 h postinjection. *A*, Individual (*open circles*) and mean values (*horizontal line*) for the 0 and 30% Xe groups. *B*, Representative coronal section of the untreated (0% Xe) control lesion. *C*, Representative coronal section of a xenon-treated animal. Xe = Xenon.

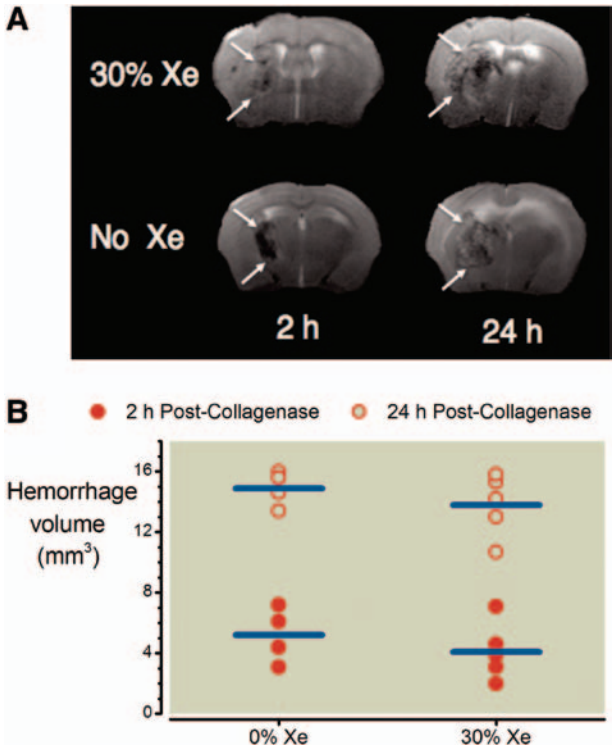

Fig. 6. Magnetic resonance volumetric imaging of collagenase-induced hemorrhage. *A*, Representative axial T2-weighted brain images were obtained at 2 h after collagenase injection but before 20 h of either 30% Xe or 0% Xe exposure and repeated at 24 h postcollagenase injection. In both groups of mice, evolution of parenchymal hemorrhage was observed over the 22-h interval. *Arrows* provide examples of lesion borders used to define hemorrhage volume. *B*, Hemorrhage volumes for individual mice (*open* and *closed circles*) were not different between groups at either the 2-h ($P = 0.42$) or 24-h ($P = 0.37$) measurement. *Horizontal bars* indicate group mean values. Mean hematoma growth over 22 h also was not different between the 0 and 30% Xe groups ($P = 0.98$). Xe = Xenon.

rats. Xe (50%) decreased brain temperature by an average of 1.3°C. Thus, the beneficial effect could be attributed to xenon, hypothermia, or a combination of these two events. In any case, long-term efficacy was not defined.

Homi *et al.*[17] studied mice subjected to 60-min MCAO. Xe (70%) or nitrous oxide (70%) was given during the ischemia insult. Pericranial normothermia was maintained. A 24-h recovery interval was allowed. Xenon was superior to nitrous oxide in two of three neurologic measures and decreased relative cerebral infarct size by approximately 35%. Efficacy of postischemic xenon treatment was not evaluated.

A common thread runs through these studies. Xenon can improve acute experimental focal ischemic outcome, but no evidence was provided that xenon can provide benefit beyond a postictal interval of 48 h. Thus, previous data had minor importance in the context of human stroke in which efficacy of interventions is typically measured at 90 days postictus.[42,43] Furthermore, it is likely that previous investigations of xenon postischemic efficacy have only been assessed in the presence of a confounding state of unregulated brain hypothermia. Thus, temperature-independent effects of xenon have yet to be explored in focal ischemic stroke.

