Neither Xenon nor Fentanyl Induces Neuroapoptosis in the Newborn Pig Brain

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ABSTRACT

Background: Some inhalation anesthetics increase apoptotic cell death in the developing brain. Xenon, an inhalation anesthetic, increases neuroprotection when combined with therapeutic hypothermia after hypoxic-ischemic brain injury in newborn animals. The authors, therefore, examined whether there was any neuroapoptotic effect of breathing 50% xenon with continuous fentanyl sedation for 24 h at normothermia or hypothermia on newborn pigs.

Methods: Twenty-six healthy pigs (<24-h old) were randomized into four groups: (1) 24 h of 50% inhaled xenon with fentanyl at hypothermia (Trec = 33.5°C), (2) 24 h of 50% inhaled xenon with fentanyl at normothermia (Trec = 38.5°C), (3) 24 h of fentanyl at normothermia, or (4) nonventilated juvenile controls at normothermia. Five additional nonrandomized pigs inhaled 2% isoflurane at normothermia for 24 h to verify any proapoptotic effect of inhalation anesthetics in our model. Pathological cells were morphologically assessed in cortex, putamen, hippocampus, thalamus, and white matter. To quantify the findings, immunostained cells (caspase-3 and terminal deoxynucleotidyl transferase–mediated deoxyuridine-triphosphate nick-end labeling) were counted in the same brain regions.

Results: For groups (1) to (4), the total number of apoptotic cells was less than 5 per brain region, representing normal developmental neuroapoptosis. After immunostaining and cell counting, regression analysis showed that neither 50% xenon with fentanyl nor fentanyl alone increased neuroapoptosis. Isoflurane caused on average a 5- to 10-fold increase of immunostained cells.

Conclusion: At normothermia or hypothermia, neither 24 h of inhaled 50% xenon with fentanyl sedation nor fentanyl alone induces neuroapoptosis in the neonatal pig brain. Breathing 2% isoflurane increases neuroapoptosis in neonatal pigs.

What We Already Know about This Topic

- Moderate hypothermia has proven clinical efficacy in protecting the brain against anoxic and ischemic injury
- Some inhalational anesthetics increase apoptotic cell death in the developing brain, whereas xenon enhances hypothermic neuroprotection in newborn rats and pigs
- The effects of xenon combined with opioids on hypothermic neuroprotection remain unknown

What This Article Tells Us That Is New

- By using a model of mechanically ventilated piglets in the absence of brain injury, the authors could show that neither 24 h of 50% inhalated xenon nor fentanyl, alone or in combination, induced apoptosis in the neonatal pig brain in normothermic or hypothermic conditions
- Isoflurane 2% was found to induce apoptosis in this experimental paradigm

PRECLINICAL findings, indicating that common general anesthetic agents may trigger or induce neuronal apoptosis in the developing brain,[1–3] have been the subject of much debate and have even been considered in a public hearing of the Anesthesia and Life Support Drugs Advisory Committee of the United States Food and Drug Administration (March 2007). However, species, age, and injury differences are major influencing factors. Programmed cell death plays a major role in normal brain development and synaptogenesis.[4] Neuronal apoptosis is the major type of programmed cell death that occurs after exposure to general anesthetics in the newborn.[3,5,6] There is a concern that anesthetics and sedative drugs may harm the immature brain by

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increased neuroapoptosis.[7–11] One important proapoptotic protease is caspase-3.[5] It is positioned at the center of the apoptotic cascade and is activated by both the intrinsic and extrinsic pathways, leading to DNA fragmentation.[12] Caspase-3 cleavage and activation occur in the pig brain after hypoxia and anesthetic exposure.[6,13]

Therapeutic hypothermia has been recommended by the international resuscitation guidelines (International Liaison Committee on Resuscitation 2010) for term newborns suffering from perinatal asphyxia.[14] Clinical trials have shown that 3 days of therapeutic hypothermia is safe and reduces mortality and long-term neurodevelopmental disability in newborns with neonatal encephalopathy.[15–18] The occurrence of death and severe disability is reduced from 66 to 50% in cooled infants, but there is evidence that neonates with the most severe forms of injury may not benefit from therapeutic hypothermia.[15,16,18] Therefore, an additional neuroprotective treatment is needed.[19]

