Xenon and Hypothermia Combine Additively, Offering Long-Term Functional and Histopathologic Neuroprotection After Neonatal Hypoxia/Ischemia

Catherine Hobbs, PhD; Marianne Thoresen, MD, PhD; Alexander Tucker, BA; Kristian Aquilina, FRCS; Ela Chakkarapani, MRCPCH; John Dingley, MD

Background and Purpose—Hypoxic/ischemic (HI) brain injury affects 1 to 6 per 1000 live human births, with a mortality of 15% to 20%. A quarter of survivors have permanent disabilities. Hypothermia is the only intervention that improves outcome; however, further improvements might be obtained by combining hypothermia with additional treatments. Xenon is a noble anesthetic gas with an excellent safety profile, showing great promise in vitro and in vivo as a neuroprotectant. We investigated combinations of 50% xenon (Xe50%) and hypothermia of 32°C (HT32°C) as a post-HI therapy.

Methods—An established neonatal rat HI model was used. Serial functional neurologic testing into adulthood 10 weeks after injury was performed, followed by global and regional brain histopathology evaluation.

Results—In the combination Xe50%/HT32°C group, complete restoration of long-term functional outcomes was seen. Hypothermia produced improvement on short- (P<0.001) and long- (P<0.001) term functional testing, whereas Xe50% alone predominantly improved long-term function (P<0.05), suggesting that short-term testing does not always predict eventual outcome. Similarly, the Xe50%/HT32°C combination produced the greatest (71%) improvement in global histopathology scores, a pattern mirrored in the regional scores, whereas Xe50% and HT32°C individually produced smaller improvements (P<0.05 and P<0.001, respectively). The interaction between the 2 treatments was additive.

Conclusions—The xenon/hypothermia combination additively confers greater protection after HI than either treatment alone. The functional improvement is almost complete, is sustained long term, and is accompanied by greatly improved histopathology. The unique safety profile differentiates xenon as an attractive combination therapy with hypothermia to improve the otherwise bleak outcome from neonatal HI. (Stroke. 2008;39:1307-1313.)

Key Words: xenon ■ neuroprotection ■ neonatal ischemia ■ hypoxia/ischemia

Hypoxic/ischemic (HI) brain injury occurs in 1 to 6 per 1000 live human births with a mortality of 15% to 20%, and a quarter of survivors have permanent disabilities.1–5 The destructive cascade lasting hours or days includes “excitotoxic” apoptosis by prolonged activation of destructive cascade lasting hours or days includes “excitotoxic” apoptosis by prolonged activation of N-methyl-D-aspartate (NMDA) glutamate receptors.6–7 The existence of such a cascade raises the possibility that a postsinsult therapy for neonates might be developed to limit the eventual damage. Currently, hypothermia is the only intervention that improves neurologic outcome after HI injury and shows promise both experimentally8–10 and clinically.11–13 One in 6 children benefit from this treatment,11 but further improvements might be gained if HT were initiated sooner after birth and/or if additional treatments were added. Xenon, a noble gas with anesthetic properties, has shown great promise as a neuroprotectant in both in vitro and in vivo experimental studies.14–20 It is attractive as a combination therapy with hypothermia owing to its lack of chemical reactivity, lack of clinical side effects,21–24 previous use in neonates,25 rapid reversibility, and lack of fetotoxicity.26,27 Furthermore, xenon was approved as an anesthetic drug in Russia in 2002,28 in Germany in 2005, and extended through Europe in March 2007. Xenon is an NMDA antagonist.29,30 Interestingly, the neuroprotective effects appear to exceed those of specific NMDA receptor antagonists,17 suggesting additional mechanisms of action, such as inhibition of 2 other subtypes of glutamate receptor channels, ie, the α-amino-3-hydroxy-5-methyl-4-isoxazololene propionate and kainate receptors,31 a general reduction in neurotransmitter release,14 and effects on other ion channels.32

Having previously demonstrated short-term neuroprotection by xenon in an established HI model,20,33 we hypothesized that this mixture of “ideal” properties would make the xenon-hypothermia combination a superior post-HI therapy to either treatment alone and would produce better long-term functional outcomes. Allowing rat pups to survive to adult-
hood permits both short- and long-term behavioral testing (2, and 8 to 11 weeks of age), as well as regional histopathologic evaluation. Therefore, in this study, we investigated combinations of xenon-hypothermia in an in vivo model of neonatal HI. Herein we report that their effects are additive, that greater neuroprotection is produced by the combination than from either treatment alone, and that this is sustained long term on both behavioral and histopathology evaluations.