Our experiments, therefore, were designed and executed with the major goal being definition of long-term stroke outcome effects of xenon treatment in the context of regulated brain temperature. When we began, there was little evidence regarding optimal xenon dose or treatment duration in MCAO. The first two studies were designed to define these criteria before embarking on a long-term outcome efficacy analysis. We observed an overall dose–response effect of xenon, most notably with 30% Xe. Furthermore, our

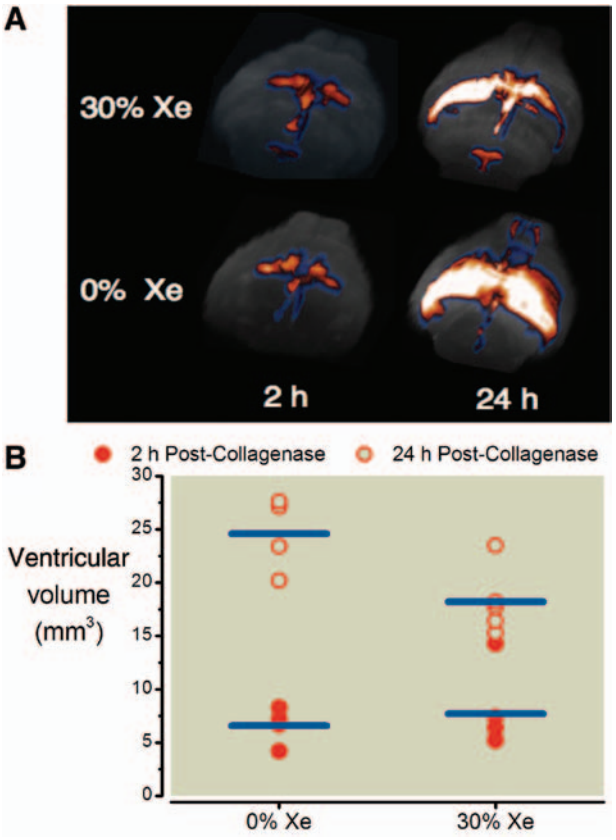

Fig. 7. Magnetic resonance volumetric ventricular imaging after collagenase-induced hemorrhage. *A*, Representative 3D volume measurements of the ventricular systems at 2 and 24 h postcollagenase injection in mice randomized to 30% Xe or 0% Xe treatment. Fused 3D renderings of brain and ventricles revealed hydrocephalus predominantly involving the lateral ventricles, with relative sparing of the third and fourth ventricles, suggesting a noncommunicating pattern of volume increase. No etiology of aqueductal obstruction, however, was observed on the magnetic resonance images obtained. *B*, Hydrocephalus was greater in animals receiving 0% Xe. *Horizontal bars* indicate group mean values. Ventricular volumes for individual mice (*open* and *closed circles*) were not different between groups at 2 h (P = 0.6). At 24 h, ventricular volumes were greater in the 0% Xe *versus* 30% Xe treatment groups (P = 0.02). Xe = Xenon.

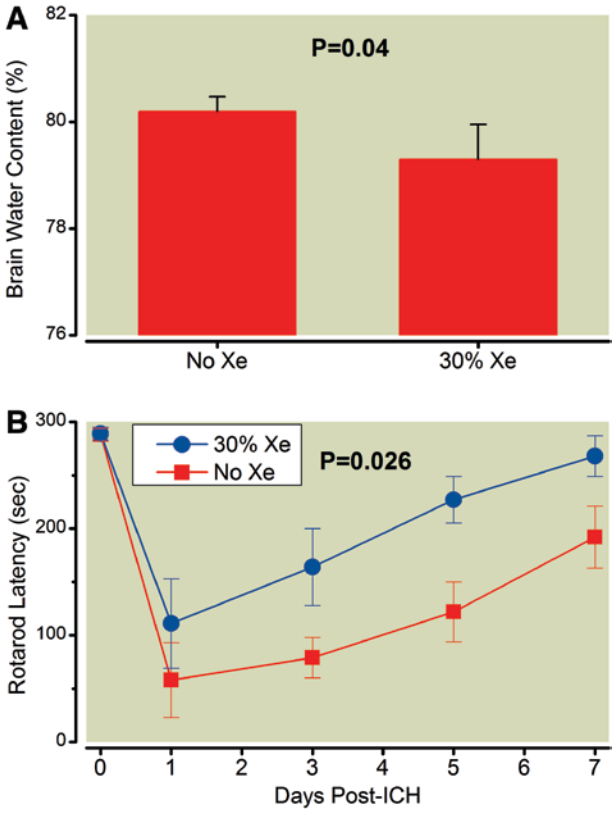

Fig. 8. Effects of Xe on brain water content and functional recovery after collagenase-induced ICH. Mice were subjected to unilateral intrastriatal collagenase injection and allowed to recover 24 h or 7 days. Two hours postcollagenase, mice were exposed to 0 or 30% Xe for 20 h. *A*, Brain water content (mean ± SD) as a function of xenon treatment measured 24 h postcollagenase injection. *B*, Rotarod function over a 7-day recovery interval. Values presented as mean ± SEM for clarity. ICH = intracerebral hemorrhage; Xe = Xenon.