We and others have shown that the noble gas such as xenon enhances hypothermic neuroprotection in immature rats and pigs.[20–23] Xenon is an inhalation anesthetic with sedative and analgesic effects and no documented clinical adverse effects. It exerts its neuroprotective properties mainly by partial inhibition of *N*-methyl-D-aspartate and other glutamate receptors.[24–26] Other general anesthetics, which act as *N*-methyl-D-aspartate antagonists, have been shown to induce neuronal injury and cell death in the healthy newborn brain.[2,3,27,28] Even though xenon is a very potent neuroprotective drug after experimental newborn hypoxic-ischemic injury, reports in neonatal rodents have shown contradictory effects of xenon administration on neuronal cell death.[22,29]

Because newborns undergoing intensive care treatment, not only during hypothermia after perinatal asphyxia but also during surgery and transport to theatre, receive opioids as background sedation and analgesia, it is very important to assess whether the combination of opioids with or without an anesthetic gas induces neuroapoptosis. Our aim was to examine whether xenon in combination with fentanyl or fentanyl alone or in combination with hypothermia treatment induces cell death in healthy newborn pigs at normal body temperature (38.5°C for pigs). As a control for the sensitivity of the model, we also needed to examine whether the newborn pig developed neuroapoptosis after exposure to isoflurane as seen convincingly in the immature rodent.[30]

Material and Methods

Animals and Experimental Groups

All experiments were conducted according to the United Kingdom Home Office license guidelines and were approved by the University of Bristol Ethical Review Panel (Bristol, United Kingdom). Thirty-one crossbred Landrace/Large White term-born pigs of either sex with a median (interquartile range) age of 11 h (8.5–13) and a median (interquartile range) weight of 1.67 kg (1.36–1.87) were used. Twenty-six animals were randomly assigned to the following treatments: (1) 24 h of 50% inhaled xenon with iv fentanyl sedation at hypothermia (rectal temperature 33.5°C, HTXeFe), (2) normothermia (rectal temperature 38.5°C, NTXeFe) conditions, (3) nonventilated instrumented pigs with 24 h of iv fentanyl sedation at normothermia (NTFe, control sedation), or (4) nonventilated juvenile control pigs without any sedation at normothermia (NTCTR, juvenile control).

To verify the proapoptotic effect of inhalation anesthetics in this animal model, a nonrandomized group comprised five pigs was added afterwards and ventilated with 2.0% inhaled isoflurane at normothermia (NTIso; fig. 1).

Animal Preparation, Baseline Data and Management of All Pigs

The juvenile control group (group (4), n = 7) was not intubated and had no central lines inserted. Blood samples were taken by venopuncture. The remaining 24 animals underwent unrestrained induction in a closed plexiglas box with 2% isoflurane, 65% nitrous oxide, and 33% oxygen, followed by oral intubation with a 3.0-mm cuffed endotracheal tube (Mallinckrodt Medical, Athlone, Ireland). After intubation, animals were mechanically ventilated (SLE 2000; SLE, Surrey, United Kingdom) with 2% isoflurane, 77% nitrous oxide, and 21% oxygen until the end of the baseline period (≤ 90 min). Fentanyl infusion was started in groups (1) to (3). Transcutaneous arterial oxygen saturation (Nonin Medical, Plymouth, MN) and end-tidal carbon dioxide were monitored (Tidal Wave SP; Respironics Novametrix, Wallingford, CT), and ventilator settings were adjusted to maintain transcutaneous arterial oxygen saturation between 95 and 98% and the end-tidal carbon dioxide between 4.0 and 6.0 kPa. If required, the oxygen fraction in the gas mixture or ventilator settings was adjusted to keep transcutaneous arterial oxygen saturation and end-tidal carbon dioxide in the target range. After attaining iv access by cannulation of an ear vein, umbilical arterial and venous catheters were inserted to allow continuous monitoring of mean arterial blood pressure, blood sampling, and infusion of maintenance fluids and drugs. Blood sampling was undertaken at preset time points, as well as when clinically indicated. Blood gases (RapidLab 248 pH/blood gas analyzer; Siemens Healthcare Diagnostics, Surrey, United Kingdom), blood glucose (Optium; MediSense, Abingdon, United Kingdom), and lactate (Lactate Pro; Arkray, Kyoto, Japan) were measured. Intensive care management of the animals was performed as previously described.[20] Pigs in groups (1), (2), and (5) received iv 5% dextrose and 0.45% saline to maintain a fluid intake of 10 $ml \cdot kg^{-1} \cdot h^{-1}$. Pigs in group (3) received iv 5% dextrose and 0.45% saline of 5 $ml \cdot kg^{-1} \cdot h^{-1}$, in addition to being bottle fed with pig formula (Pig formula milk "Baby Lactal"; Peter Möller A/S, Oslo, Norway) to a rate of 10 $ml \cdot kg^{-1} \cdot h^{-1}$. Pigs in group (4) were bottle fed every 2–3 h with pig formula and maintained a fluid intake of 10 $ml \cdot kg^{-1} \cdot h^{-1}$. Hypotension (mean arterial blood pressure <40 mmHg for ≥10 min) was first treated

