Combined Treatment of Xenon and Hypothermia in Newborn Rats - Additive or Synergistic Effect?

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Abstract

Background: Breathing the inert gas Xenon (Xe) enhances hypothermic (HT) neuroprotection after hypoxia-ischemia (HI) in small and large newborn animal models. The underlying mechanism of the enhancement is not yet fully understood, but the combined effect of Xe and HT could either be synergistic (larger than the two effects added) or simply additive. A previously published study, using unilateral carotid ligation followed by hypoxia in seven day old (P7) rats, showed that the combination of mild HT (35°C) and low Xe concentration (20%), both not being neuroprotective alone, had a synergistic effect and was neuroprotective when both were started with a 4 h delay after a moderate HI insult. To examine whether another laboratory could confirm this finding, we repeated key aspects of the study.

Design/Methods: After the HI-insult 120 pups were exposed to different post-insult treatments: three temperatures (normothermia (NT) NT37°C, HT35°C, HT32°C) or Xe concentrations (0%, 20% or 50%) starting either immediately or with a 4 h delay. To assess the synergistic potency of Xe-HT, a second set (n = 101) of P7 pups were exposed to either HT35°C+NTE0%, NT+XE20%, or a combination of HT35°C+XE20%, starting with a 4 h delay after the insult. Brain damage was analyzed using relative hemispheric (ligated side/unligated side) brain tissue area loss after seven day survival.

Results: Immediate HT32°C (p = 0.042), but not HT35°C significantly reduced brain injury compared to NT37°C. As previously shown, adding immediate XE50% to HT32°C increased protection. Neither 4 h-delayed XE20% nor XE50% at 37°C significantly reduced brain injury (p > 0.050). In addition, neither 4 h-delayed HT35°C alone, nor HT35°C+XE20% reduced brain injury. We found no synergistic effect of the combined treatments in this experimental model.

Conclusions: Combining two treatments that individually were ineffective (delayed HT35°C and delayed XE20%) did not exert neuroprotection when combined, and therefore did not show a synergistic treatment effect.

Introduction

Therapeutic hypothermia (HT) has been shown to be safe and reduces brain injury after hypoxia ischemia (HI) in human newborns [1,2,3,4,5,6] and animal models [7,8]. Since 2010, HT has become standard clinical treatment after perinatal asphyxia [9]. Outcome data show that HT has the potential to improve long term outcome [10,11,12]. However, 50% of cooled newborns still suffer death or severe disability [12,13,14] and improved neuroprotective treatment is sought. In theory, such additional treatment might have either an additive or a synergistic effect with HT.

We and others have previously shown in different animal species that the noble gas xenon (Xe) alone is neuroprotective after hypoxia-ischemia [8,13,16], and that neuroprotection is increased when combined with HT [7,8,17,18]. However, the exact mechanism by which Xe is neuroprotective is not yet fully understood. Xenon is an anesthetic and is often referred to as an “ideal anesthetic” as it has been shown to be safe, providing rapid onset and offset characteristics with no adverse hemodynamic or other effects [19]. Xenon is not only neuroprotective, but has also been shown to be cardio- and nephroprotective [20,21,22].

When combining two treatments, one needs to document the potency of the combination, as drugs administered during the neonatal period may have toxic effects on the newborn body and brain. Two treatments combining to give an effect greater than the sum of the two treatment effects alone are synergistic, where as an additive effect is the sum of two individual treatments. There are opposing views on whether combining Xe and HT demonstrates a synergistic or an additive effect. We have previously shown in neonatal rats and newborn pigs suffering hypoxia-ischemia, that both Xe and HT have individual neuroprotective abilities and that their combined neuroprotection is additive [7,8]. In contrast, Ma and colleagues have found that the neuroprotective effects of Xe
and HT are synergistic [17,23]. However, they have used a different setup to ours to prove this synergistic effect. Therefore, this current study was carried out to examine the potential synergistic effect of Xe and HT in newborn rats using an experimental setup with their design, which has previously suggested a synergistic effect between Xe and HT [17].

**Materials and Methods**

**Procedures**

All procedures were carried out under Home Office license in accordance with UK regulations and approved by the University of Bristol’s animal ethical review panel.