Materials and Methods
We used the standard neonatal HI rat model that has been in use for >25 years.34 There are inherent variabilities in the degree of brain injury between litters and strains. Specifically, within any discrete group of rat pups simultaneously receiving the same insult, there will be a wide distribution of damage, from no injury to severe. Consequently, relatively large numbers of strictly randomized animals must be used to convincingly demonstrate an effect.35 We reduced variability by using 3 experienced researchers and performing the carotid ligations in parallel, hence, a short duration of anesthesia (<6 minutes) and similar durations between ligation and insult. Our new design of the chamber for hypoxia ensures similar temperature, O2, and CO2 levels in each compartment.

Procedures
All procedures were conducted under Home Office license in accordance with UK guidelines. A total of 119 seven-day-old rat pups were randomized to juvenile control (n=28) or experimental (n=91) groups. The experimental pups underwent left common carotid artery ligation under general anesthesia. After <1.5 hours of recovery with their dam, pups were exposed to 8% O2 for 90 minutes in a temperature-controlled chamber with maintenance of rectal temperature at 36°C; without treatment, this gives ~60% unilateral brain injury in this model.8,20 The 81 pups that survived this hypoxic challenge were then paired by weight and sex before randomization among 4 groups (n=19 to 22) to recover for 3 hours at normothermia (NT37°C) or hypothermia (HT32°C) with or without 50% xenon (Xe50%) in the breathing gas. Rectal temperature was continuously measured in additional “sentinel” pups in each chamber by using a calibrated (~0.1°C deviation) temperature probe (TF-21, Physitemp Instruments, Clifton, NJ) inserted 0.5 cm rectally. Continuous rectal temperature recordings allowed the target temperature to be maintained by varying the chamber temperature. These sentinel rats were excluded from analysis, as the stress of carrying a probe has been shown to affect outcome.36

Chamber Design
Ideally, the core temperature of all rats in any group should be identical, as differences in rectal temperature during the insult affect outcome. In a new chamber design (by J.D.) with even heat distribution and CO2 scavenging, each rat pup had its own enclosure with an individual gas supply. A closed-loop gas-recirculation system was used to conserve xenon. The chamber design is shown in Figure 1.

Figure 1. Chamber design. Pups were placed on a heat-conductive “floor” (A) in individual enclosures (B). This was inserted into a gas-tight container (C). The floor (A) was then in thermal connection to a heated/ cooled mat (D) beneath, allowing even temperature control of the floor (A). Soda-lime in a tube (E) absorbed CO2. Gases recirculated around a closed-loop circuit: Chamber gases entered at (F), passed through a CO2 absorber (E), and exited through a port (G) to a roller pump, returning to the chamber through a divided tube (H) so that each pup had its own fresh gas supply port. The thermal mat (D) contained fluid circulating through ports (I), supplied by a heater/ cooler unit (Tecotherm, Inspiration Healthcare Ltd Leicester, UK).

Figure 2. Short- and long-term testing methods. a, Negative geotaxis test. Pups were placed at the midpoint of a sloping platform (A) facing downward. The time taken to rotate to face upward was recorded. b, Montoya staircase test. The pups (now 8–11 weeks old) entered the chamber through an opening at the rear and innately mounted the platform (A). On either side of platform (A) was a “staircase” (B) comprising 7 steps, each baited with 3 sugar pellets. This staircase was in a groove that was just wide enough for a paw to enter. Consequently, the task became increasingly difficult as the pellets were consumed. The pup had to fully grasp and manipulate the tiny pellets to successfully retrieve them.
Early Behavioral Testing (Negative Geotaxis Test)

At 2 weeks of age, 1 week after the HI insults, the animals underwent the "negative geotaxis" test, which examines the time taken to rotate 180° from a head-down to a head-up position when placed head-down on a 45° slope (Figure 2a). This is an innate postural response that develops in the second week of life in normal pups. Because we wished to examine whether this early test was a reliable predictor of long-term functional and pathologic outcomes, a long-term testing regime then followed.

