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## Mild hypothermia fails to protect infant macaques from brain injury caused by prolonged exposure to Antiseizure drugs

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### Abstract

Barbiturates and benzodiazepines are GABA<sub>A</sub>-receptor agonists and potent antiseizure medications. We reported that exposure of neonatal macaques to combination of phenobarbital and midazolam (Pb/M) for 24 h, at clinically relevant doses and plasma levels, causes widespread apoptosis affecting neurons and oligodendrocytes. Notably, the extent of injury was markedly more severe compared to shorter (8 h) exposure to these drugs. We also reported that, in the infant macaque, mild hypothermia ameliorates the apoptosis response to the anesthetic sevoflurane. These findings prompted us explore whether mild hypothermia might protect infant nonhuman primates from neuro- and gliotoxicity of Pb/M. Since human infants with seizures may receive combinations of benzodiazepines and barbiturates for days, we opted for 24 h treatment with Pb/M.

Neonatal rhesus monkeys received phenobarbital intravenously, followed by midazolam infusion over 24 h under normothermia ( $T > 36.5$  °C-37.5 °C;  $n = 4$ ) or mild hypothermia ( $T = 35$  °C-36.5 °C;  $n = 5$ ). Medication doses and blood levels measured were comparable to those in human infants. Animals were euthanized at 36 h and brains examined immunohistochemically and stereologically.

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Author contributions

K.K.N contributed experimental design, immunohistochemistry, stereology and manuscript preparation; N.A.F contributed stereological analysis. S.H.W. contributed immunohistochemistry; S.C.III, K.R.B. and S.L. contributed veterinary supervision for macaque experiments; K.C. contributed macaque experiment coordination and execution; H.A.S. and A.F.M. contributed macaque perfusion; C.I. contributed experimental design, analysis of data and manuscript preparation.

CRedit authorship contribution statement

**Chrysanthy Ikonomidou:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Visualization, Supervision, Funding acquisition. **Sophie H. Wang:** Methodology, Data curation. **Nicole A. Fuhler:** Methodology, Data curation. **Shreya Larson:** Resources, Supervision. **Saverio Capuano:** Resources, Supervision. **Kevin R. Brunner:** Resources, Supervision. **Kristin Crosno:** Resources, Project administration. **Heather A. Simmons:** Methodology, Resources. **Andres F. Mejia:** Methodology, Resources. **Kevin K. Noguchi:** Methodology, Formal analysis, Writing – review & editing, Visualization, Supervision, Funding acquisition.

Treatment was well tolerated. Extensive degeneration of neurons and oligodendrocytes was seen at 36 h in both groups within neocortex, basal ganglia, hippocampus and brainstem. Mild hypothermia over 36 h (maintained until terminal perfusion) conferred no protection against the neurotoxic and gliotoxic effects of Pb/M. This is in marked contrast to our previous findings that mild hypothermia is protective in the context of a 5 h-long exposure to sevoflurane in infant macaques.

These findings demonstrate that brain injury caused by prolonged exposure to Pb/M in the neonatal primate cannot be ameliorated by mild hypothermia.

## Keywords

Antiseizure; Sedative; Brain injury; Apoptosis; Development; Barbiturate; Benzodiazepine

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## 1. Introduction

Millions of human infants are exposed to antiseizure medications each year, many of which potentiate the action of the major inhibitory neurotransmitter GABA ( $\gamma$ -aminobutyric acid) at GABA<sub>A</sub> receptors. GABA regulates brain development at molecular, cellular and systems level (Daniel et al., 1998; Nicholls and Attwell, 1990; Seeburg, 1993). Interference with this neurotransmitter system may trigger adverse effects in the immature brain. Over the past two decades, we and many other investigators reported that compounds which positively modulate GABA<sub>A</sub> receptors, including barbiturates, benzodiazepines, alcohol and volatile anesthetics, trigger apoptosis of neurons and oligodendroglia in the rodent and nonhuman primate brain (Ikonomidou et al., 2000; Brambrink et al., 2012; Brambrink et al., 2010; Noguchi et al., 2021), suppress neurogenesis (Stefovska et al., 2008), inhibit normal synapse development and maturation (Forcelli et al., 2012; Jevtovic-Todorovic et al., 2013) and cause long-lasting behavioral and cognitive impairments (Fredriksson et al., 2007; Jevtovic-Todorovic et al., 2003; Stefovska et al., 2008; Paule et al., 2011), when exposure occurs during a period of rapid brain growth. In humans, this brain growth spurt period starts in the third trimester of gestation and extends to the third year of life (Dobbing and Sands, 1979). Notably, several clinical studies raise concerns that prenatal or early postnatal exposure of fetuses and children to antiseizure drugs may contribute to adverse neuro-developmental outcomes (Farwell et al., 1990; Meador et al., 2009, 2012; Ikonomidou et al., 2007). Most concerning are recent findings by Walsh et al. (2021) who reported that preterm infants exposed to surgical anesthesia demonstrated decreased relative white matter volumes at term age and lower cognitive and motor composite scores at 2-year follow up. Notably, those with longer surgical exposure demonstrated the greatest decrease in white matter volumes and lowest cognitive and motor scores.

We studied the neuropathological sequelae resulting from exposure of infant macaques to a combination of phenobarbital and midazolam at doses commonly used in pediatric intensive care. We reported that Pb/M cause widespread apoptosis affecting neurons and oligodendrocytes. Pattern and severity of cell death differed depending on treatment-duration, with substantially enhanced neurodegeneration detected following the longer 24 h exposure. The findings allowed to conclude that brain toxicity of Pb/M in the neonatal

primate brain becomes more severe with longer duration of treatment and expands trans-synaptically (Noguchi et al., 2021).

The combination of high doses of phenobarbital (Pb), benzodiazepines (lorazepam, midazolam, diazepam) and other antiseizure medications constitutes the recommended standard of care for medical management of prolonged, recurrent seizures and status epilepticus in infancy (Glass and Shellhaas, 2019; Soul, 2018). Combinations of barbiturates, benzodiazepines and opiates are used for sedation in the intensive care for days to weeks, and might constitute an independent confounder which influences neurocognitive outcomes of these children. Despite all concerns, there is no ethically and medically acceptable alternative to the use of these agents for treatment of seizures and status epilepticus, in the context of anesthesia for surgical procedures or long-term sedation of critically ill infants in pediatric medicine. Notably, clinical data clearly demonstrate that seizures and status epilepticus also negatively impact neurocognitive outcomes and must therefore be addressed (Lalgudi Ganesan and Hahn, 2019; Payne et al., 2014). Thus, we posed the question whether effective and safe protective therapies can be developed to help prevent or minimize iatrogenic injury to the immature human brain, while allowing medications to exert their desired actions.

