Asynchronous administration of xenon and hypothermia significantly reduces brain infarction in the neonatal rat

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Background. Neonatal asphyxia causes long-term neurological and behavioural impairment in the developing brain. Concurrent administration of xenon and hypothermia synergistically reduces long-term damage in a rat model of neonatal asphyxia. This study sought to investigate whether asynchronous administration of xenon and hypothermia is capable of combining synergistically to provide neuroprotection.

Methods. Seven-day-old rats were subjected to right common carotid artery occlusion followed by 90 min hypoxia with 8% oxygen. After a 1 h recovery period, rats received asynchronous administration of mild hypothermia (35°C) and xenon (20%) with a 1 or 5 h gap between interventions, xenon (20%) alone, or mild hypothermia (35°C) alone. Infarct volume in the brain was measured 4 days after injury.

Results. Administration of hypothermia or xenon alone, 1 and 6 h after the hypoxic ischaemic insult, respectively, provided no neuroprotection. Asynchronous administration of xenon and hypothermia at a 1 h interval produced a significant reduction in infarct volume [93 (7) vs 74 (8); P<0.05]. Reduction in infarct volume was also present when hypothermia and xenon were asynchronously administered with an intervening gap of 5 h [97 (5) vs 83 (3); P<0.05].

Conclusions. This finding provides a rationale for investigating the combined use of hypothermia and xenon in a progressive manner for the management of neonatal asphyxia. Thus, hypothermia can be administrated at the site of delivery and xenon can be administered later.


Keywords: anaesthetics gases, xenon; hypothermia, asynchronous; hypoxia-ischaemia; neonate; rat

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Perinatal hypoxic ischaemia (HI) is a devastating complication of childbirth; estimates suggest that 2–4 per 1000 term infants will suffer such an episode during labour.1 Of the infants that develop HI encephalopathy, 15–20% will die and of the survivors, a quarter will develop chronic neurological deficits such as cerebral palsy.2 3 Although significant progress has been made in understanding the pathogenesis of the neuronal damage, the development of neuroprotective strategies has proved largely unsuccessful. However, subgroup analysis from the Cool Cap Study showed significant benefit of mild hypothermia (34–35°C) initiated within 6 h and administered for 72 h in mild to moderately injured sufferers of perinatal HI.4 Furthermore, a second large randomized control trial of neonates with HI encephalopathy due to acute perinatal asphyxia demonstrated that systemic hypothermia (33.5°C) for 72 h reduces death and moderate or severe disability by 18% when followed-up at 18 to 22 months of age.5 Any intervention that could augment the protection afforded by hypothermia would be of great significance.

We have recently demonstrated that xenon potentiates hypothermic neuroprotection in in vitro and in vivo models of perinatal HI injury in a synergistic manner6 with efficacy present even when therapy was delayed up to 6 h after the insult. However, as xenon administration is

1Declaration of interest. Professors Maze and Franks are paid consultants to Air Products, a company that is interested in developing clinical applications for medical gases, including xenon. In addition, Air Products have funded, and continue to fund, work in the authors’ laboratories that bears on the actions of xenon as an anaesthetic and neuroprotectant.
currently expensive and the delivery systems capable of mitigating the costs not likely to be widely available, clinical translation of xenon-hypothermia combination therapy may be more feasible if the therapies are started asynchronously with hypothermic therapy initiated at the primary care centre and maintained during transport to a tertiary referral centre where xenon can be delivered. Herein, we report that in *in vivo* models of neonatal HI, xenon and hypothermia combine synergistically to afford neuroprotection even when administered asynchronously.

**Methods**

This study conformed to the United Kingdom Animals (Scientific Procedures) Act of 1986 and was approved by the Home Office (UK). Every effort was made to minimize animal suffering and the number of animals used. Twelve pups of either sex per dam were used and housed with a 12 h light/12 h dark schedule in a temperature- and humidity-controlled colony room.

**Animal model of hypoxic-ischaemic injury**

Seven-day-old Sprague-Dawley rat pups of either sex, weighing between 10 and 14 g were subjected to a model of HI injury as previously described by Rice and colleagues. In brief, rat pups were anaesthetized with isoflurane 2%, unilateral right common carotid artery ligation was then performed through a midline neck incision using 5.0 silk suture. After completion of surgery, pups recovered from the anaesthesia before being returned to their dams for 1 h. The environment was maintained at a constant temperature (23°C) and humidity (48%) for 1 h.