**Animal Experiments**

1. **Control Experiments of added neuroprotection by Xe-HT.** The first set of experiments was set out to verify our previously published finding of additive neuroprotection between 50% Xe and HT at 32°C (HT32°C) in this neonatal animal model, [7] using hemispheric area loss after 1 week survival as the outcome measure [24].

   Forty-five P7 Wistar rat pups of both sexes underwent a left common carotid ligation under general anesthesia as previously described [24]. After a maximum recovery of 180 mins, whilst pups were with their dams, pups were exposed to 8% oxygen for 90 min at a rectal temperature (Trectal) of 36.0°C in a temperature-controlled chamber, resulting in a moderate hypoxic-ischemic (HI) insult with ~40% brain area loss. This HI insult has been shown to be of the same severity as that of the original paper by Hobbs et al. [7,24]. The temperature of the animals was continuously recorded by animals carrying a rectal (IT-21, Physitemp Instruments, Clifton, New Jersey, USA) or skin (CritiCool, MTRE, Charter Kontron Ltd, Milton Keynes, UK) temperature probe. Animals carrying a temperature probe were excluded from further analysis, as they respond differently to hypoxia ischemia [25]. Subsequently after the HI surviving pups from different litters were randomized equally to each group, and matched for sex and weight (Figure 1A).

A total of 38 pups survived the insult (7 died). Six probe animals were excluded from further analysis and 32 pups were randomized to 3 treatments lasting 5 h starting immediately: normothermia at 37°C (NT37°C, n = 11), HT at 32°C (HT32°C, n = 10) or combined HT at 32°C with 50% Xe (HT32°C+Xe50%, n = 11). The temperature was continuously measured in the additional “probe animals” in each chamber with a rectal temperature probe and a skin probe on the abdomen. Both probes were calibrated to ±0.1°C over a range of 20.0 to 40.0°C against a certified mercury-in-glass thermometer (BS593, Zeal, London, UK). The temperatures were maintained with a servo-controlled mat (CritiCool, MTRE, Charter Kontron Ltd, Milton Keynes, UK). Rectal temperature correlates within 0.1°C with brain temperature in P7 rats [26]. For the HT groups, a Trectal of 32.0°C±0.2°C was achieved within 15 mins. The stable Xe concentration was achieved and measured within the chamber using our previously described closed re-circulating system to conserve Xe [27]. The set Xe concentration (20% or 50%) was achieved within 15 mins and maintained, for the 5 h treatment period. After the treatment period, pups were immediately removed from the chamber and returned to their dams. No clinical seizures were observed. While with their dam, the temperature of the probe animals was intermittently measured. All animals were kept in a 12:12 h dark/light cycle at 22°C environmental temperature with adequate food and water, and weights were checked daily until the end of the survival period at P14.

2. **Different Temperatures and Xenon Concentrations with and without delay.** The second set of experiments was performed to assess the effect of different temperatures (Part 1) or different Xe concentrations (Part 2) on brain area loss at one week survival. During the different treatments the temperature was continuously measured in additional “probe animals” as described above.

Part 1: 96 P7 pups of both sexes from 10 litters underwent a moderate HI insult as described above. If untreated, the NT group, this insult results in ~40% relative hemispheric area loss after one week. [24,26] Three pups died during the insult and 12 pups were excluded from further analysis as they carried a temperature probe. Pups were randomized to 6 groups and
A set of experiments was carried out to assess whether the concentration (Xe20%) is neuroprotective when started immediately for 5 h in 21% oxygen at NT to different Xe groups. Pups were randomized to 6 groups to be exposed either immediately or after a 4 h delay: HT32°C (n = 13), HT35°C (n = 13) or NT37°C (n = 14) (Figure 1B–C). During the 4 h delay pups were kept with their dams. For the HT groups, a Tu_{rectal} of 32.0°C±0.2°C or 35.0°C±0.2°C was achieved within 15 minutes. After the treatment period, pups were immediately removed from the chamber and returned to their dams until P14.

Part 2: 99 P7 pups of both sexes from 10 litters underwent the moderate HI insult as described above. Five pups died during the insult and 12 pups carrying a temperature probe were excluded. Pups were randomized to 6 groups to be exposed either immediately for 5 h in 21% oxygen at NT to different Xe concentrations: Xe0% (n = 14), Xe20% (n = 14) or Xe50% (n = 14) or after a 4 h delay: Xe0% (n = 13), Xe20% (n = 14) or Xe50% (n = 13) (Figure 1D–E). After the treatment period, pups were immediately removed from the chamber and returned to their dams until P14.