Much experience has been gained with the use of hypothermia (HT) in human neonates in the context of hypoxic ischemic encephalopathy (HIE). HT has been shown to be safe and effective in reducing death and disability from HIE at 22 months and at 6–7 years of age (Edwards et al., 2010; Guillet et al., 2012; Holzki, 2011; Rutherford et al., 2010; Shankaran et al., 2012). We studied the protective effects of HT against neonatal neurotoxicity of a 5 h long anesthesia with sevoflurane in infant macaques and found it to significantly reduce apoptosis of neurons and oligodendroglia (Ikonomidou et al., 2019). Interestingly, the temperature window within which a protective effect could be achieved was narrow (35–36.5 °C), with protection lost at temperatures below 35 °C. Here we investigated whether neuroprotection with mild hypothermia can be achieved following longer exposures of infant macaques to phenobarbital midazolam, a frequently encountered scenario in neonatal medicine.

## 2. Materials and methods

### 2.1. Animals

All animal procedures were approved by the Wisconsin National Primate Research Center and the University of Wisconsin - Madison Institutional Animal Care and Use Committees (IACUC) and were conducted in full accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Infant rhesus macaques ( $n = 9$ ) received a loading dose of phenobarbital (40 mg/kg) over 30 min followed by intravenous infusion of midazolam (loading dose of 0.5 mg/kg followed by 2–5 mg/kg and hour for 23.5 h). Midazolam infusion rate was adjusted to maintain a moderate level of sedation. Phenobarbital maintenance doses (2.5 mg/kg) were given at 6 and 18 h, with the goal to maintain a therapeutically relevant phenobarbital plasma level over 36 h.

Normothermia ( $T > 36.5$  °C– $37.5$  °C;  $n = 4$ ) was maintained using a servo-controlled warming blanket. Oxygen saturations were monitored with continuous pulse oximetry, vital signs with a cardiorespiratory monitor. Blood gases, blood glucose, electrolytes, lactate and hemoglobin were monitored on venous blood (femoral vein) at 0, 2, 4, 6, 12 and 24 h.

Animals were deeply sedated and transcardially perfused at 36 h. Perfusion fixation was performed with heparinized saline followed by 4% paraformaldehyde in phosphate buffer by a board-certified Veterinary Pathologist and according to the latest AVMA Guidelines for the Euthanasia of Animals.

**Controls:** The historical control group ( $n = 5$ ; 2 males and 3 females) consisted of 4–6 day old rhesus macaques who were separated from their mothers but not exposed to medications. These animals were returned to their mothers after a physical examination identical in nature to how the treatment animals were handled prior to medication administration. These animals were also deeply sedated and transcardially perfused. Perfusion fixation was performed with 4% paraformaldehyde in phosphate buffer by a trained pathologist according to institutional euthanasia standards. This is the same control group we used in previously published work (Ikonomidou et al., 2019; Noguchi et al., 2021).

## 2.2. Hypothermia

Five infant macaques were included in the HT group. HT was induced with a servo-controlled infant cooling system, which consists of a Blanketrol III hypothermia unit, neonatal cooling blankets and an esophageal/rectal temperature probe (Cincinnati Sub-Zero, OH, USA). Using feedback from the animal's rectal temperature sensor, the proprietary control algorithm responds by modifying water temperature such that infant target temperature is achieved precisely. This enables management of body temperature in a non-invasive, effective and precise manner. The design of the study was to target for a temperature of 35–36.5 °C ( $n = 5$ ; mild hypothermia) for 36 h until euthanasia.

All animals were monitored continually to ensure that their vital statistics (e.g., heart rate, respiratory rate), blood gases, and multiple metabolic parameters remained within acceptable limits.

## 2.3. Histopathology studies

Brains were serially sectioned in the coronal plane using a vibratome at 70  $\mu$ m across the entire rostrocaudal extent and every 64th section immunolabeled for activated caspase-3 (AC3; CAT#9661 L; Cell Signaling Technology, Danvers, MA) as a marker of apoptosis. For immunolabeling, sections were first immersed in citrate buffer (pH 6.0) and subjected to heat in a pressure cooker for 10 min for antigen retrieval. They were then quenched in 3% hydrogen peroxide in absolute methanol for 10 min, immersed for 1 h in a blocking solution (2% Bovine Serum Albumin, 0.2% Dry Milk, 0.8% TX-100 in PBS), and incubated overnight at 4 °C with a 1:1000 dilution of AC3. The next morning, sections were incubated with a biotinylated secondary antibody (goat anti-rabbit; Vector Labs, Burlingame, CA), reacted with an avidin-biotin conjugate kit (ABC kit), and visualized using the chromogen VIP (Vectastatin Elite ABC kit and Vector VIP kits; Vector Labs, Burlingame, CA).

The chromogenic AC3 staining method permits comprehensive quantification of cells irreversibly committed to apoptotic death and provides a permanent record of the cell death throughout the brain. This includes both cell bodies and processes that allows one to distinguish early from late stages of degeneration. Neurons and oligodendrocytes were easily distinguished by their location in white or grey matter in addition to each cell's unique morphological profile. In primate tissue, the AC3 antibody will produce non-specific staining in the nucleus of neurons but, in apoptotic cells, engulfs the entire cell including soma and processes making them easy to distinguish. Apoptotic oligodendrocytes are distinguished by their immuno-labeled soma that is surrounded by a halo of particulate debris. Histological photocomposites were made using a Leica DM 4000B microscope equipped with a Leica DFC310FC camera and Surveyor software (Version 9.0.2.5, Objective Imaging, Kansasville, WI).

#### 2.4. Analysis of neuronal and glial cell death

Immunofluorescence was also performed to identify apoptotic cells by co-labeling AC3 with antibodies that identify neurons (NeuN, EMD Millipore, Burlington, MA; MAB377, 1:100), astrocytes (GFAP, Sigma-Aldrich, St Louis, MO; G3893, 1:400), and oligodendrocytes (Myelin Basic Protein, EMD Millipore, Burlington, MA; MAB395, 1:200). Tissue was blocked and incubated in primary antibodies as described above. Finally, tissue was immersed in appropriate secondary antibodies (Invitrogen, Carlsbad, CA; A21428, A11001, or A1106) for one hour before cover-slipping with mounting medium containing DAPI.