rats were visibly sedated at 45% Xe, whereas sedation was not evident at 30% Xe. Sedation might not be tolerated in humans recovering from stroke. Hence, we performed subsequent studies with 30% Xe.

We then examined xenon treatment duration. A range of 8–40 h was studied. This was primarily based on our study of Mn porphyrin oxidoreductants in a similar MCAO model, in which long-term outcome efficacy was only achieved with sustained Mn porphyrin treatment.[44] Similarly, postischemic hypothermia provides enduring protection only when sustained for more than 12 h.[45] Despite this, we found little difference between treatment duration groups. This may indicate that the pharmacologic effect of xenon is only relevant in the first few hours postischemia. This is consistent with the purported mechanism of action, that is, impedance of ischemia-induced Ca^{2+} influx through the NMDA ionophore.[18] NMDA antagonists have a narrow therapeutic window,[46] consistent with normalization of extracellular glutamate concentrations shortly after restoration of circulation from MCAO.[47]

Our data showed a beneficial effect of xenon begun after transient MCAO that was reproducible and independent of temperature in a 7-day recovery model. We then conducted a longer-term (28 days) MCAO outcome efficacy analysis. On the basis of reports that the combination of xenon and hypothermia offered superior efficacy to xenon alone in neonatal hypoxia–ischemia models,[48–50] we chose to also evaluate that combination in the long-term outcome MCAO study. We studied a subtherapeutic magnitude of hypothermia that could potentially be used clinically in the absence of mechanical ventilation.[28,51] Neither 36.0°C nor xenon alone provided sustained benefit. In contrast, long-term benefit was observed when 30% Xe was combined with pericranial temperature maintained at 36.0°C in conscious rats. This encourages further experimental investigation of this therapeutic approach in focal ischemic stroke. It remains to be determined whether