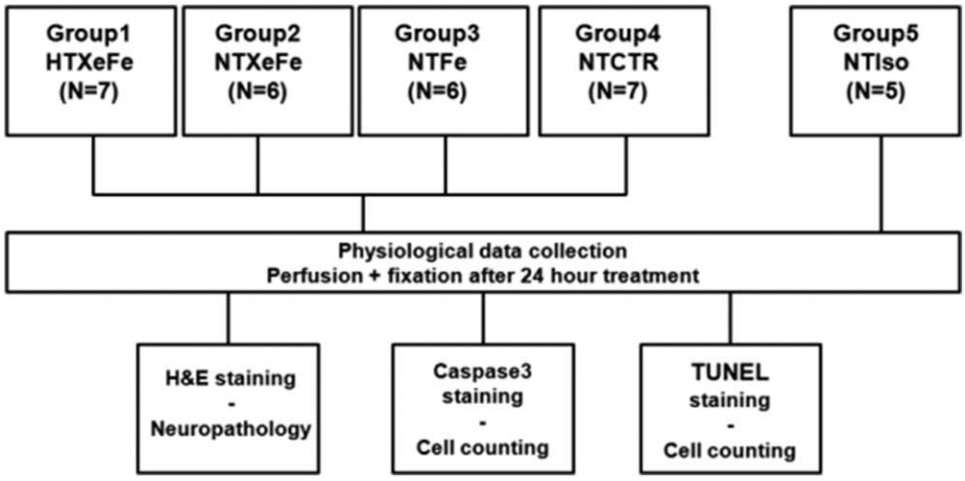

Fig. 1. Experiment design. Twenty-six newborn pigs were randomized into four groups: (1) 24 h of 50% inhaled xenon with fentanyl sedation at hypothermia (HTXeFe, rectal temperature [T_{rec}] = 33.5°C; n = 7); (2) 24 h 50% Xe with fentanyl sedation at normothermia (NTXeFe, T_{rec} = 38.5°C; n = 6); (3) 24 h of fentanyl sedation at normothermia (NTFe, n = 6); or (4) normothermia control (NTCTR, n = 7). Five subsequently added pigs inhaled 2% isoflurane at normothermia (NTIso, n = 5) for 24 h. After 24 h of assigned treatment, all animals were brain perfusion-fixed, and brain pathology was assessed and cells counted. H&E = hematoxylin and eosin; HTXeFe = 24-h ventilation with 50% xenon and iv fentanyl at hypothermia; NTCTR = juvenile controls at normothermia; NTFe = 24-h iv fentanyl at normothermia; NTIso = 24-h ventilation with isoflurane at normothermia; NTXeFe = 24-h ventilation with 50% xenon and iv fentanyl at normothermia; TUNEL = terminal deoxynucleotidyl transferase–mediated deoxyuridine-triphosphate nick-end labeling.

with two separate boluses of crystalloids (10 ml/kg, 0.9%, saline), followed by continuous dopamine infusion (5–20 $\mu g \cdot kg^{-1} \cdot min^{-1}$), which applied to one pig in group (1) and four pigs in group (5). Tracheal suction was performed every 8 h or as clinically indicated. Hence, pH, blood glucose, and lactate values were kept between 7.35 and 7.47 (analyzed at actual body temperature), 3.0 and 8 mM, and less than 3.5 mM, respectively (table 1).