#### 2.5. Quantification of apoptosis

**2.5.1. Whole brain counts**—Whole brain counts were performed using Stereoinvestigator Software (v 2019.1.3, MBF Bioscience, Williston, Vermont, USA) running on a Dell Precision Tower 5810 computer connected to a QImaging 2000R camera and a Labophot-2 Nikon microscope with electronically driven motorized stage. A rater, blind to treatment, traced each hemisection and stereologically quantified the number of neurons and oligodendrocytes using the unbiased optical fractionator method. This information was then used to estimate the number of apoptotic profiles per hemisphere which was multiplied by two to get total number per brain. Stereoinvestigator software was also used to generate cell plots of the regional distribution of neurons and oligodendrocytes using the meander scan option.

**2.5.2. Regional counts**—AC3 immunolabeled sections used for whole brain counts were also used to quantify regional apoptosis in the ventral cortex, hippocampus, and thalamus using Stereoinvestigator software meander scans. A subset of sections containing the hippocampus were used for counting all three regions. The hippocampi (including CA regions, dentate gyrus, and subiculum) on each section were outlined and all apoptotic cells counted within its borders. The ventral cortex roughly included the region ventral to the Sylvian fissure. More specifically, a line was drawn from the hippocampus to the point where the Sylvian fissure meets the insular cortex and all apoptotic profiles counted in the region ventral to this line (not including the hippocampus). The thalamus was also outlined and apoptotic cells counted within its borders. Finally, a density (apoptotic profiles per

square millimeter) was calculated by dividing the total number of apoptotic profiles in each region by the total area.

## 2.6. Statistical analysis

Data are presented as means  $\pm$  standard error of the mean (SEM). One sided ANOVA with Bonferroni post-hoc test for multiple comparisons was used to compare  $>2$  groups. Statistical comparisons between normothermia and hypothermia groups were performed using Student's *t*-test (GraphPad Software, La Jolla, CA).

## 3. Results

Treatment with Pb/M was tolerated well in both groups. Vital signs, venous blood gases, lactate, hemoglobin and blood glucose remained within physiological levels (Table 1). Phenobarbital level on day 1 was drawn at 4 h and on day 2 at 24 h. Plasma concentrations of phenobarbital on day 1 were  $28.00 \pm 9.58$   $\mu\text{g/ml}$ ,  $36.93 \pm 6.25$   $\mu\text{g/ml}$  on day 2 in the normothermia group and  $34.20 \pm 10.62$   $\mu\text{g/ml}$  at 4 h and  $40.52 \pm 7.60$   $\mu\text{g/ml}$  at 24 h in the hypothermia group. There were no statistically significant differences in the Pb plasma levels between the normothermic and hypothermic groups at the 4 h or the 24 h time point (Student's *t*-test).

Plasma levels for midazolam were drawn at 4 h and at 24 h, at the end of the midazolam infusion. Midazolam plasma levels were  $2302 \pm 1094$  ng/ml on day 1 and  $6018 \pm 2379$  ng/ml on day 2 of treatment in the NT group and  $1230 \pm 574.90$  ng/ml at 4 h and  $2702 \pm 921.40$  ng/ml at 24 h in the hypothermia group (Table 1). The midazolam plasma levels in the normothermic and hypothermic groups did differ significantly at the 4 h or the 24 h time point (Student's *t*-test).

A profound apoptotic response to Pb/M was detected in the brains of both, normothermic and hypothermic infants at 36 h following the 24 h long Pb/M exposure period (Fig. 1). This was most prominent in several divisions of the neocortex, especially the temporal cortex (layers II and V) and the primary visual cortex (layers II and V), insular cortex (Fig. 2), but also involved the caudate, globus pallidum, hippocampus, thalamus and diffusely the subcortical white matter. In addition, findings consistent with Wallerian degeneration were captured within the subiculum, anterior thalamus and several pontine nuclei. Overall, at this timepoint neurodegeneration was much more profound compared to degeneration of oligodendrocytes.

Immunohistochemistry confirmed that neurons and oligodendrocytes but not astrocytes were affected by the apoptosis process (Fig. 3).

Stereological analysis of the pattern and severity of the degenerative changes revealed that, at this time point, apoptosis affected primarily neurons (Fig. 4). One way ANOVA comparing the control, the NT and HT groups revealed that treatment with Pb/M had a significant effect on apoptotic neurons [ $F(3,13) = 14.22$ ,  $P = 0.0012$ ] and total profiles [ $F(3,13) = 8.684$ ,  $P = 0.0065$ ] but not oligodendrocytes [ $F(3,13) = 0.8540$ ,  $p > 0.05$ ]. Post hoc analysis using Bonferroni test revealed that there were no significant differences in apoptotic

neurons, oligodendrocytes or profiles between the hypothermia and normothermia groups (Figs. 4 & 5). Student's *t*-test comparing the NT and HT groups also revealed no significant differences in the severity of apoptosis affecting neurons, oligodendrocytes and profiles. Notably, apoptotic oligodendroglia were significantly enhanced in Pb/M treated animals at 8 h (Noguchi et al., 2021) but appeared to have been removed by 36 h, so that we only detected a trend at this time point. In contrast, neurodegeneration was ongoing and markedly enhanced at 36 h compared to shorter exposure times.

Statistical comparisons of regional apoptotic cell densities between the control and the Pb/M groups (NT and HT) by means of one-way ANOVA revealed that treatment with Pb/M significantly increased densities of apoptotic neurons in the ventral cortex [ $F(3,14) = 9.377, p = 0.0042$ ], hippocampus [ $F(3,14) = 7.975, p = 0.0072$ ] but not thalamus. No significant effect on oligodendrocyte apoptosis was detected in any of these regions at 36 h. Treatment with Pb/M significantly increased densities of apoptotic profiles in the ventral cortex [ $F(3,14) = 8.698, p = 0.0054$ ], hippocampus [ $F(3,14) = 5.559, p = 0.0215$ ] and thalamus [ $F(3,14) = 5.093, p = 0.0272$ ]. No significant differences were detected between the normothermia and hypothermia groups by means of Student's *t*-test in any of these brain regions.