3. Combined Experiments of Different Temperatures and Xenon Concentrations with and without delay. The third set of experiments was carried out to assess whether the combination of mild hypothermia (HT35°C) and a low Xe concentration (Xe20%) is neuroprotective when started with a 4 h delay.

One hundred and one P7 pups of both sexes from 20 litters underwent a left common carotid ligation under general anesthesia. All pups were exposed to 8% oxygen for 90 min at Tu_{rectal} of 36.0°C in a temperature-controlled chamber. Three animals died during the insult and 16 probe animals were excluded from further analysis, leaving 82 pups for randomization matched for litter, sex and weight (Figure 1F) to 4 groups. After a 4 h delay with the insult and 16 probe animals were excluded from further analysis, leaving 82 pups for randomization matched for litter, sex and weight on brain area loss in any of the groups. Additionally linear regression did not show a significant effect of sex and weight at P7. In the different treatment groups regarding sex and weight at P7. In the different treatment groups had a significantly reduced mean brain area loss compared to animals treated in air (Xe20% 38.4% (HT32°C 41.6% (HT35°C 42.0% (NT37°C 42.6% (p = 0.042). Immediate HT35°C did not significantly reduce brain area loss, when compared to NT37°C (HT32°C 31.5% (p = 0.272). (Figure 2B). The ANOVA did not show any significant difference between the treatment groups (p = 0.163).

After a 4 h delay, neither HT32°C nor HT35°C significantly reduced brain area loss when compared to NT37°C (HT32°C 33.1% (p = 0.272). (Figure 2C). The ANOVA did not show any significant difference among the treatment groups (p = 0.502).

In the second part of the study we found that immediate Xe20% did not significantly reduce mean brain area loss when compared to animals treated in air (Xe20% 39.4% (HT32°C 42.2% (p = 0.327 and HT35°C 41.6% (NT37°C 42.6% (p = 0.382) (Figure 2E). The ANOVA did not show significant differences between the groups (p = 0.452). After a 4 h delay neither Xe20% nor Xe50% significantly reduced brain area loss when compared to animals treated in air (Xe20% 35.5% (HT32°C 52.2% (p = 0.0619) (Figure 2D). In addition the ANOVA did not show significant differences between the groups (p = 0.418). Additional linear regression did not show a significant effect of sex and weight on brain area loss in any of the groups.

3. Combined Experiments of Different Temperatures and Xenon Concentrations with and without delay

In the combined experiments, we found that none of the different treatment groups had a significantly reduced mean brain area loss, compared to the NT37°C group. Mean (±SD) area loss

Statistical analyses were performed with SPSS version 18 (SPSS Inc., Chicago, IL). For two-group comparisons the t-test was used. One way ANOVA was used to compare the different treatment groups. To assess a possible effect of sex and weight of pups on brain area loss and to assess combined effects of the two treatments (HT and Xe), linear regression analysis was used. Two-sided testing with p<0.05 was considered statistically significant. Descriptive data are presented as mean ± standard deviation (±SD).

Results

1. Control Experiments of added neuroprotection by Xe-HT

When repeating our previously published experiments and using brain area loss instead of a neuropathology score as an outcome parameter, we found that mean (±SD) area loss in the NT37°C group was 54.2% (±5.56). There was a significant reduction in mean brain area loss in the immediately cooled animals (HT32°C 36.0% (±21.83), p = 0.015) compared to the NT37°C group. Also, as previously shown, adding Xe50% to HT32°C significantly reduced mean brain area loss (19.9% (±11.32) compared to NT37°C (p<0.001) and HT32°C alone (p = 0.045). ANOVA p=0.001) (Figure 2A). This shows that we were able to confirm our findings of additional neuroprotection combining 50% Xe with HT at 32°C, using brain area loss at 1 week survival as the outcome.

2. Different Temperatures and Xenon Concentrations with and without delay

In this part of the study we found that immediate HT32°C significantly reduced mean brain area loss when compared to NT37°C (HT32°C 25.8% (±17.37) vs NT37°C 41.6% (±25.38), p = 0.042). Immediate HT35°C did not significantly reduce brain area loss, when compared to NT37°C (HT35°C 31.5% (±19.00) vs NT37°C, p = 0.272. (Figure 2B). The ANOVA did not show any significant difference between the treatment groups (p = 0.163).

