Background and Purpose—Brain injury after hypoxic-ischemic insults evolves via an apoptotic/necrotic cascade. Glutamate over release and N-methyl-D-aspartate (NMDA) receptor over activation (excitotoxicity) are believed to trigger this process. Xenon is a nontoxic anesthetic gas that reduces neurotransmitter release and functionally antagonizes NMDA receptors. Administering xenon to hypoxic-ischemic newborns might be clinically effective if the neurotoxic processes continue evolving after delivery. We sought to determine whether xenon administration after the initial hypoxic-ischemic insult was neuroprotective.

Methods—Fifty 7-day-old rats received a 90-minute hypoxic insult after unilateral carotid ligation. They were then randomized to breathe 1 of 2 gas mixtures for 3 hours: 50% Xe/30% O2/20% N2 or 30% O2/70% N2.

Results—One week after hypoxic-ischemic survival, significant global protection was seen in the xenon group (80% less injury); cortex/white matter (88% versus 25%), hippocampus (62% versus 0%), basal ganglia (81% versus 25%), and thalamus (50% versus 0%; percentage of global damage score in nonxenon versus xenon groups, respectively).

Conclusions—Three hours of xenon administration commenced after hypoxia-ischemia in neonatal rats provides short-term neuroprotection. This finding suggests that treatment with xenon after perinatal asphyxia would also be neuroprotective. Because xenon does not cause other neurotoxic effects and has demonstrated minimal side effects in extensive anesthesia studies, it would make an ideal candidate for the treatment after human perinatal hypoxia-ischemia. (Stroke. 2006;37:501-506.)

Key Words: anesthesia ■ neuroprotection ■ xenon ■ hypoxia ■ ischemia ■ newborn ■ asphyxia ■ rats ■ N-methyl-D-aspartate

The development of therapies to reduce brain injury secondary to perinatal hypoxia-ischemia (HI) is important because of the severity of disability that may result. Theencephalopathy that follows an hypoxic-ischemic insult reflects an evolving process characterized by an initial primary injury followed by a self-sustaining cascade of harmful biochemical events leading to further brain damage.1,2 This involves apoptotic and necrotic processes.3,4 The evolving injury involves the process of excitotoxicity in which excess neurotransmitter release causes receptor overstimulation and cell death. Cooling is the only intervention that has proven to be effective, both experimentally in neonatal models of HI5–8 and in recent clinical trials of either selective head cooling with mild systemic hypothermia9 or total body cooling.10,11 From these human studies, it has been estimated that it will be necessary to treat 6 neonates to achieve a more favorable outcome in 1 of them. Therefore, more effective treatments are desirable. Hypothermia as clinical neuroprotection is still regarded as an experimental treatment, and adverse cardiovascular effects may limit its applicability in the preterm infant.

An ideal neuroprotectant should be effective some hours after the initial hypoxic insult, with minimal adverse effects, no toxicity, and fast onset. The noble gas xenon may be such an agent. It is an almost chemically inert apolar atom that rapidly reaches equilibrium with the brain when inhaled. It is an effective anesthetic with a very rapid onset, no metabolism by the body, and no proven adverse hemodynamic clinical side effects, unlike many other inhaled agents.12–15 However, xenon is expensive, which has limited its clinical use. Xenon has been shown recently to be neuroprotective in vitro and in vivo in rats when administered during hypoxia.16,17 However, in the newborn infant born with evidence of brain injury, only treatment starting after birth is feasible.

Recent randomized clinical trials show that hypothermia provides neuroprotection after hypoxic-ischemic encephalopathy.10,11 It may be that combinations of therapies emerge as the optimum clinically applicable neuroprotective strategy.

Therefore, the aim of this study was to investigate whether 3 hours of xenon inhalation was neuroprotective in an established rat model of neonatal HI18 when administered after the HI insult.
Materials and Methods

Animal Preparation

The protocol was conducted under Home Office license according to UK guidelines. A total of 76 7-day-old (P7) Wistar rat pups of either gender from 8 size-culled litters were used (Barn 1, Langford, Somerset, UK). Twenty-six pups were used for pilot experiments or temperature monitoring. Fifty were studied in the main experiment. These were monitored before, during, and after a severe unilateral HI insult followed by a period of xenon administration in half the animals. Rats were allowed to survive 7 days (P14) after the procedure.