#### 4. Discussion

This study was designed to explore whether mild hypothermia has the potential to ameliorate neurotoxicity of combined treatment with a barbiturate and a benzodiazepine, at doses, plasma concentrations and duration that are clinically relevant for the management of human infants. We wanted to model the clinical situation of administering Pb/M for recurrent neonatal seizures or status epilepticus, whereby treatment durations of 24 h and longer are common (Glass and Shellhaas, 2019).

The key finding of our study is that mild hypothermia, reported to prevent toxicity of shorter exposure to sevoflurane, failed to influence the neuroapoptosis response to prolonged treatment with Pb/M.

These results suggest that human infants subjected to hypothermia for treatment of hypoxic/ischemic encephalopathy may not be protected from brain toxicity of GABA<sub>A</sub> receptor agonist medications. They also indicate that HT, contrary to our expectation, may not be suitable as an adjunctive therapy aiming to prevent neurotoxicity of antiseizure medications in human infants.

The strong proapoptotic properties of barbiturates, benzodiazepines and many anesthetics have been known for years. Disturbances in physiologic excitatory neurotransmission is thought to underlie this toxicity which is confined to developmental stages of ongoing physiological programmed cell death in the brain. During this time, synaptic activity critically determines the fate of cells which are trying to integrate and network. Synaptic activity regulates intracellular survival pathways and related gene transcription and its impairment may lead to self-elimination of unsuccessful neurons and immature oligodendrocytes. This elimination occurs via the intrinsic apoptosis pathway in a bax-

and caspase-3 dependent manner. Down regulation of Bcl<sub>xL</sub>, mitochondrial injury and extra-mitochondrial leakage of cytochrome *c* are critical steps of the proapoptotic cascade involved (Bittigau et al., 2002; Hansen et al., 2004; Léveillé et al., 2010; Olney et al., 2001, 2002; Yon et al., 2005; Young et al., 2003).

Reactive oxygen species and oxidative stress may decisively contribute to this cell elimination process, as impaired synaptic activity, primarily through synaptic NMDA receptors, has been shown to impair intracellular antioxidative defenses (Papadia et al., 2008). Furthermore, using microarray analysis Lui and colleagues demonstrated that differential expression of many genes involved in multiple pathways directly related to brain function is significantly altered in rat brains following exposure to anesthetics (Liu et al., 2013). This agrees with our own studies, which have demonstrated that GABA<sub>A</sub> agonists cause long-term dysregulation of proteins associated with apoptosis, oxidative stress, inflammation, cell proliferation and neuronal circuit formation in the brain proteome of infant mice (Kaindl et al., 2008). In addition, some antiseizure drugs may lead to a persistent decrease of synapses in several brain regions in rodents and suppress neurogenesis (Forcelli et al., 2012; Jevtovic-Todorovic et al., 2013; Stefovskaja et al., 2008).

Many of these pathways can be influenced by HT in ways that let us anticipate a protective effect. HT may suppress AC3, protein synthesis, mitochondrial permeability transition, and inhibit apoptosis pathways (Drury et al., 2012; Yenari and Han, 2012). In vitro, mild HT directly suppressed neuronal apoptosis induced by serum deprivation, with reduced activation of caspases-3, -8, and -9 after 24 h, and reduced cytochrome *c* translocation. HT may slow the extrinsic and intrinsic apoptotic pathways, reduce BAX and increase BCL-2 expression, prevent pro-apoptotic PKC $\delta$  activation and anti-apoptotic PKC $\epsilon$  degradation, reduce production of free radicals, and decrease activation of immune transcription factors (Drury et al., 2012; Yenari and Han, 2012). Creeley and Olney first reported protective effect of HT in mice treated with ketamine or isoflurane. The protection measured was described as very robust but was measured at 5 h after administration of either anesthetic (Creeley and Olney, 2010).

Our group studied effects of mild HT in infant macaques and found it to significantly reduce apoptosis of neurons and oligodendroglia following a 5 h long treatment with sevoflurane. Remarkably, in our study only mild HT (35–36.5 °C) was effective, while protection was lost at lower temperatures. Our explanation for this discrepancy was that at body temperatures below a certain threshold, the negative impact of hypothermia itself on physiological neurotransmission and the resulting compromise of its trophic and survival promoting effects on neurons and oligodendrocytes outweigh the inhibition of proapoptotic mechanisms conferred by HT. In agreement with this explanation, a series of studies has addressed the effects of cooling and rewarming on neuronal activity and excitatory postsynaptic potential (EPSP) thresholds. Aihara and colleagues investigated effects of cooling on pyramidal neurons in guinea pig hippocampal slices and reported reduction of the excitatory postsynaptic potential (EPSP) slope in a temperature dependent manner between 37° and 30 °C (Aihara et al., 2001). Amplitudes of NMDA and AMPA mediated EPSPs decreased in a temperature dependent manner in the 37–33 °C temperature window, regardless of cooling rate, in rat olfactory cortex slices. Whether and how HT impacts



synaptic activity in the primate brain has not been evaluated (Mokrushin et al., 2014). In a study performed by Hicks and Poole (1981), the effects of hypothermia on the EEG of 326 human infants and children undergoing hypothermic perfusions were analyzed. Below 35 °C there was progressive slowing of the dominant rhythm with amplitude reductions becoming evident below 32 °C.

Considering the above scientific evidence, a potential explanation for the failure of mild hypothermia to ameliorate Pb/M neurotoxicity could be that its long duration, as was applied in this experimental setting, may have critically compromised synaptic function and triggered anti-survival signals strong enough to overcome HT-dependent inhibition of proapoptotic pathways. To some extent this conforms with our previous finding that, while neuroprotective effects did predominate following a short period of mild cooling in the sevoflurane experiments, deeper hypothermia was ineffective (Ikonomidou et al., 2019). There we hypothesized that it was the depth of hypothermia that critically enhanced anesthesia-induced synaptic failure and allowed anti-survival signals to eliminate HT-dependent antiapoptotic effects.

Alternatively, it is also possible that a protective effect of prolonged mild HT was masked by a rebound cell death phenomenon which started upon rewarming and evolved during the subsequent 12 h of normothermia.