Long-Term Behavioral Testing (Staircase Test)

Throughout weeks 8 to 10, the rats underwent long-term "staircase" testing. This is a functional test of the ability to pick up and manipulate sugar pellets from steps, where each pellet was more difficult to reach than the previous one, making this a sensitive test of fine motor dexterity (Figure 2b). Ligation of the left common carotid artery followed by hypoxia impairs the function of the right paw. In brief, sucrose pellets (three 45-mg pellets per step; BioServ, Frenchtown, NJ) were placed on each of the 7 descending steps of either 1 of 2 staircases straddling a central platform. By baiting 1 staircase at a time, fine motor dexterity of each forepaw may be assessed independently. Rats underwent 1 trial per day with each staircase baited, 5 days per week, for 3 weeks. During the first 5 days of testing, rats were allowed 7 1/2 minutes to retrieve the pellets from each baited staircase. After the first week, this testing period was reduced to 3 1/2 minutes. Because the first 2 steps may be reached

Figure 3. Outcomes. Short- (b) and long- (c, d) term functional effects of each treatment. a, Rectal temperatures from "sentinel" rats during 90-minute hypoxia in a chamber with 8% O2 followed by 3-hour recovery at 37°C or 32°C. b, Negative geotaxis test (at 2 weeks of age, 1 week after insult): time to rotate 180° (head placed downhill to spontaneous turn head uphill). c, "Staircase" test (end of week 10): retrieving sugar pellets with the right paw from the staircase with increasing difficulty; the mean number of pellets retrieved during the last 3 days are shown (the right paw tests the function of the left hemisphere). d, Number of pellets retrieved with the right paw during the 15-day testing period (8–11 weeks age) for each treatment group illustrates the emerging performance advantage of the Xe50%/HT32°C combination.

Hobbs et al Xenon With Cooling Gives Sustained Neuroprotection

1309

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with the tongue, retrieval of pellets from these steps does not reflect fine motor control. Thus, retrieval of these pellets was not included in the analysis. The number of pellets (a maximum of 15 pellets possible beyond the first 2 steps per side) retrieved during this time was recorded.

**Histopathology**

Transcardiac perfusion with 10% phosphate-buffered (0.1 mol/L) formaldehyde was performed at 11 weeks of age under deep halothane/fentanyl anesthesia. The brains were held in 4% formaldehyde until further processing. Coronal 3-mm blocks were cut according to a standard matrix for uniformity through the brain (ASI Instruments Inc, Warren, Mich) and then embedded in paraffin. Blocks were sectioned at 6 μm and stained with hematoxylin and eosin. Four areas of the brain were examined (cortex, basal ganglia, thalamus and hippocampus) by an investigator blinded to 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 adapted from a scoring scale used to assess injury 1 week after HI that has been validated against cell counting previously by us and used by others.20,36,39 – 41

**Statistics**

Data are given as mean±SEM in the figures. To investigate the nature of any interaction between xenon and hypothermia, we used a full factorial linear model. We investigated between-subject effects for each of 3 dependent variables: negative geotaxis, staircase testing, and global pathology. The design comprised intercept+HT_{32°C}+Xe_{50%}+HT_{32°C}×Xe_{50%}, where the term HT_{32°C}×Xe_{50%} represents the question, does the combined effect of these 2 treatments differ from the sum of the effects of each when given alone? We also investigated between-subject effects in a separate full factorial linear model of similar design with the pathology scores from each of the 4 brain regions as the dependent variables. A probability value <0.05 was considered statistically significant.

**Results**

Rectal temperature was maintained close to target values in all groups (Figure 3a). Hypothermia alone produced a functional improvement observed on both short- (negative geotaxis) and long- (staircase) term testing (P<0.001 and P<0.001, respectively), whereas Xe_{50%} alone showed more modest improvement, only evident in the long term (negative geotaxis P=0.865, staircase P<0.05). This suggests that short-term testing in this model is not always a reliable predictor of long-term outcome. The combined Xe_{50%} and HT_{32°C} treatment produced the greatest improvement on both short- and long-term functional testing, indistinguishable from the results observed in the juvenile control group, with almost complete restoration of function (Figures 3b and 3c).