After a 4 h delay, neither HT32°C nor HT35°C significantly reduced brain area loss when compared to NT37°C (HT32°C 33.1% (±17.65) vs NT37°C 42.2% (±26.83), p = 0.327 and HT35°C 41.6% (±17.36) vs NT37°C 42.6% (±26.85), p = 0.392) (Figure 2C). The ANOVA did not show any significant difference among the treatment groups (p = 0.502).

In the second part of the study we found that immediate Xe20% did not significantly reduce mean brain area loss when compared to animals treated in air (Xe20% 39.4% (±21.42) vs Xe0% 39.7% (±18.43), p = 0.671). In addition, immediate Xe50% did not significantly reduce mean brain area loss when compared to animals treated in air (Xe50% 35.5% (±23.97) vs Xe0% 39.7% (±10.43), p = 0.619) (Figure 2D). In addition the ANOVA did not show significant differences between the groups (p = 0.872).

After a 4 h delay neither Xe20% nor Xe50% significantly reduced mean brain area loss when compared to animals treated in air (Xe20% 53.2% (±15.60) vs Xe0% 49.7% (±18.70), p = 0.624 and Xe50% 45.8% (±16.11) vs Xe0% 49.7% (±18.78), p = 0.589) (Figure 2E). The ANOVA did not show any significant difference between the different treatment groups (p = 0.560).

Table 1 shows that there was no significant difference between the different treatment groups regarding sex and weight at P7. In addition linear regression did not show a significant effect of sex and weight on brain area loss in any of the groups.
was 39.7% (\pm 20.88) for the NT37\textdegree C group, 40.1% (\pm 20.91) for the NT37\textdegree C + Xe20% group (p = 0.905) and 33.2% (\pm 16.90) for the HT35\textdegree C group (p = 0.310). When combining both treatments (HT35\textdegree C + Xe20%) mean (\pm SD) brain area loss was 42.4% (\pm 16.62), which was not significantly different from the NT37\textdegree C group (p = 0.599). The ANOVA did not show any significant difference between the treatment groups (p = 0.594). In the regression analysis, the coefficient of the interaction term between cooling and Xe was small and non-significant.

Combining the treatments, Xe20% and NT37\textdegree C, that individually were not effective was not neuroprotective indicating that there was no synergistic effect between the two treatments. There was no significant difference between the treatment groups (p = 0.594). In the regression analysis, the coefficient of the interaction term between cooling and Xe was small and non-significant.

Combining the treatments, Xe20% and NT37\textdegree C, that individually were not effective was not neuroprotective indicating that there was no synergistic effect between the two treatments.

There was no significant difference between the different treatment groups regarding sex and weight at P7 (Table 1). In addition linear regression did not show a significant effect of sex and weight on brain area loss in any of the groups.

4. Animal Model Variability

Figure 3 shows the scatter plots of percentage area loss for the four groups from the combined experiments of different temperatures and Xe concentrations. It shows the variability in injury pattern in this animal model within each treatment group, which is well known [7,24,28,29,30,31]. This variability has been observed throughout all performed experiments and therefore explains the large standard deviations within each treatment group and necessitates the need for large numbers in each group.

Discussion

The main finding of this study is that combining two treatments (HT35\textdegree C and Xe20%), both not individually neuroprotective, did not result in neuroprotection. We were unable to replicate the results of a previously published study [17], despite using the same animal model, treatment temperature, Xe concentration and delay before start of treatment. We recognize that a significant weakness of the model is the variability of injury within treatment groups (with associated large standard deviations), therefore we used larger group sizes. However, we were unable to show a synergistic effect between the two treatments (HT35\textdegree C + Xe20%).