Operative Procedures

At P7, the pups were subjected to the combined hypoxic-ischemic insult (unilateral carotid ligation followed by hypoxia; inhalation of 8% oxygen as described by Rice et al in 1981). Pups were anesthetized with halothane (3.5% induction, 1.5% maintenance) in nitrous oxide and oxygen (1:1). The left common carotid artery was cut between double ligatures of prolene sutures (6.0). The pups were left to recover with the dam for ~1 hour followed by exposure to hypoxia (8% O₂, 92% N₂) for 90 minutes. During this period, 4 of 50 pups died. Pups were paired for body weight and sex; within each pair, they were randomized to breathe a xenon/nitrogen/oxygen mixture (xenon group) or an oxygen/nitrogen mixture (nonxenon group) for 3 hours starting immediately after the hypoxic insult. Operation order was balanced between each treatment group.

One pup in the nonxenon group died during this 3-hour post-HI treatment period. Four nonligated pups had a rectal temperature probe placed for monitoring and were not used for further examination. A total of 45 pups (22 in the xenon group and 23 in the xenon group) survived to P14 and their brains were examined.

Temperature Monitoring

Calibrated (<0.1°C deviation) temperature probes (IT-21; Physitemp) were inserted 0.5 cm rectally. Rectal temperature was maintained as close to 37.0°C as possible by varying the chamber/gas temperature. As shown previously in this model, rectal temperature is representative of the brain temperature.

Gas Delivery

After the exposure to a 90-minute HI insult, the xenon group was given a breathing mixture of 50% xenon/20% nitrogen/30% oxygen, and the nonxenon group was given a mixture of 30% oxygen/70% nitrogen in their respective chambers for 3 hours before being returned to the dam. A 50% xenon/30% oxygen/20% nitrogen mixture has 2.8× the density but almost the same viscosity as air. We chose to use 50% xenon in our treatment group for 2 reasons: First, although xenon might potentially be more neuroprotective than 50%, it would leave no margin for increasing the inspired oxygen percentage, a likely clinical requirement. Second, in our pilot studies, some neonatal rats did not tolerate breathing such a dense gas mixture for 3 hours, and any additional anesthetic depressant effect would be greater at 70% than 50%.

The O₂/N₂ mixture was delivered at 200 mL/min. The chamber design included an adjustable temperature-controlled circulating water jacket through which the tubing carrying the breathing gas to the chamber first passed to keep the gas temperature equal to that of the water jacket.

Xenon, oxygen, and nitrogen were premixed in a large (50 L) metallic bag before commencement of the experiment (Hans Rudolph, Inc.) and were delivered at 200 mL/min by an electric pump (Bedfont Scientific Ltd.). Both chambers were temperature controlled via the circulating water jacket such that the rectal temperature of the “thermometer” rat in each chamber remained as close to 37°C as possible.

Survival Period

Pups were returned to their dams after the 3-hour xenon or nonxenon exposure and weighed daily.

Histopathology

On P14 (1 week of recovery), the rats were weighed and anesthetized with 20 mg/kg pentobarbital intraperitoneally and the brain perfused (transcardiac) with 5 mL of isotonic NaCl and fixed with 4% formaldehyde (6 to 8 mL/min for 5 minutes). The brains were dissected, weighed, and kept in 4% formaldehyde before embedding.

Neuropathology Scoring

Blocks were sectioned at 6 μm and stained with hematoxylin and eosin. Ligated and nonligated sides of the brain was examined and scored. Four areas of the brain were examined (cortex/white matter, hippocampus, basal ganglia, and thalamus) by a pathologist (H.P.) blinded to the treatment allocation. The severity of damage was graded from 0.0 (no injury) to 4.0 (maximum injury), with 0.5 intervals for each of the 4 regions, giving a 9-step scale that has been validated previously by us and used by others (Table 1). Results are presented for each individual region and as an average of the sum of these scores from these regions (global pathology score; Table 2). There was no injury on the nonligated side of the brain in any of the animals.

Data Analysis

Data were analyzed using Statview v.5.0.1 (Adept Scientific) and displayed as mean (SD) or median (95% CI).