During daily staircase testing from 8 to 11 weeks, the untreated pups (Xe_{0%}NT_{37°C}) reached an early performance plateau while all treated groups continued to demonstrate further improvement, suggesting that the normal ability of immature animals to continuously learn was impaired by HI. In the Xe_{50%} or HT_{32°C} only groups, this function was partially restored, and in the case of the combination Xe_{50%}HT_{32°C} treatment group, it was fully restored (Figure 3d). No sex differences were noted in the control or any treatment group

**Figure 4. Pathology.** a, Treatment effects at 10-week survival. Global neuropathology score was assessed from hematoxylin and eosin–stained sections of the HI-injured (left) hemisphere. Injury severity was graded from 0.0 to 4.0 (4=75% to 100% injury). Representative brain sections of each global score (0–4) are shown. b, Regional pathology scores. Global pathology score was the average of the 4 brain regional scores shown (see Table 2 for statistics). All results are expressed as mean±SEM.
with the “negative geotaxis test” performed at P14; however, the staircase test showed significantly better performance (by 1.8 pellets) by female rats in both the control and all treatment groups. This improved performance was independent of lesion size or type of treatment. Because our experimental groups had a balanced sex distribution, this finding did not affect the overall results.

After 10 weeks (ie, long-term) of survival, the HI group without postinsult treatment (Xe0% NT37°C) had the greatest global histopathologic injury scores (Figure 4a). Xe50% and HT32°C were both individually neuroprotective (P<0.05 and P<0.001, respectively), HT32°C having the greater effect. The greatest neuroprotection was seen with the Xe50% HT32°C combination (Figure 4a). Furthermore, these findings were observed not only in the global pathology scores but also in the pathology scores for each individual brain region (Figure 4b). HT32°C alone was protective in all brain regions (P≤0.003 in all areas), whereas Xe50% alone was protective in the cortex only (P<0.01, Figure 4b). Again, the greatest reduction in injury across all areas was seen with Xe50% and HT32°C in combination (Figure 4b).

We particularly wished to determine whether there were any neuroprotective effects of the combined use of xenon and hypothermia that were in excess of the sum of those seen when each was used in isolation (ie, synergism).18,19 Consequently, Tables 1 and 2 also show the results of an ANOVA that investigated possible interactions between each of the 2 treatments in isolation? Residuals were approximately normally distributed. Global neuropathology score was assessed from hematoxylin and eosin–stained sections of the HI-injured (left) hemisphere. Injury severity was graded from 0.0 to 4.0 (4=complete infarction).

Results from multivariate tests of a full factorial linear model investigating between-subject effects for each of 3 dependent variables: Negative geotaxis, staircase, and global pathology scores. Design: Intercept+HT32°C+Xe50%+HT32°C×Xe50%. The term HT32°C×Xe50% represents the question, does the combined effect of these 2 treatments differ from the sum of the effects of each treatment when given in isolation? Residuals were approximately normally distributed. Global neuropathology score was assessed from hematoxylin and eosin–stained sections of the HI-injured (left) hemisphere. Injury severity was graded from 0.0 to 4.0 (4=complete infarction).

Table 1. Treatment Effects up to 10-Week Survival on Short- (Negative Geotaxis) and Long- (Staircase) Term Functional Testing Results and Global Pathology Scores

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Results from multivariate tests of a full factorial linear model investigating between-subject effects with the pathology scores from each of the 4 brain regions as the dependent variables (see Figure 4b). Design: intercept+HT32°C+Xe50%+HT32°C×Xe50%. The term HT32°C×Xe50% represents the question, does the combined effect of these 2 treatments on each brain region differ from the sum of the effects of each treatment when given in isolation? Residuals were approximately normally distributed. Injury severity score was graded from 0.0 to 4.0 (4=complete infarction).

With the xenon-hypothermia combination (Table 1, HT32°C×Xe50%) was not different from the sum of the effects of each treatment alone and that this was true for both short- and long-term functional testing scores, as well as the global histopathology scores. Similarly, there was no evidence of interaction between Xe50% and HT32°C on the regional brain pathology scores (Table 2). Thus, we found that the presence of 1 treatment neither enhanced nor diminished the neuroprotective effect of the other and that the overall effect of the combination was additive.