Table 1. Baseline Parameters for All Treatment Groups

	HTXeFe (n = 7)	NTXeFe (n = 6)	NTFe (Self Ventilating, n = 6)	NTCTR (Self Ventilating, n = 7)	NTIso (n = 5)
Median weight, kg (IQR)	1.42 (1.35–1.59)	1.73 (1.45–1.76)	1.75 (1.63–1.86)	1.36 (1.29–1.42)	1.90 (1.78–1.96)
Sex					
Male	4	1	4	3	3
Female	3	5	2	4	2
Median age, h (IQR)	9 (6.5–19.5)	10 (8–14.2)	10 (9–11.7)	10 (9.5–11)	13 (11–13)
Median heart rate, min^{-1} (IQR)	123 (108–126)	139 (131–146)	159 (153–164)	n/a	180 (161–188)
Median arterial blood pressure, mmHg (IQR)	49 (46.6–49.7)	49 (47.4–51.3)	53 (46.8–55)	n/a	44 (43.5–45)
Median tcSO2, % (IQR)	99 (98–99.5)	100 (99.3–100)	98 (97–99)	n/a	100 (97–100)
Median pH (IQR)	7.35 (7.34–7.36)	7.40 (7.36–7.44)	7.41 (7.38–7.45)	7.46 (7.42–7.50)	7.42 (7.38–7.46)
Median glucose, mM (IQR)	6.9 (5.6–7.9)	5.2 (4.5–5.9)	5.9 (5.4–6.1)	3.9 (3.4–4.3)	6.5 (5.3–7.1)
Median lactate, mM (IQR)	2.1 (1.5–3.0)	1.5 (1.3–1.7)	2.1 (1.4–3.1)	3.3 (2.9–3.5)	2.9 (2.8–3.5)
Median fentanyl dosage, $\mu g \cdot kg^{-1} \cdot h^{-1}$ (IQR)	55.0 (50–60)	50.0 (42.5–75.3)	17.5 (15–20)	0	0

Median (IQR) baseline parameters (weight, sex, and age), heart rate, arterial blood pressure, tcSO2, pH, blood glucose, and lactate levels of all the 31 animals in the different treatment groups and median fentanyl dosage for 19 pigs in the treatment groups receiving iv fentanyl. NTIso group was subsequently added to verify an apoptotic effect of inhalation anesthetics in newborn pigs. It was therefore not randomized and is separated by double border from the other groups.

HTXeFe = 24-h ventilation with 50% xenon and iv fentanyl at hypothermia; IQR = inerquartile range; iv = intravenous; NTCTR = juvenile controls at normothermia; NTFe = 24-h iv fentanyl at normothermia; NTIso = 24-h ventilation with isoflurane at normothermia; NTXeFe = 24-h ventilation with 50% xenon and iv fentanyl at normothermia; n/a = data was not applicable, as animals nor were they cardiovascularly monitored as they were noninstrumented NTCTR; tcSO2 = transcutaneous oxygen saturation.

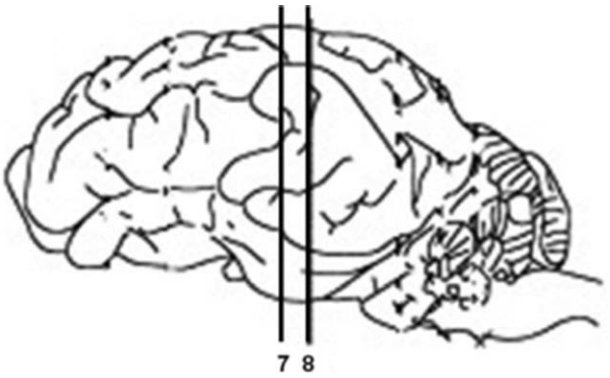

Fig. 2. Shows the two adjacent blocks (7 and 8) that were chosen for histological analysis, best presenting the cerebral cortex grey matter, white matter, hippocampus, basal ganglia (including putamen), and thalamus in the newborn pig brain.