After the 1 h recovery period, pups were exposed to hypoxia by placing them in purpose-built chambers, partially submerged in a 37°C water bath. The animals were exposed to systemic hypoxia by a continuous flow of 8% oxygen balanced with nitrogen for 90 min. This mixture was monitored every 15 min using a Datex Ohmeda (Bradford, UK) analyser. The duration of hypoxia was determined by preliminary experiments which showed that such a period of hypoxia produced maximal HI damage, as measured by hemispheric weight.

Following HI, pups were returned to their dam for a second 1 h recovery period. Littermates were then divided randomly into control and treatment groups. Treatment groups were subjected to one of the following treatment strategies: asynchronous administration of mild hypothermia (35°C) and xenon (20%) with a 1 or 5 h interval between the completion of mild hypothermia and the initiation of xenon, xenon (20%) alone, or mild hypothermia (35°C) alone (Fig. 1). Each treatment strategy was performed on a separate litter with littermates randomly divided into control and treatment groups.

**Treatment strategies**

Pups subjected to the asynchronous treatment strategy of hypothermia and xenon initially underwent 2 h of mild hypothermia (35°C). A pup was selected at random, and under isoflurane and local anaesthesia, a temperature probe was inserted into the cortex and held in place with superglue. Pups were placed in chambers (as before) with a continuous flow of 30% oxygen balanced with nitrogen. The water bath was maintained at a temperature that maintained the cortical temperature at 35°C, as measured with a telemetry system (VitalView, Mini-Mitter, OR, USA). (The pup with the cortical temperature probe was killed upon completion of hypothermia and was not used for analysis.) After treatment, the pups were returned to their dam for an interval of 1 or 5 h. Pups were subsequently placed into the chambers (as before), with the water bath maintained at 37°C, for 90 min. The gas mixture was maintained at 20% xenon, 30% oxygen, and 50% nitrogen via a purpose-built closed system which minimized
xenon leakage. Following 90 min of treatment with xenon, the pups were returned to their dam for 4 days before being killed.

Pups subjected to a treatment strategy of xenon (20%) alone underwent HI then were returned to their dams for 6 h. They were then placed in the chambers and treated with 20% xenon with the water bath maintained at 37°C for 90 min as described earlier. Pups were again returned to their dam for 4 days before being killed. Previous studies have demonstrated that 20% xenon administered alone does not attenuate infarction in the neonatal rat. The interval of 6 h was chosen as it represented treatment with 20% xenon at 3 h without the preceding period of hypothermia. Pups treated with mild hypothermia (35°C) were subjected to the same experimental protocol as described in the asynchronous treatment group, but once the pups were returned to their dam, following mild hypothermia, they underwent no further interventions until killed after 4 days.

In all treatment strategies, control pups were littersmates subjected to surgery and hypoxia but not to the experimental interventions, thereby acting as positive controls. When experimental animals underwent treatment with hypothermia or xenon, control animals were placed in identical chambers with the water bath maintained at 37°C and a continuous flow of 30% oxygen balanced with nitrogen.

Tissue preparation
Brains were harvested 4 days following the end of the experimental protocol. Pups were killed via an intraperitoneal injection of pentobarbital (100 mg kg⁻¹), perfused transcardially with heparin 2 u ml⁻¹ in phosphate buffer solution (PBS) followed by formaldehyde 4%, 200 ml in PBS, and whole brains removed. Brains were dehydrated using a Histokinette 2000 tissue embedding processor (Leica UK Ltd., Milton Keynes, UK) and then embedded in paraffin wax blocks. Coronal sections (thickness of 6 μm) were taken at regular intervals of 500 μm, using a microtome (Leica, Germany). Sections were then stained with cresyl violet. Five sections, relative to the bregma (+2 mm, +1, 0, −2, −4, and −5) based on their morphological appearance were selected (Fig. 2A). Once identified, the slice was photographed and the area of the healthy brain matter in both unlesioned and lesioned hemispheres was measured using analysis software (ImageJ v1.31, NIH image software, Bethesda, MD, USA) by one investigator who was blinded to the experimental treatment. The difference between two areas was used to indicate the brain infarct size (mm²) in each animal at the different anatomical level.