We conducted this present study with translation into the clinical setting in mind, given that HT is a recognized effective treatment for perinatal asphyxia, is well tolerated and applied in neonatal medicine worldwide. Phenobarbital continues to be most popular as a first line antiseizure medications for neonatal and infantile seizures with target plasma levels in the range of 20–40 µg/ml. Midazolam is added to phenobarbital for the treatment of intractable seizures and administered in humans as a continuous infusion at rates of up to 0.5 mg/kg/h (Glass and Shellhaas, 2019). Midazolam plasma concentrations in infants have not been systematically studied, but available data from neonates on ECMO treated with midazolam infusions have revealed plasma levels of up to 4 µg/ml (4000 ng/ml) within 48 h, at an infusion rate of 0.25 mg/kg/h (Mulla et al., 2003). Thus, the medication plasma levels measured in the macaque neonates in our study are comparable to levels measured in human infants treated with these same medications.

The degree of brain injury caused by prolonged exposure of the neonatal macaque brain to Pb/M was several-fold higher than that following 5 h exposure to sevoflurane (Ikonomidou et al., 2019) or 8 h exposure to Pb/M (Noguchi et al., 2021). Also, at 36 h neuroapoptosis dominated while oligoapoptosis was marginal. We interpret this as a result of overwhelming synaptic failure due to prolonged action of the drugs. The pattern of neuropathological sequelae detected at 36 h additionally points towards degeneration of distant synaptic partners which were not affected at earlier time points (Noguchi et al., 2021). The failure of mild HT to protect from this more severe and widespread neuroapoptosis pattern indicates that a very fine balancing between HT-induced protective mechanisms and HT-dependent synaptic malfunction is necessary in order to achieve meaningful therapeutic effects.