To verify our own previous findings [7] we first showed that the immediate combined treatment of HT32\textdegree C + Xe50% significantly reduced relative brain area loss, compared to HT32\textdegree C alone. We have previously shown that brain area loss correlates well with our
previously used global pathology score [24]. It is a quick, reliable and reproducible way to assess brain injury in this animal model as long as the groups are large. Rice and Vannucci introduced the unilateral carotid ligation “rat hypoxia-ischemia-model” in 1981 [30]. In their original paper they used hemispheric brain weight ratio (ligated/non-ligated side) to assess brain injury. Many authors initially used this weight ratio in their studies [32,33,34]. As it is technically not very easy to cut an injured brain in the midline, it has been stated that brain weight assessment can be used for screening purposes only [35] and histopathology will remain the gold standard. We have validated our brain area loss from histological section versus pathology scoring [24].

As shown in Figure 3 and mentioned in many other papers [7,24,28,29,30,31] the variability in degree of injury in this animal model is large. Standard deviation typically is ~20%, which necessitates the need for large groups. Nevertheless this experimental fact strengthens the translational value of the model, as it reproduces variability observed in human neonatal hypoxia-ischemia [29].

Xenon has been shown to offer additional neuroprotection when combined with HT after hypoxia-ischemia in newborn rats and pigs [7,8,18]. However, whether this effect is additive or synergistic remains to be fully elucidated. Ma and colleagues have previously suggested that the effect of combining the two treatments is synergistic [17,23]. In contrast, we have found no evidence of synergy, but only additive effects when the two treatments were combined both in rats and newborn pigs [7,8]. In our previous papers we did not use the same temperatures and Xe concentrations as Ma et al., so we have replicated those conditions here. In addition to the in vivo work we have repeated in this paper, their conclusion about synergy is partly based on cell culture work [17]. Ma et al. present results obtained from in vivo

| Table 1. Mean (± SD). |
|-----------------------|
| A Immediate Treatment | NT<sub>37</sub> C | HT<sub>32</sub> C | HT<sub>32</sub> C+Xe50% |
| N (male)              | 11 (6)           | 10 (5)           | 11 (4)           |
| Weight at P7 [g]     | 15.46 (±1.50)    | 14.84 (±1.32)    | 15.61 (±1.12)    |
| Weight gain at P14 [g]| 5.95 (±3.47)     | 7.84 (±3.37)     | 6.16 (±5.21)     |

B Immediate Treatment | NT<sub>37</sub> C | HT<sub>35</sub> C | HT<sub>32</sub> C |
| N (male)              | 14 (6)           | 13 (5)           | 14 (5)           |
| Weight at P7 [g]     | 16.02 (±1.25)    | 15.93 (±1.99)    | 15.54 (±1.59)    |
| Weight gain at P14 [g]| 14.82 (±2.91)    | 14.53 (±2.48)    | 12.81 (±3.43)    |

C 4 h delayed Treatment | NT<sub>37</sub> C | HT<sub>35</sub> C | HT<sub>32</sub> C |
| N (male)              | 14 (7)           | 13 (9)           | 13 (8)           |
| Weight at P7 [g]     | 15.78 (±1.41)    | 16.17 (±2.19)    | 16.37 (±1.76)    |
| Weight gain at P14 [g]| 14.27 (±2.51)    | 14.70 (±2.14)    | 14.47 (±2.13)    |

D Immediate Treatment | Xe0% | Xe20% | Xe50% |
| N (male)              | 14 (7) | 14 (6) | 14 (7) |
| Weight at P7 [g]     | 15.14 (±2.09)    | 14.48 (±1.59)    | 14.80 (±1.88)    |
| Weight gain at P14 [g]| 14.20 (±2.08)    | 12.88 (±2.78)    | 14.51 (±2.27)    |

E 4 h delayed Treatment | Xe0% | Xe20% | Xe50% |
| N (male)              | 13 (8) | 14 (7) | 13 (5) |
| Weight at P7 [g]     | 17.34 (±1.32)    | 16.64 (±1.44)    | 16.32 (±1.40)    |
| Weight gain at P14 [g]| 13.52 (±2.88)    | 13.89 (±2.03)    | 13.62 (±1.92)    |

F 4 h delayed Treatment | NT<sub>37</sub> C | NT<sub>37</sub> C+Xe20% | HT<sub>35</sub> C |
| N (male)              | 21 (10) | 20 (9)  | 20 (10)  |
| Weight at P7 [g]     | 13.83 (±2.66)    | 14.24 (±1.24)    | 13.94 (±1.50)    |
| Weight gain at P14 [g]| 8.52 (±2.67)     | 7.99 (±3.09)     | 8.27 (±2.21)     |

There was no significant difference between the treatment groups regarding sex, weight at 7-days of age (P7) or weight gain at 14-days of age (P14). Experimental setup (A–E) as described in Figures 1+2.