Statistical analysis
The results were expressed as mean (SEM). Infarct size in each anatomical region was plotted together and the area under the curve (AUC), representing infarct volume, was then calculated for further statistical analysis using unpaired Student’s t-test. A P-value of <0.05 was considered statistically significant.

Results
Seven of the 50 animals subjected to 90 min of hypoxia after right common carotid artery ligation died during exposure to hypoxia and before intervention (brain samples from these subjects were not analysed further). No delayed mortality occurred in any of the groups. After returning the animals to their dam, all recovered well and started feeding from their mothers. Ninety minutes of HI insult caused a readily noticeable brain infarct in the right hemisphere (Fig. 2A). This was reduced with asynchronous administration of xenon and hypothermia at a 1 h interval after 90 min period of HI (Fig. 2C).

Administration of 20% xenon alone 6 h after the HI insult afforded no neuroprotection (Fig. 3A) and the infarct volume did not differ significantly in control vs treatment groups [88 (4) vs 85 (9); P=0.38] (Fig. 4). Administration of 2 h of 35°C hypothermia 1 h following the HI insult also afforded no neuroprotection (Fig. 3B). The mean infarct volume (AUC) showed no statistically significant difference between control and treatment groups [control, 89 (8) vs treatment, 94 (1); P=0.42] (Fig. 4). Asynchronous administration of xenon and hypothermia at a 1 h interval demonstrated a statistically significant reduction in infarct volume between control and treatment groups [93 (7) vs 74 (8); P<0.05] (Figs 3C and 4). This reduction in infarct volume was still afforded when xenon and hypothermia were administered at an interval of 5 h [97 (5) vs 83 (3); P<0.02] (Figs 3D and 4).
Discussion

This study aimed to investigate whether the synergistic neuroprotection afforded by intervention with concurrent xenon and hypothermia was retained if these interventions were administered asynchronously in an in vivo model of HI. Asynchronous administration of xenon and hypothermia at intervals of 1 and 5 h significantly reduced infarction in a neonatal rat model of HI. This neuroprotective effect was not seen when xenon or hypothermia were administered alone. Previously we have shown that xenon and hypothermia exhibit synergistic neuroprotective properties when administered together and the enthalpy change produced by this combination has been shown by van’t Hoff plots to far exceed the change that would be anticipated by a physical interaction of these two treatments. As the synergistic neuroprotection is retained even when xenon and hypothermia are administered asynchronously, this indicates that the synergism is not purely physical; as the two treatments were administered asynchronously this negates the possibility that hypothermia simply increases binding of xenon to its receptor.

Both excitotoxicity and apoptosis are involved in the pathogenesis of neonatal HI injury. Hypothermia and xenon have been shown to have both anti-excitotoxic and anti-apoptotic actions. Hypothermia acts presynaptically to reduce glutamate release and acts proximally in the apoptotic cascade to prevent neurotoxicity. Hypothermic neuroprotection preserves Akt activity while inhibiting pro-apoptotic proteins such as c Jun N-terminal kinase activation and forkhead transcription factor, proximal to cytochrome C release. Xenon acts postsynaptically to reduce excitotoxicity, likely via inhibition of the NMDA subtype of the glutamate receptor and prevents apoptosis by actions preceding translocation of Bax. Furthermore, xenon has recently been shown to be efficacious in various models of pharmacological induced apoptosis including protein kinase inhibitor-induced and isoflurane-induced apoptosis; also, xenon upregulates anti-apoptotic proteins in a preconditioning paradigm. The asynchronous administration of the two interventions suggests combined efficacy at preventing evolving neuronal injury, which is likely to be apoptotic in nature. Further investigation of the cross-talk between the neuroprotective cascades of xenon and hypothermia will allow optimization of therapeutic strategies incorporating both interventions.

We suggest that this treatment strategy correlates with the likely clinical scenario that the high cost and specialist equipment for xenon administration would demand. If
hypothermia were initiated at a primary care centre, transporta-
tion to a tertiary referral centre could then be com-
pleted for xenon therapy in a specialized unit. Indeed, it is
even possible that the therapeutic effect of the combi-
nation could be augmented if the period of cooling was
extended to 72 h as employed in recent clinical trials.45

In summary, xenon and hypothermia combine synergis-
tically to significantly reduce HI injury in the neonatal rat
brain when administered asynchronously. This provides an
insight to the mechanism through which xenon and
hypothermia afford neuroprotection and may make such a
treatment strategy more adherent to a clinical scenario.

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