### TABLE 1. Definition of Neuropathology Scoring System (reproduced with permission)

<table>
<thead>
<tr>
<th>Brain area</th>
<th>Grading</th>
<th>Percent Area Affected</th>
<th>Morphologic Changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex, thalamus, and basal ganglia</td>
<td>1</td>
<td>≤10</td>
<td>Small, patchy, complete, or incomplete infarcts</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>20–30</td>
<td>Partly confluent complete or incomplete infarcts</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>40–60</td>
<td>Large confluent complete infarcts</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>&gt;75</td>
<td>In cortex; total disintegration of the tissue, in thalamus and basal ganglia; large complete infarcts</td>
</tr>
<tr>
<td>Hippocampus*</td>
<td>1</td>
<td>≤20</td>
<td>Necrotic neurons only in the most lateral areas. (i.e, CA3 and CA4)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>50</td>
<td>Patchy areas of necrotic neurons in sectors CA1–CA4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>75</td>
<td>More extensive areas of necrotic neurons in sectors CA1–CA4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>100</td>
<td>Complete infarction of hippocampus including the dentate gyrus</td>
</tr>
</tbody>
</table>

Grade 0 indicates no histopathologic damage.

*The grading system is defined differently for grades 1 through 3 in the hippocampus because selective neuronal necrosis is the typical damage in this area.
Eosin–Stained Sections From 4 Brain Regions and Between-Group Comparisons* for Hematoxylin and Eosin–Stained Sections From 4 Brain Regions

<table>
<thead>
<tr>
<th></th>
<th>Nonxenon-Treated (n = 22)</th>
<th>Xenon-Treated (n = 23)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuropathology cerebral</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cortex/white matter</td>
<td>3.5 (1.0–4.0)</td>
<td>1.0 (0–3.0)</td>
<td>0.023</td>
</tr>
<tr>
<td>Neuropathology hippocampus</td>
<td>2.5 (1.0–4.0)</td>
<td>0 (0–2.0)</td>
<td>0.048</td>
</tr>
<tr>
<td>Neuropathology basal ganglia</td>
<td>3.25 (0.5–4.0)</td>
<td>1.0 (0–3.0)</td>
<td>0.045</td>
</tr>
<tr>
<td>Neuropathology thalamus</td>
<td>2.0 (0.5–3.6)</td>
<td>0 (0–1.5)</td>
<td>0.059</td>
</tr>
<tr>
<td>Global neuropathology score</td>
<td>2.6 (0.5–3.6)</td>
<td>0.5 (0–2.4)</td>
<td>0.033</td>
</tr>
</tbody>
</table>

*Mann–Whitney U test.

A P value of <0.05 was considered significant. The Mann–Whitney U test was used for comparison of nonparametric data and Student t test (unpaired) for comparing parametric data within and between groups.

Results

Pilot Experiments
Two pilot studies were performed to develop aspects of equipment and experimental design.

Methods of Gas Delivery
Ten rat pups were subjected to a hypoxic gas mixture of 50% xenon/42% nitrogen/8% oxygen for 120 minutes, without carotid ligation. The gases were supplied to the chamber in a closed recirculating loop with carbon dioxide removal by soda-lime to conserve xenon and contain costs.

It proved difficult to accurately control the gas composition, and also, carbon dioxide removal was inefficient. Three animals died toward the end of the insult, the remaining 7 surviving to P14. A nonrecirculating gas supply system was devised for future experiments.

Intrahypoxic-Ischemic Xenon
In this pilot experiment, the left carotid was ligated in 12 rat pups before they were randomized into 2 groups, 1 receiving 50% xenon/42% nitrogen/8% oxygen and the other a gas mixture of 92% nitrogen/8% oxygen in separate chambers. An additional 2 ligated pups were placed in the xenon chamber for blood gas analysis at the end of the 90-minute exposure period. After 90 minutes of hypoxia, 2 had died and 4 survived to P14 of the 6 study animals in each group.

The presence of xenon 50% compared with nitrogen during hypoxia did not alter survival either way. At P14, body weight was not significantly different in either group. The open gas delivery circuit used in this experiment was more practical with no CO2 build up in either chamber. The 2 additional pups in the xenon chamber had blood gases taken at the end of the 90-minute breathing period, confirming that there was no elevation of blood PaCO2 versus control values.

Main Experiments
Two identical temperature-controlled chambers with nonrecirculating fresh gas supply systems were used for each group after the 90-minute HI insult. The mean time for carotid ligation was 8.4 minutes.