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Figure 3. Shows the large variability in mean area loss within each group from the 4 h delayed combination experiments. Each circle (o) represents one of the animals from the individual groups used for analysis.

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cultures of neuronal and glial cells exposed to experimental ischemia in the form of oxygen-glucose deprivation for 75 minutes, followed by a 16-hour recovery period. During the oxygen-glucose deprivation and the recovery period, different cultures were exposed to five different temperatures from 37°C to 20°C, or five different concentrations of Xe in the atmosphere from 0% to 75%, alone and in combination. The amount of injury was quantified by the release of lactate dehydrogenase (LDH). LDH release decreased with decreasing temperature and with increasing concentrations of Xe, showing that both interventions protected the neuronal cells in this cell culture model. Using results obtained from using 12.5% Xe and 33°C as their example, the authors argue that the combination acts synergistically. This conclusion is based on an application of isobolographic analysis [36]. However, there are considerable problems both with both the application of isobolographic analysis to this kind of data, as well as the assumption that the method is appropriate in the first place. One general problem is that the degree of HT (relative to 37°C) is treated as if it were similar to the concentration of a drug. However, there is one important difference. The concentration of a drug can never have a value below zero, while the degree of hypothermia may well be negative (for temperatures below 37°C). In addition, when the dose-effect relationship of the individual agents (temperature and Xe) has a different maximum, as they have in the reported experimental results, the isobole of additivity is not a straight line, but a downwards curve [37]. Furthermore, the 50% of maximum response points appear to not have been correctly plotted in their Figure 1G. The 12.5% Xe point should perhaps be moved to the right to about 17%, and the other point should perhaps be moved vertically from 4°C to about 6°C. These two corrections to their Figure 1G, a downward movement of the middle section of the curved isobole of additivity and a movement upwards and to the right of the experimental points, will likely bring the experimental points well within a 95% confidence band around the curved isobole of additivity. Our conclusion is therefore that no synergistic effect of temperature and Xe has been shown in these cell culture experiments. However, in their paper, they also present results from in vivo experiments, which they claim give direct evidence of a synergistic effect between HT and Xe [17]. As our previous experiments have suggested an additive, rather than synergistic, effect, we have repeated the experimental conditions described by Ma et al. Like many other researchers in this field, they used the Rice and Vannucci rat model to produce unilateral brain injury. Brain weight ratio was used as their outcome parameter seven days after the insult.

Considering that a raw screening tool (brain weight) was used as an outcome parameter, it is interesting to note both the small number of rats in each treatment group (n = 6–8), and that statistically significant differences were obtained. This would suggest very small standard deviations within the data [17]. Most error bars in their figures are presented as standard error of the mean, being 7% or less. This would correspond to standard deviations less than 20%. These standard deviations are substantially smaller than what we, and many other researchers, report in the same experimental model [7,24,29,30]. For this reason, and because we have not observed any indication of synergistic effects between HT and Xe in our own experiments, we wanted to repeat the experiments following their experimental protocol, but using a higher number of animals in each group. Larger group numbers were also required according to our own statistical power calculations.

There are some limitations to our study. First, Ma et al. have also shown improvement of functional outcome thirty days after HT [17]. Our animals only survived 1 week. We have however shown in a previous study, where animals were randomized to short (7d) or long (30d) survival, that neuropathology after 7d correlated strongly with both 30d pathology and functional outcome in this animal model [7]. It is very important to study the underlying mechanisms of the evolution of brain injury and neuroregeneration. This will require a different design with a sequence of survival times.

We did not find any evidence for a synergistic effect of HT and Xe, when using the experimental protocol of Ma et al. With a 4 h delay, none of the different individual treatments were effective, nor was the combined treatment of HT/35°C+Xe/20%. This suggests that these two conditions do not provide a synergistic effect when combined.

**Author Contributions**

Conceived and designed the experiments: MT LW JD. Performed the experiments: HS ES XL. Analyzed the data: MT LW HS. Contributed reagents/materials/analysis tools: HS ES XL. Contributed to the writing of the manuscript: HS MT LW.