**Discussion**

This study investigated whether xenon and hypothermia offered greater neuroprotection in combination than either intervention alone and whether any such effects were sustained in the long term in a well-known in vivo HI model. The xenon-hypothermia combination conferred greater protection after HI than either treatment alone. The functional improvement was almost complete, was sustained long term, and was accompanied by greatly improved histopathology. Also, to a lesser extent, both Xe50% and HT32°C individually reduced the
functional deficit and histopathologic brain damage after HI. In combination, we found that these 2 effects were additive. Despite a great deal of research, the mechanisms underling neuroprotection by hypothermia are still not well understood. Hypothermia is known to reduce glutamate release, which would be expected to reduce excitotoxic apoptosis. Hypothermia also reduces glycine release, and this may further reduce hyperexcitation, as glycine promotes the effect of glutamate on NMDA receptors. Furthermore, activation of the Akt/protein kinase B pathway is known to be neuroprotective, and hypothermia may protect against ischemic damage by preserving Akt activity, which in turn inhibits some proapoptotic proteins.

In addition to NMDA receptor antagonism, the mechanisms whereby xenon is neuroprotective may include (1) a general reduction in neurotransmitter release; (2) inhibition of 2 other subtypes of glutamate receptor channels, i.e., the α-amino-3-hydroxy-5-methyl-4-isoxazolole propionate and kainate receptors; (3) reduction in cytosolic proapoptotic Bak protein expression and increased Bcl-xL expression (which binds Bak and so is, in effect, antiapoptotic); (4) activation of 2-pore domain K+ channels by xenon, which will also enhance neuroprotection; (5) inhibition of calcium/calcmodulin-dependent protein kinase II, conferring protection against excitotoxicity in vitro; and (6) increased phosphorylation of transcription factor cAMP-response element binding protein, which may, in turn, upregulate cAMP-response element binding protein-dependent post-synaptic genes.

Complete NMDA receptor blockade may actually be proapoptotic in the developing brain. If so, then it may be noteworthy that even when xenon is applied at a concentration as high as 80%, this only produces partial antagonism of the NMDA receptor, reducing NMDA activated currents by ≈60%. This is a higher xenon concentration than would ever be used clinically, as the xenon/oxygen mixture would be expected to reduce excitotoxic apoptosis. Although a synergistic interaction in vivo between xenon and hypothermia in similar situations has been suggested, we can only report additive effects from short-term function, long-term function, global histopathology, and regional histopathology analyses.

When hypothermia treatment first showed early promise for neonatal neuroprotection, there was concern when it was found to offer only transient short-term neuroprotection, something that had also been seen with other agents trialed in this role. Only 1 previous experimental hypothermia trial had randomized between short (1 week) and relatively long (6 week) survival, and it showed protection (35%) on the basis of histologic analysis. In this study, where the injury overall was very severe, a ranking score from 4 functional tests was correlated with a morphology score; however, this was significant for female pups only. In 2 more recent, large clinical studies with examination at 18 months of age, a reduction in motor deficits such as cerebral palsy without improvement in mental function was found. Eleven-week survival, the point at which we performed our histopathology evaluations, in rats is far longer than 18 months for a human, as rats reach sexual maturity by 9 weeks of age. Therefore, this study is the first to show real long-term functional and pathologic protection in an experimental model of HI, which is crucial in the context of clinical utility.

In conclusion, our investigations demonstrated that Xe50 in combination with HT2°C additively conferred greater protection after HI than either treatment alone. Moreover, the benefit was sustained, with complete restoration of long-term functional outcomes and greatly improved regional histopathology, the xenon-hypothermia combination producing a 71% reduction in the global pathology score. The known safety profile, lack of toxicity, rapid brain penetration, and easy reversal options make xenon a uniquely suitable therapy to combine with hypothermia. This combination has the potential to improve the otherwise bleak outcome after perinatal asphyxia, implying that testing on larger in vivo models, followed by proof-of-concept studies in humans, are now warranted.

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Disclosures
Dr Dingley is currently a board member of a University of Wales College of Medicine spin-out company that is involved in the development of delivery systems for medical gases, including xenon, with relevant intellectual property. None of these systems were used in this study. No other author has a declaration of interest except as researchers on this project funded by the grants mentioned.

References


Xenon and Hypothermia Combine Additively, Offering Long-Term Functional and Histopathologic Neuroprotection After Neonatal Hypoxia/Ischemia
Catherine Hobbs, Marianne Thoresen, Alexander Tucker, Kristian Aquilina, Ela Chakkarapani and John Dingley

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