This study has several limitations. Firstly, we used historical controls which we also presented in previous studies (Ikonomidou et al., 2019; Noguchi et al., 2021). This was done because nonhuman primate resources are very limited and costly. We also want to draw attention to the fact that we administered mild hypothermia in this study (35–36.5 °C) at a temperature range that is higher than the one used to cool human infants with hypoxic ischemic encephalopathy (33.0–33.5 °C). Thus, there remains the theoretical possibility that deeper hypothermia might still protect from Pb/M toxicity. We consider this possible but unlikely, given that hypothermia below 35 °C failed to protect neonatal macaques from sevoflurane-induced neuro- and gliotoxicity (Ikonomidou et al., 2019).

Finally, this study did not evaluate whether prolonged HT might prevent neurotoxicity of antiseizure medications in the context of seizures. Our studies pertain to effects of GABA<sub>A</sub>-mimetic medications in the healthy developing non-human primate brain and not in a neonatal seizure model. Notably, any toxicity in the healthy brain needs to be balanced against the harm that would arise from untreated seizure activity.

In conclusion, we are showing that the combination of a barbiturate and a benzodiazepine, at clinically relevant doses, plasma concentrations and treatment duration, causes profound neurodegeneration in the neonatal macaque brain which is not reduced by mild HT. Thus, anti-seizure medications given to treat neonatal seizures in the context of whole-body cooling likely maintain their full neurotoxic potential.

## Acknowledgements

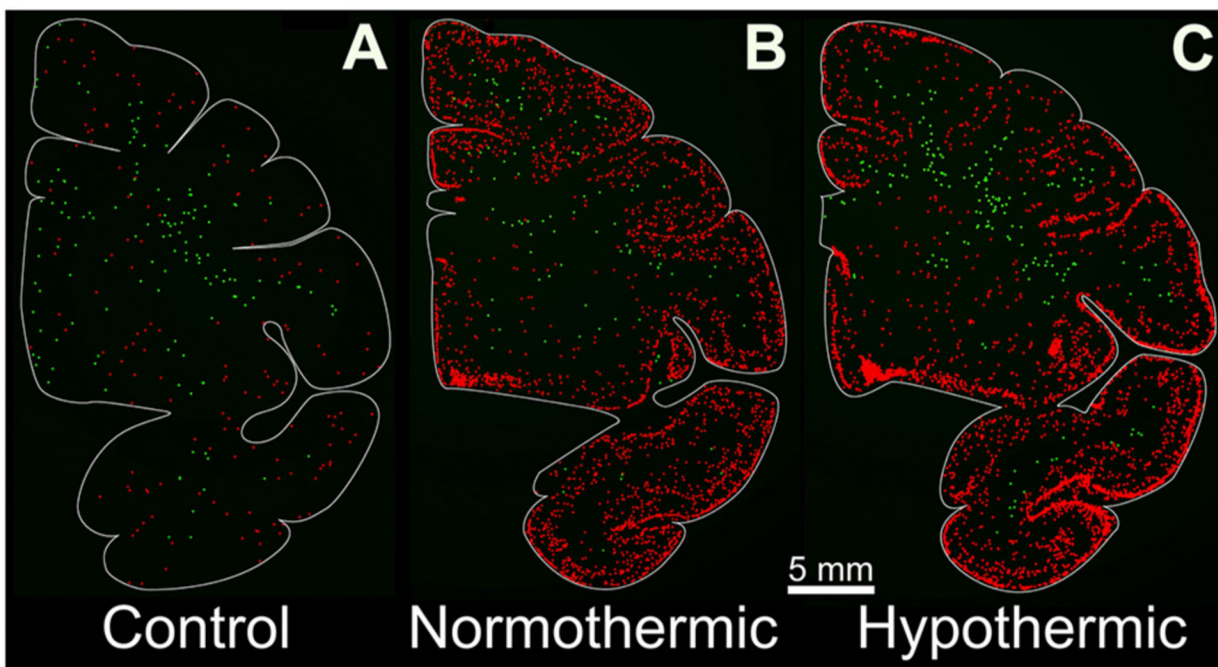
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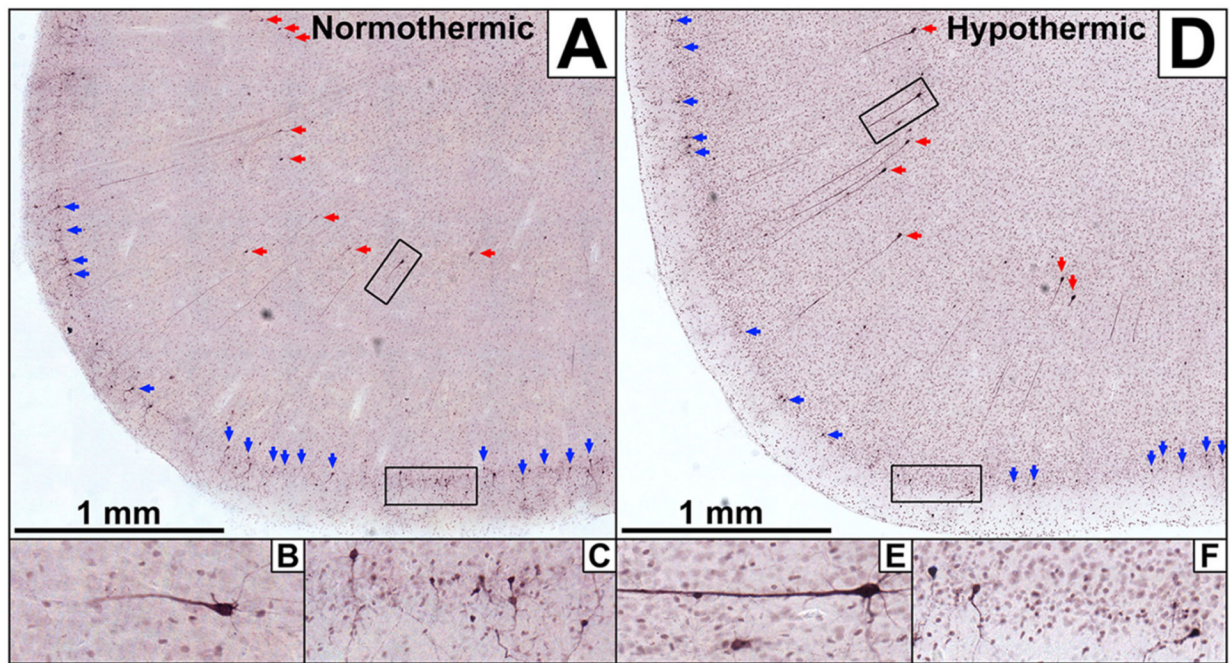
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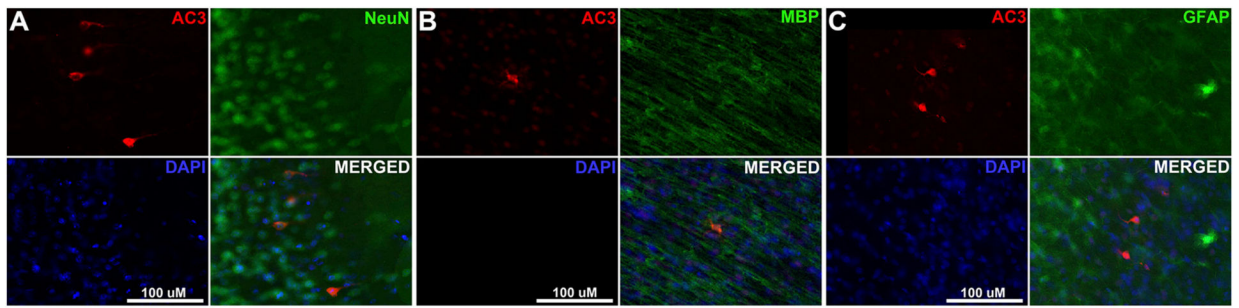
**Fig. 1.**