Temperature measurements were undertaken with a rectal probe (reusable YSI 400 series, CritiCool; MTRE, Yavne, Israel), inserted 6 cm into the rectum, and a skin probe (CritiCool), sited on the ear lobe. Both probes were calibrated before use within ±0.1°C over a temperature range of 20°–40°C against a certified mercury-in-glass thermometer (BS593; Zeal, London, United Kingdom). For the normothermia group, a rectal temperature (T_{rec}) of 38.5° ± 0.2°C was maintained, using the overhead heater of an open Giraffe incubator (Giraffe, Omnibed; GE Healthcare, Bucks, United Kingdom) or a wrap containing circulating water, servo controlled to 38.5°C (CritiCool). For the hypothermia group, a T_{rec} of 33.5° ± 0.2°C was achieved within 40 min after start cooling and thereafter maintained with a body wrap containing circulating water, servo controlled to T_{rec} 33.5°C (CritiCool).

Fentanyl Sedation and Xenon Inhalation

Fentanyl infusion was started immediately after intubation and iv access for groups (1), (2), and (3) with a bolus of 10 μg/kg followed by maintenance infusion. This iv sedation was adjusted to achieve adequate responsiveness for sedation. The median (interquartile range) fentanyl dosage was 55.0 $\mu g \cdot kg^{-1} \cdot h^{-1}$ (50.0–60.0) in group (1), 50.0 $\mu g \cdot kg^{-1} \cdot h^{-1}$ (42.5–75.3) in group (2), and 17.5 $\mu g \cdot kg^{-1} \cdot h^{-1}$ (15.0–20.0) in group (3). Pigs in group (3) were extubated after instrumentation under anesthesia to be self ventilating in air. Therefore group (3) received less iv fentanyl sedation than pigs in the ventilated treatment groups (1) and (2), who needed deeper sedation. Pigs in group (4) were always self ventilating and not sedated.

After experimental preparation, baseline physiological parameters were recorded for 60 min before the gas intervention was started. In groups (1) and (2), a xenon–oxygen–nitrogen mixture (50% Xe–30% O_2–20% N_2) was delivered using an automated version of a previously described closed-circuit delivery system for a period of 24 h.[31] Xenon was maintained at a concentration of 50% throughout the whole 24 h.

Isoflurane Inhalation

Pigs in group (5) were ventilated for 24 h with 2% isoflurane and 21% oxygen–nitrogen, following the same experimental and monitoring protocol as pigs in groups (1) and (2). The isoflurane concentration was maintained throughout the whole 24 h at normothermia, providing adequate sedation for ventilation at normothermia, and therefore no fentanyl was added.

Neuropathology Assessment

After 24 h of allocated treatment, pigs were deeply anesthetized with isoflurane (after intubation in groups (3) and (4)). Brains were slowly flushed with 0.9% saline through the common carotid arteries followed by perfusion fixation with 10% neutral buffered formalin and dissected out. In addition, an autopsy of full body was performed. The right hemisphere of the brain was coronally cut into 5-mm blocks and paraffin processed. Two adjacent blocks were chosen, containing the cerebral cortex, subcortical white matter, hippocampus, basal ganglia (including putamen), and thalamus (fig. 2). In addition, two representative blocks of the cerebellum were chosen. Hematoxylin and eosin–stained sections were assessed at ×20 magnification by a neuropathologist blinded to the randomization and clinical details. Cells in the cerebral cortex grey matter, subcortical white matter, hippocampus, basal ganglia (including putamen), thalamus, and Purkinje neurons of the cerebellum were scored as apoptotic when showing typical morphology of apoptosis.[5]