Body Weight and Temperature
The mean body weights and brain weights in both groups did not differ significantly at any postnatal age. The bodyweights of rats in both groups were almost identical at P7 (xenon 11.98 g [1.63]; nonxenon 12.0 g [1.41]) and also at P14 (xenon 25.03g [3.84]; nonxenon 24.78 g [3.64]). The ratio of brain weight to body weight at P14 was slightly lower in the xenon group, but this was not statistically significant (xenon 0.052 [0.009]; nonxenon 0.05 [0.008]).

Recordings of rectal temperatures and corresponding chamber air temperatures during the HI insult and subsequent exposure to xenon- or nonxenon-containing gas mixture are shown in Figure 1. During the HI insult, the mean rectal temperature of the rats was 37(0.43)°C, and the corresponding mean environmental chamber temperature was 35.3(0.56)°C, a difference of 1.7°C. During the subsequent treatment phase, this difference was significantly higher in the xenon group at 2.05°C than the difference in the nonxenon-treated group, which was 1.68°C (P<0.05).

Evaluation of Brain Damage
Posthypoxic administration of xenon significantly reduced (P<0.05) brain injury evaluated by the global neuropathology score (Table 2). In each of the 4 regions examined, cortex/white matter, hippocampus, basal ganglia, and thalamus, the brain injury was reduced by xenon. This reduction was significant (P<0.05) in the cortex/white matter, hippocampus, and basal ganglia (Table 2).

A column scatter plot shows the distribution of the global neuropathology score for each animal in the xenon and nonxenon groups (Figure 2). The scores are not normally distributed in either group. In the xenon group, the scores are skewed toward the mild end of the severity scale compared with the nonxenon group (median 0.5 and 2.6, respectively).

The macroscopic appearance of a typical brain in the xenon group (median global pathology score 2.6) and xenon-treated group (0.5) is shown in Figure 3.

Discussion
Our findings indicate that a nonanesthetic (50%) dose of the noble gas xenon administered after a hypoxic-ischemic insult to a spontaneously breathing neonatal rat model offers uni-
formally 80% neuroprotection, as assessed by neuropathology of the major areas of the brain. The dose of xenon used would be clinically achievable in the human neonate because it would allow for the use of up to 50% oxygen in the inhaled gas mixture. It has been shown previously in adult rat HI models that xenon is neuroprotective when applied during the insult phase (intraischemic neuroprotection). This is not an option for sick newborns that are typically diagnosed after delivery, so we ideally require therapies that are effective after birth.

Our findings are consistent with the view that the processes leading to apoptosis triggered by HI are ongoing for a period of time after the initial insult. This would explain why xenon is still protective even when given after rather than during an HI insult.

Our assessment took place 7 days after the insult, and so it is a possibility that damage might continue to evolve in the longer term. However, in many neuroprotection studies of similar design, usually involving cooling, any attenuation of the initial benefits derived from the neuroprotective treatment has usually occurred by 5 to 7 days after insult. Future studies using xenon should not only investigate whether xenon neuroprotection is sustained in the longer term but also define the maximum window of opportunity after which treatment may be futile.

The P7 rat pup model has been used very extensively as a model of the near-term human newborn. There is some controversy about this maturity correlation because some believe it more closely represents a mildly preterm ≈34-week infant. However, aspects of brain maturation in the rat have different rates of maturation, and overall, most believe that the model is indeed a valid representation of a near-term human infant.

In general, hypothermia has a beneficial effect on the HI neonatal brain. The results obtained depend on the timing of initiation of hypothermia, its depth, and the duration for which it is applied. For example, previous studies with this and other models have shown significant protection with a 3- to 5-hour intervention, although a study in which very mild hypothermia was used for 3 hours, long-term protection did not occur. Two human trials have demonstrated benefit from 48 hours of hypothermia applied within 6 hours of birth. There is probably an ideal balance between speed of commencement of therapy and degree and duration of cooling, but it appears that more work in this area is required.