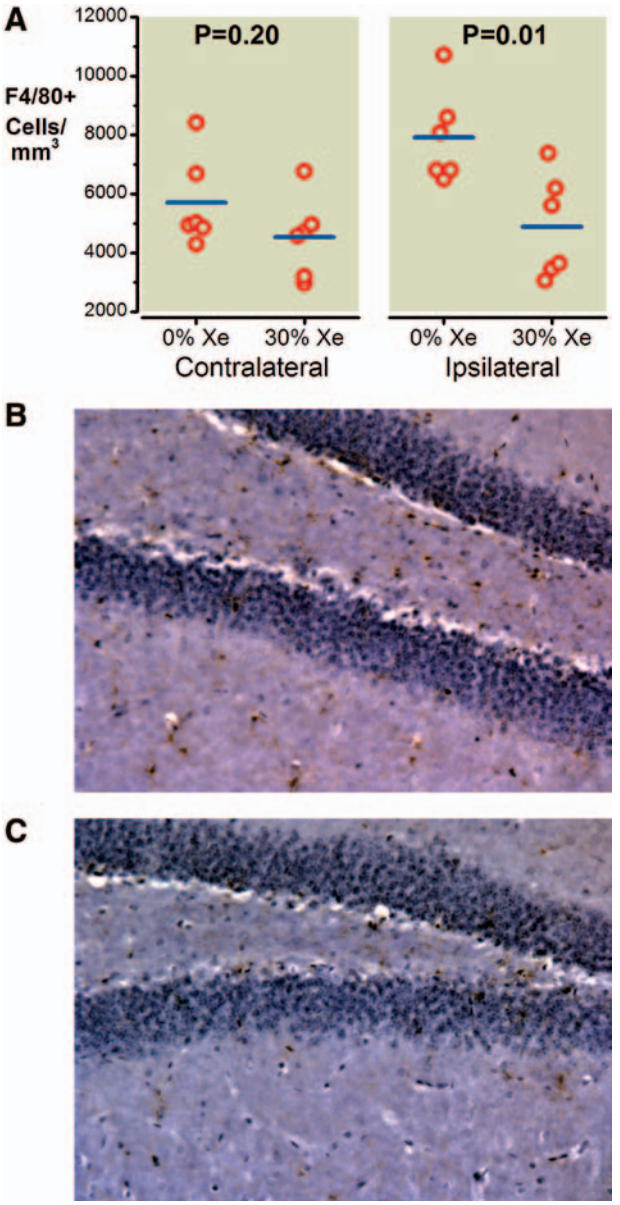

Fig. 9. Effects of xenon (Xe) on microglial activation after intracerebral hemorrhage. Microglial activation (F4/80 positive cells) was stereologically assessed in the hippocampus 7 days after intrastriatal collagenase injection. *A*, Individual (*open circles*) and mean values (*horizontal line*) for the 0 and 30% Xe groups. *B*, Representative F4/80 immunohistochemistry in mouse not treated with xenon. *C*, Representative F4/80 immunohistochemistry in mouse treated with 30% Xe for 20 h.

our observed histologic effect size (30% decrease in cerebral infarct volume) and the corresponding magnitude of improvement in neurologic deficit resulting from xenon and subtherapeutic hypothermia will be sufficient to translate to a heterogeneous human stroke population.

Because xenon has negligible hemodynamic and intracranial pressure effects and because 30% Xe causes only moderate sedation,[52,53] it could be proposed that xenon treatment could begin early in the course of medical care, even before scanning is performed to rule out spontaneous ICH. If so, it would be necessary to ensure that xenon does not potentiate ICH. To address this, we subjected mice to an ICH insult. We found that, to the contrary, xenon improved ICH outcome. This could either be attributable to advantageous properties of xenon in the context of ICH or to an artifact of an interaction between xenon and collagenase, as used to initiate hemorrhage in our experiments. Both factors may be relevant. While xenon attenuated microglial activation and decreased hydrocephalus and brain water content, xenon also binds with the active sites of some serine proteases.[54]

David *et al.*[55] examined interactions between xenon and tissue plasminogen activator, a serine protease. Xenon inhibited tissue plasminogen activator thrombolysis in a rat thromboembolic MCAO model. This work may portend limitations for use of xenon in ischemic stroke victims eligible for tissue plasminogen activator therapy. In contrast, when xenon treatment was begun after tissue plasminogen activator thrombolysis was achieved, major reduction of infarct size was observed, but temperature was not measured, and the outcome observation interval was only 24 h in both studies.[55]

Clostridial collagenase is, in part, activated by serine proteases.[56] Xenon may have improved ICH outcome by decreasing collagenase activity and subsequent hemorrhage in our experiments. Hematoma expansion is approximately 75% evolved at 3 h postcollagenase injection, with full expansion complete at 24 h.[57] Thus, there was some opportunity for xenon to interact with collagenase-induced hematoma expansion. To further explore this, we repeated our work using MR imaging allowing each animal to serve as its own control. Xenon did not affect hemorrhage volume and evolution, consistent with reports from others who failed to find an increase in collagenase-induced ICH hematoma volume in rats treated with a serine protease inhibitor.[58] A fundamental question was answered in both of our ICH studies. Xenon does not worsen outcome in experimental ICH. Further exploration of this newly described therapeutic potential for xenon is warranted. Sustained efficacy in a longer-term recovery model should be explored.

We conclude that xenon alone offers limited and transient improvement in outcome from experimental focal ischemic stroke. This was independent of treatment duration, with 30% Xe providing optimal efficacy. Subtherapeutic pericranial hypothermia also offered no long-term benefit. However, the combination of 30% Xe and subtherapeutic hypothermia produced sustained outcome improvement. In collagenase-induced ICH, xenon decreased brain edema, hydrocephalus, neurologic deficits, and microglial activation. Further investigation of xenon in ischemic and hemorrhagic stroke should serve to better define potential clinical value. Such work might investigate effects of age, sex, and coexisting diseases common to stroke patients, including insulin resistance and hypertension, on efficacy.

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