**References**

1. Gluckman PD, Wyatt JS, Azzopardi D, Ballard R, Edwards AD, et al. (2005) Selective head cooling with mild systemic hypothermia after neonatal encephalopathy: multicentre randomised trial. Lancet 365: 663–670.
2. Shankaran S, Laptook AR, Ehrenkranz RA, Tyson JE, McDonald SA, et al. (2005) Whole-body hypothermia for neonates with hypoxic-ischaemic encephalopathy. N Engl J Med 353: 1574–1584.
3. Jacobs S, Hunt R, Tarnow-Mordi W, Inder T, Davis P (2007) Cooling for hypoxic-ischemic encephalopathy. Cochrane Database Syst Rev: CD003511.
4. Azzopardi DV, Strohm B, Edwards AD, Dyet I, Halliday HL, et al. (2009) Moderate hypothermia to treat perinatal asphyxial encephalopathy. N Engl J Med 361: 1499–1508.
5. Simbleur G, Mittal RA, Rohmann F, Muche R (2010) Systemic hypothermia after neonatal encephalopathy: outcomes of neo.NEURO network. RCT. Pediatrics 126: e771–778.
6. Zhou WH, Cheng GQ, Shao XM, Liu XZ, Shan RB, et al. (2010) Selective head cooling with mild systemic hypothermia after neonatal hypoxic-ischemic encephalopathy: a multicenter randomized controlled trial in China. J Pediatr 157: 367–372, 372 e361–363.
7. Hobbs C, Thoresen M, Tucker A, Aquilina K, Chakkarapani E, et al. (2008) Xenon and hypothermia combine additively, offering long-term functional and histopathologic neuroprotection after neonatal hypoxia/ischemia. Stroke 39: 1307–1313.
8. Chakkarapani E, Dingley J, Liu X, Hoque N, Aquilina K, et al. (2010) Xenon enhances hypothermic neuroprotection in asphyxiated newborn pigs. Ann Neurol 68: 330–341.
9. Perlman JM, Wyllie J, Kattwinkel J, Atkins DL, Chameides L, et al. (2010) Part 11: Neonatal resuscitation: 2010 International Consensus on Cardiopulmonary Resuscitation and Emergency Cardiovascular Care Science With Treatment Recommendations. Circulation 122: S516–530.
10. Guillet R, Edwards AD, Thorsson M, Ferreira DM, Gluckman PD, et al. (2012) Sevoflurane seven- to eight-year follow-up of the CoolCap trial of head cooling for neonatal encephalopathy. Pediatr Res 71: 205–209.
11. Shankaran S, Pappas A, McDonald SA, Vohr BR, Hintz SR, et al. (2012) Childhood outcomes after hypothermia for neonatal encephalopathy. N Engl J Med 366: 2083–2092.
12. Jacobs SE, Berg M, Hunt R, Tarnow-Mordi WO, Inder TE, et al. (2013) Cooling for newborns with hypoxic ischaemic encephalopathy. Cochrane Database Syst Rev 1: CD003511.
13. Edwards AD, Broderick P, Gonn AJ, Halliday H, Jussaume E, et al. (2010) Neurological outcomes at 18 months of age after moderate hypothermia for perinatal hypoxic ischaemic encephalopathy: synthesis and meta-analysis of trial data. BMJ 340: c636.
14. Tagin MA, Woodcott GG, Vinicer MJ, Whyte RK, Stinson DA (2012) Hypothermia for neonatal hypoxic ischaemic encephalopathy: an updated systematic review and meta-analysis. Arch Pediatr Adolesc Med 166: 558–566.
15. Dinlley J, Tooley J, Porter H, Thoresen M (2006) Xenon provides short-term neuroprotection in neonatal rats when administered after hypoxia-ischemia. Stroke 37: 501–506.

16. Zhuang L, Yang T, Zhao H, Fidalgo AR, Vizcaychipi MP, et al. (2012) The protective profile of argon, helium, and xenon in a model of neonatal asphyxia in rats. Crit Care Med 40: 1724–1730.

17. Ma D, Hossain M, Chow A, Arshad M, Battson RM, et al. (2005) Xenon and hypothermia combine to provide neuroprotection from neonatal asphyxia. Ann Neurol 58: 182–193.