An HI insult can trigger an over-release of the transmitter glutamate. Excessive synaptic glutamate levels cause overactivation of N-methyl-D-aspartate (NMDA)-type glutamate receptors, flooding cells with Ca²⁺, causing generation of NO/peroxynitrite, and a neurotoxic cascade is set up that results in cell death. Activation of lipases and proteases, including those in the proinflammatory cytokine cascades, also contributes to cell injury and death. Experimentally, NMDA blockade exerts some neuroprotective effect, and it has been noted that this becomes ineffective if delayed >1 to 2 hours after HI insult because these downstream events become self-sustaining. The window of opportunity for starting neuroprotective interventions therefore appears to be a few hours. In most xenon neuroprotection studies, the xenon has been administered during the insult, with occasional exceptions including a study suggesting that xenon pretreatment may also be beneficial.

It is a finding in animal models that there is a spontaneous fall in temperature during hypoxic brain injury. This has been called anapryxia and is mediated via dopamine D1 receptors. This explains why, in our study, the chamber temperature needed to be gradually increased to maintain a constant rectal temperature during the HI insult phase (Figure 1).

Evidence exists in animals and humans that postinsult hyperthermia increases brain injury. We avoided this by maintaining the rectal temperature of the rats in both chambers close to the target value and close to each other, although the nonxenon group was slightly and nonsignificantly cooler by 0.2°C. Whole body cooling is known to be neuroprotective in its own right, and if 0.2°C cooling affected outcome in this study, it would have reduced any apparent neuroprotective benefit of xenon by favoring the nonxenon group.
The difference between rectal and chamber gas temperature was significantly higher in the xenon than the non-xenon group, 2.05°C versus 1.68°C, and this may be related to the differing heat transfer properties of the 2 gas mixtures. Xenon has one third of the thermal conductivity and approximately one sixth of the specific heat capacity of nitrogen and oxygen. This confirms the need to measure rectal temperature in such experiments to ensure similar core temperatures.

In the first 2 weeks of life in the neonatal rat, neurons expressing NMDA receptors become very sensitive to glutamate-triggered excitotoxicity and cell death by apoptosis. However, complete NMDA blockade during this period in rats also induces apoptosis. Although there is evidence that NMDA antagonists can be protective in models of neuronal injury, equally, there is evidence that they can also be neurotoxic.

Most anesthetics inhibit γ-aminobutyric acid type A receptors or reduce excitation via NMDA receptors. With the exception of xenon, drugs acting by either mechanism have been found to induce widespread apoptosis in the immature rat brain. If complete NMDA receptor blockade induces apoptosis and yet overstimulation causes excitotoxic apoptosis, then it is perhaps noteworthy in this context that xenon produces a partial rather than complete blockade of the NMDA receptor; at a concentration of 80%, it reduces NMDA activated currents by −60%. In addition to the inhibition of NMDA receptor activation by xenon, other possible neuroprotective mechanisms of action may exist. For example, in glutamatergic and dopaminergic cell cultures, xenon reduces hypoxia-induced release of these neurotransmitters to a greater extent than pure NMDA receptor blockers, suggesting other modes of action for xenon than NMDA blockade alone. This protective effect has been shown to require functioning intracellular calcium homeostasis and calmodulin systems.

In vivo studies have also demonstrated xenon neuroprotection. In a rat brain injury model, arcuate nucleus injury provoked by xenon was reduced in a dose-dependent manner by xenon. In a rat cardiopulmonary bypass model, neurocognitive dysfunction was reduced by xenon delivered during cardiopulmonary bypass. The reduction was greater than that produced by the NMDA antagonist drug MK801, again suggesting >1 mechanism of neuroprotection for xenon.

Xenon has the advantage over oral or intravenous agents because the partial pressure in the brain will closely follow that delivered to the lungs. Xenon crosses the blood–brain barrier effectively because it is a general anesthetic and is notably free from side effects. This may be a major advantage because many potential neuroprotectants do not cross the blood–brain barrier, and adverse side effects profiles have prevented many from reaching clinical use. Although xenon is expensive, cost-efficient methods of delivery are being developed. There is a need for effective neuroprotective strategies in many areas of medicine, and we suggest that neonatology should be a high priority, given the current limited effective alternatives and the profound impact of neonatal brain damage on the future life of the affected baby and the consequent financial impact on society.


Xenon Provides Short-Term Neuroprotection in Neonatal Rats When Administered After Hypoxia-Ischemia

John Dingley, James Tooley, Helen Porter and Marianne Thoresen

*Stroke.* 2006;37:501-506; originally published online December 22, 2005; doi: 10.1161/01.STR.0000198867.31134.ac

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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