Apoptosis in the neonatal rhesus macaque brain following Pb/M exposure under normothermic and hypothermic conditions. A - C show computer generated plots of neuroapoptosis (red dots) and oligoapoptosis (green dots) in the brains of a rhesus control infant (left), a normothermic infant exposed to Pb/M for 24 h and euthanized at 36 h (middle) and a hypothermic infant exposed to Pb/M for 36 h and euthanized at 36 h (right). There is substantial amount of neuro- and oligoapoptosis in the brains of both treated infants. At this level and at both time points there is homogeneous pattern of neuro and oligoapoptosis in the caudate, extending into the thalamus, hypothalamus, corpus callosum and subcortical white matter. A laminar pattern of neuronal apoptosis appears within the cingulate, frontal somatosensory, insular, temporal, entorhinal cortices and subiculum. There is no apparent difference in the amount of injury when comparing the normothermic and hypothermic infants. Scale bar = 5 mm.

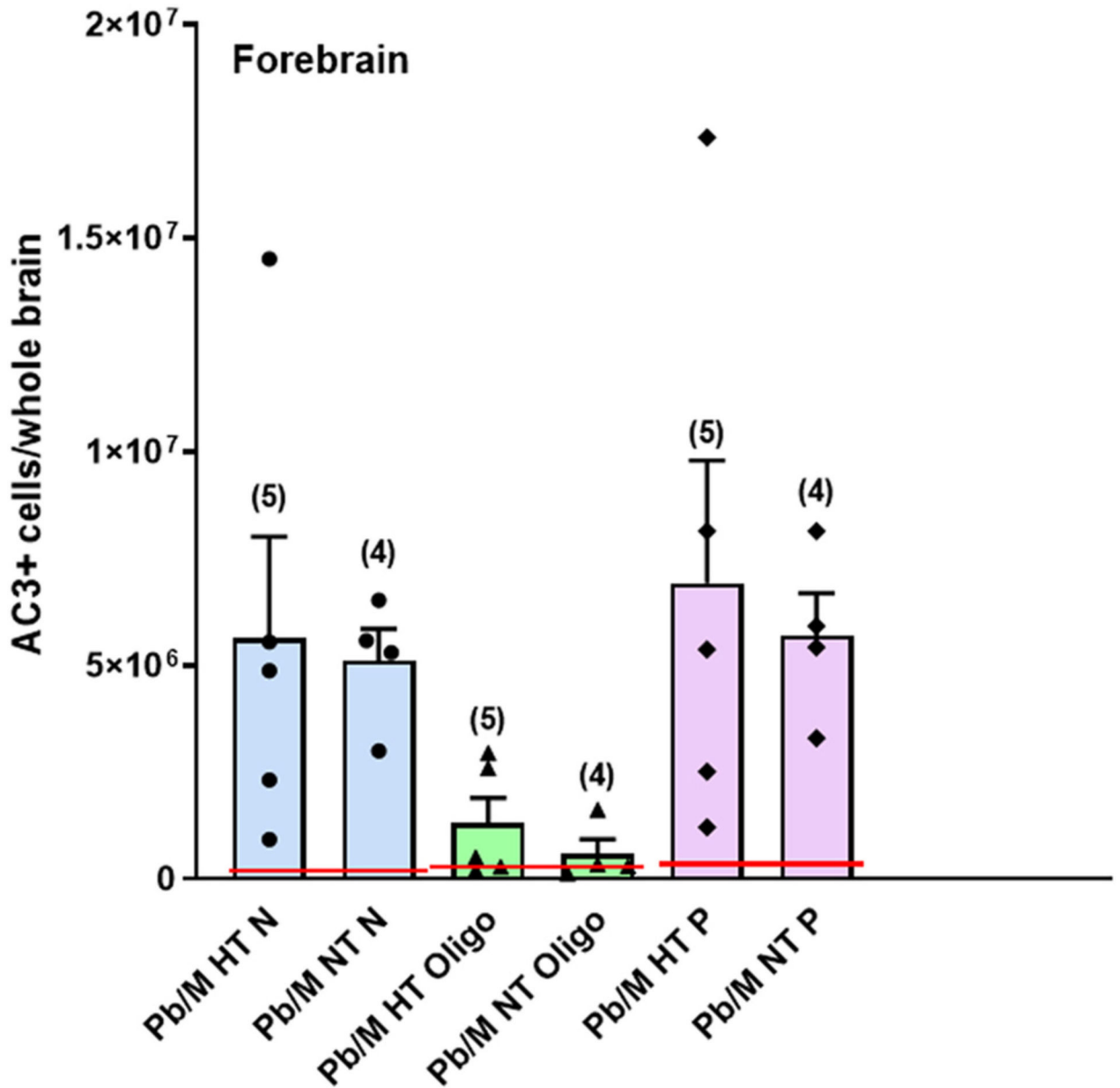


**Fig. 2.**

Light micrographs depicting AC3 positive profiles (red arrows) within the insular cortex in a normothermic rhesus infant exposed to Pb/M (A) and a hypothermic infant exposed to the drug combination +36 h of hypothermia (D). Perfusion fixation occurred for both animals at 36 h. Note that AC3 immuno-positive neurons are present at high density in layer II of the cortex and that neuronal processes are prominently stained. B, C, E and F show magnified views of the black rectangles in A and D.



**Fig. 3.** Apoptosis induced by Pb/M affects neurons and oligodendrocytes. Composites A-C show confocal images of NeuN, MBP, GFAP, AC3 and DAPI stained sections. There is colocalization of NeuN with AC3 and MBP with AC3 indicating neuronal and oligodendroglia apoptosis respectively. In C, GFAP staining (green) does not colocalize with AC3 (red) indicating that astroglia are not affected by the proapoptotic effect of Pb/M.



**Fig. 4.** Mild hypothermia does not protect from apoptosis induced by Pb/M in the neonatal macaque brain. Pb/M induces widespread apoptosis in the infant macaque brain affecting primarily neurons at 36 h. The graphs illustrate numbers of apoptotic neurons (blue), apoptotic oligodendrocytes (green) and apoptotic profiles (purple) counted in the brains of infants treated with Pb/M under normothermic (NT:  $n = 4$ ) or hypothermic (HT:  $n = 5$ ) conditions and euthanized at 36 h. The red lines demonstrate mean numbers of apoptosis reported previously in control animals for each cell type (Noguchi et al., 2021; N:  $95,476 \pm 21,807$ ; Oligo:  $247,372 \pm 81,510$ ; P:  $342,849 \pm 96,592$ ;  $n = 5$ ). Columns represent means  $\pm$  SEM of total numbers of neurons (N; blue), oligodendrocytes (Oligo; green) and total apoptotic profiles (P; purple), counted in the whole brain. Statistical comparisons between NT and HT groups were performed by means of Student's test-test.



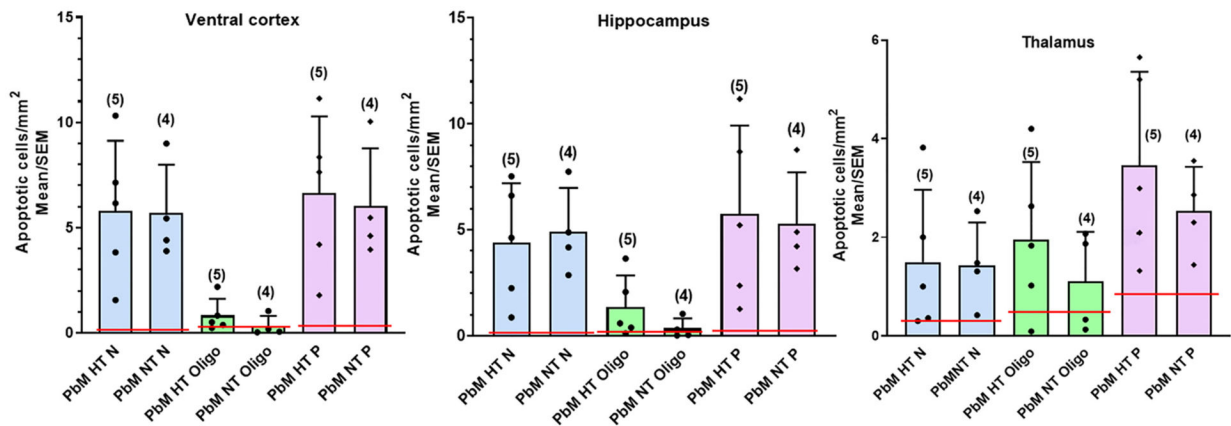
There were no statistically significant differences between the two groups. Abbreviations: Pb/M: phenobarbital/midazolam; HT: hypothermia; NT: normothermia; N: neurons; Oligo: oligodendrocytes; P: profiles.