18. Faulkner S, Bainbridge A, Kato T, Chandrasekaran M, Kapetanakis AB, et al. (2011) Xenon augmented hyperthermia reduces early lactate/N-acetylaspartate and cell death in perinatal asphyxia. Ann Neurol 70: 133–150.

19. Sabir H, Bishop S, Cohen N, Maes E, Liu X, et al. (2013) Neither xenon nor fentanyl induces neuroapoptosis in the newborn pig brain. Anesthesiology 119: 345–357.

20. Preckel B, Mullenheim J, Moloschavij A, Thamer V, Schlack W (2000) Xenon administration during early reperfusion reduces infarct size after regional ischemia in the rabbit heart in vivo. Anesth Analg 91: 1327–1332.

21. Baumert JH, Hein M, Gerets C, Baltus T, Hecker KE, et al. (2007) The effect of xenon anesthesia on the size of experimental myocardial infarction. Anesth Analg 105: 1200–1206, table of contents.

22. Ma D, Lim T, Xu J, Tang H, Wan Y, et al. (2009) Xenon preconditioning protects against renal ischemic-reperfusion injury via HIF-1alpha activation. J Am Soc Nephrol 20: 713–720.

23. Martin JL, Ma D, Hossain M, Xu J, Sanders RD, et al. (2007) Asynchronous administration of xenon and hypothermia significantly reduces brain infarction in the neonatal rat. Br J Anaesth 98: 236–240.

24. Sabir H, Scall-Brown E, Liu X, Thoresen M (2012) Immediate hypothermia is not neuroprotective after severe hypoxia-ischemia and is deleterious when delayed by 12 hours in neonatal rats. Stroke 43: 3364–3370.

25. Thoresen M, Bagenholm R, Loberg EM, Apricena F, Kjellmer I (1996) Posthypoxic cooling of neonatal rats provides protection against brain injury. Arch Dis Child Fetal Neonatal Ed 74: F3–9.

26. Thorensen M, Bagenholm R, Loberg EM, Apricena F, Kjellmer I (1996) Posthypoxic cooling of neonatal rats provides protection against brain injury. Arch Dis Child Fetal Neonatal Ed 74: F3–9.

27. Chakkarapani E, Thoresen M, Hobbs CE, Aquilina K, Liu X, et al. (2009) A closed-circuit neonatal xenon delivery system: a technical and practical neuroprotection feasibility study in newborn pigs. Anesth Analg 109: 451–460.

28. Charriaut-Marlangue C, Bonnin P, Leger PL, Renolleau S (2013) Brief update on hemodynamic responses in animal models of neonatal stroke and hypoxia-ischemia. Exp Neurol 240: 316–320.

29. McQuillen PS, Ferriero DM (2004) Selective vulnerability in the developing central nervous system. Pediatr Neurol 30: 227–235.

30. Rice JE 3rd, Vannucci RC, Brierley JB (1981) The influence of immaturity on hypoxic-ischemic brain damage in the rat. Annu Neurol 9: 131–141.

31. Scafidi J, Fagel DM, Meut LR, Vaccarino FM (2009) Modeling premature brain injury and recovery. Int J Dev Neurosci 27: 863–871.

32. Feng Y, Paul IA, LeBlanc MH (2006) Nicotinamide reduces hypoxic ischemic brain injury in the newborn rat. Brain Res Bull 69: 117–122.

33. Young RS, Olginski TP, Yagel SK, Towfighi J (1983) The effect of graded hypothermia on hypoxic-ischemic brain damage: a neuropathologic study in the neonatal rat. Stroke 14: 929–934.

34. Zhao P, Zuo Z (2004) Isoflurane preconditioning induces neuroprotection that is inducible nitric oxide synthase-dependent in neonatal rats. Anesthesiology 101: 695–705.

35. Andine P, Thordstein M, Kjellmer I, Nordborg C, Thiringer K, et al. (1990) Evaluation of brain damage in a rat model of neonatal hypoxic-ischemia. J Neurosci Methods 35: 235–260.

36. Geuner PK (1995) Isobolographic analysis of interactions: an update on applications and utility. Toxicology 105: 161–179.

37. Tallarida RJ (2006) An overview of drug combination analysis with isobolograms. J Pharmacol Exp Ther 319: 1–7.