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**Fig. 5.**

Effects of Pb/M at 36 h on apoptosis of neurons (N), oligodendrocytes (Oligo) and total profiles (P: N + Oligo) in the ventral cortex, hippocampus and thalamus under normothermic (NT) and hypothermic (HT) conditions. Within the ventral cortex and the hippocampus, Pb/M exposure had a significant effect on neuronal apoptosis (blue columns) and total profiles (purple) but not on oligoapoptosis (green columns). Hypothermia did not protect from neuro- or oligoapoptosis. Columns represent means  $\pm$  SEM of densities (cells per square millimeter) of neurons (blue), oligodendrocytes (Oligos; green) and total apoptotic profiles (purple). Numbers in parentheses represent numbers of animals in each group. The red lines depict mean densities previously reported in control animals (Noguchi et al., 2021: *Ventral Cortex*: N:  $0.104 \pm 0.019$ ; Oligo:  $0.140 \pm 0.040$ ; P:  $0.246 \pm 0.043$ ; *Hippocampus*: N:  $0.156 \pm 0.042$ ; Oligo  $0.138 \pm 0.042$ ; P:  $0.292 \pm 0.049$ ; *Thalamus*: N:  $0.400 \pm 0.144$ ; Oligo:  $0.484 \pm 0.234$ ; P:  $0.886 \pm 0.253$ ;  $n = 5$ ). No significant differences were detected for any cell type between the normothermia and hypothermia groups (Student's *t*-test). Abbreviations: Pb/M: phenobarbital/midazolam; HT: hypothermia; NT: normothermia; N: neurons; Oligo: oligodendrocytes; P: profiles.

**Table 1**

Physiologic variables in infant rhesus monkeys subjected to Pb/M for 24 h under normothermia (NT;  $n = 4$ ) or hypothermia (HT;  $n = 5$ ).

	Weight (kg) (mean $\pm$ SEM)	Age (days) (mean $\pm$ SEM)	Sex distribution	0.5 h (mean $\pm$ SEM)	2 h (mean $\pm$ SEM)	4 h (mean $\pm$ SEM)	6 h (mean $\pm$ SEM)	12 h (mean $\pm$ SEM)	24 h (mean $\pm$ SEM)
Pb/M NT; (n = 4) T 36.98 $\pm$ 0.32	0.48 $\pm$ 0.05	5.75 $\pm$ 1.60	2 M, 2 F						
pH (venous)				7.332 $\pm$ 0.03	7.354 $\pm$ 0.02	7.383 $\pm$ 0.02	7.360 $\pm$ 0.001	7.338 $\pm$ 0.02	7.286 $\pm$ 0.02
HR (beats/min)				214.50 $\pm$ 6.30	197.50 $\pm$ 9.82	186.00 $\pm$ 6.67	187.00 $\pm$ 3.76	197.00 $\pm$ 7.38	180.80 $\pm$ 13.40
pCO <sub>2</sub> (mmHg)				48.9 $\pm$ 4.90	44.75 $\pm$ 3.45	48.85 $\pm$ 3.91	43.28 $\pm$ 1.90	37.53 $\pm$ 0.70	42.58 $\pm$ 3.88
SaO <sub>2</sub> (%)				97.99 $\pm$ 2.12	97.00 $\pm$ 1.92	97.00 $\pm$ 1.09	96.50 $\pm$ 1.32	97.50 $\pm$ 1.50	98.25 $\pm$ 1.03
Lactate (mM)				3.54 $\pm$ 0.98	2.17 $\pm$ 0.65	1.08 $\pm$ 0.11	1.15 $\pm$ 0.06	1.32 $\pm$ 0.29	1.31 $\pm$ 0.34
Hb (mg/dl)				14.55 $\pm$ 1.40	13.38 $\pm$ 0.32	13.68 $\pm$ 1.33	13.85 $\pm$ 0.93	11.80 $\pm$ 0.84	12.35 $\pm$ 0.57
Glucose (mM)				65.00 $\pm$ 3.08	57.25 $\pm$ 3.97	42.00 $\pm$ 2.80	43.00 $\pm$ 1.47	41.25 $\pm$ 6.59	60.75 $\pm$ 21.09
Phenobarbital level ( $\mu$ g/ml)						28.00 $\pm$ 9.58			36.93 $\pm$ 6.25
Midazolam level (ng/ml)						2302 $\pm$ 1104			6018 $\pm$ 2379
Pb/M HT; (n = 5) T 35.30 $\pm$ 0.16	0.48 $\pm$ 0.02	5.40 $\pm$ 0.68	2 M, 3 F						
pH (venous)				7.344 $\pm$ 0.017	7.364 $\pm$ 0.02	7.362 $\pm$ 0.03	7.337 $\pm$ 0.02	7.355 $\pm$ 0.04	7.361 $\pm$ 0.03
HR (beats/min)				223.00 $\pm$ 15.18	191.80 $\pm$ 6.46	200.80 $\pm$ 8.62	191.60 $\pm$ 7.42	184.8 $\pm$ 7.69	191.8 $\pm$ 6.14
pCO <sub>2</sub> (mmHg)				45.04 $\pm$ 1.02	46.12 $\pm$ 2.52	45.82 $\pm$ 2.48	49.36 $\pm$ 3.99	43.42 $\pm$ 0.71	44.60 $\pm$ 2.16
SaO <sub>2</sub> (%)				97.20 $\pm$ 0.58	96.80 $\pm$ 0.92	97.40 $\pm$ 0.87	96.00 $\pm$ 1.34	96.00 $\pm$ 1.00	96.60 $\pm$ 0.68
Lactate (mM)				3.42 $\pm$ 0.22	1.30 $\pm$ 0.11	1.19 $\pm$ 0.12	1.26 $\pm$ 0.09	1.29 $\pm$ 0.11	1.02 $\pm$ 0.18
Hb (mg/dl)				13.37 $\pm$ 1.11	12.24 $\pm$ 0.49	11.70 $\pm$ 0.35	11.56 $\pm$ 0.35	11.16 $\pm$ 0.63	10.82 $\pm$ 0.39
Glucose (mM)				83.80 $\pm$ 6.71	81.40 $\pm$ 8.52	76.20 $\pm$ 7.57	74.00 $\pm$ 5.87	60.40 $\pm$ 8.17	57.20 $\pm$ 7.66
Phenobarbital level ( $\mu$ g/ml)						34.20 $\pm$ 10.62			40.52 $\pm$ 7.60
Midazolam level (ng/ml)						1230 $\pm$ 574.90			2702 $\pm$ 921.40

Animals were euthanized at 36 h. Measurements were taken at 0.5, 2, 4, 6, 12 and 24 h after initiation of Pb injection and represent means  $\pm$  SEM. All values are within physiological range. HR: heart rate; pCO<sub>2</sub>: partial CO<sub>2</sub> pressure; SaO<sub>2</sub>: oxygen saturation measured by pulse oximetry; Hb: hemoglobin.