For cerebral cortex, putamen, hippocampus, thalamus, white matter, and Purkinje neurons, apoptosis was assessed by examining two adjacent sections for positive staining of Cleaved caspase-3 (Cell Signalling Technologies, New England Biolabs, Hertfordshire, United Kingdom). To assess DNA fragmentation, the adjacent sections were stained with terminal deoxynucleotidyl transferase–mediated deoxyuridine-triphosphate (dUTP) nick-end labeling (Roche, Meylan, France).[32] For immunohistochemical analysis, deparaffinized 5-μm thick sections were microwaved in 10-mM citrate buffer pH 6 at power 9 for 15 min before incubation in 3% H_2O_2 and methanol for 15 min, followed by 30-min incubation in 2.5% normal horse serum to block nonspecific staining. Sections were incubated overnight in monoclonal rabbit anti-caspase-3 antibody (1/500) at room temperature. After primary antibody incubation, sections were incubated in horse anti-rabbit biotinylated antibody (1/200) for 30 min, then ABC Elite (1/200; Vector Laboratories, Peterborough, United Kingdom) for 30 min. Diaminobenzidine-based peroxide substrate was used as a chromogen, and sections were lightly counterstained with Mayers Hematoxylin (Thermo Fisher Scientific, Runcorn, United Kingdom), dehydrated through graded alcohols to xylene, and then mounted using *p*-xylene-bis-pyridinium bromide. For each animal and each brain region, the two stained adjacent sections were counted for Cleaved caspase-3 and terminal deoxynucleotidyl transferase–mediated dUTP

nick-end labeling positive cells at ×20 magnification. The complete hemispheric section was counted. For the right hemisphere of the cerebellum, Purkinje neurons of three complete gyri were counted for Cleaved caspase-3 and terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling positive cells at ×20 magnification.

Total cell counting was performed by three independent observers blinded to the randomization and to clinical details.

Statistical Analysis

Statistical analyses were performed with SPSS version 18 (SPSS Inc., Chicago, IL). The Wilcoxon test was used (two-tailed) for the two-group comparisons. One-way ANOVA was used to compare the results of cell counting, blood gases, blood sugar, and lactate levels within the different randomized treatment groups followed by a nonparametric *post hoc* test (Tukey–Kramer) if the ANOVA showed significant differences between treatment groups. Regression analysis was used to assess the effect of the independent variables such as total fentanyl dosage during the experiment, inhalation of xenon (yes or no), sex, cooling (yes or no), and age since birth in hours on the percentage of Cleaved caspase-3 and terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling positive cells in groups (1)–(4) for each of the five brain regions. A dummy code was used to include the categorical data (cooling and xenon) into the linear regression model. Statistical significance was considered at a probability (P) value of 0.05 or more on two-sided testing. Continuous variables were presented as median (interquartile range) or mean (±SD).

Results

Physiological Data

There was no significant difference in age ($P = 0.700$), weight ($P = 0.055$), and sex distribution ($P = 0.475$) among the treatment groups (table 1). There was a significantly lower dosage use of fentanyl in group (3) as compared with groups (1) and (2) ($P < 0.001$) because all the pigs in group (3) were self ventilating with normal pCO_2 and therefore received less sedation (table 1). Table 1 summarizes the physiological parameters in the treatment groups for the 24-h duration of the experiment. Heart rate was significantly lower in the hypothermia group ($P < 0.001$), as expected because hypothermia reduces heart rate by 10 beats/1°C decrease in core temperature.[33] Mean arterial blood pressure was higher than 40 mmHg in all pigs of all the five groups throughout the 24-h treatment period, providing an adequate cerebral blood flow in newborn pigs.[34–37]

Blood gases, blood sugar, and lactate values were within the normal range in all animals, and there were no significant differences among the four groups with regard to blood gases over the whole 24-h study time ($P > 0.05$; table 1).

Histological Results

Hematoxylin and eosin–stained sections did not show significant signs of brain pathology in the regions examined in treatment groups (1) to (4). No necrotic cells were seen, and apoptotic cells were analyzed based on morphological assessment in the hematoxylin and eosin–stained sections. For each individual brain region, the maximum number of apoptotic cells within one hemisphere was not different between groups (1) and (4) and not more than five cells representing normal developmental programmed cell death in a newborn pig brain.[38] In Purkinje cells of the cerebellum, no apoptotic cell was seen.

Pigs in group (5) showed a large increase in apoptotic cells compared with other treatment groups for all assessed hematoxylin and eosin–stained brain regions, indicating isoflurane-induced neurotoxicity. To quantify this finding, Cleaved caspase-3 and terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling positive cells were counted in all assessed brain regions (tables 2 and 3 and figs. 3 and 4). The number of Cleaved caspase-3–positive cells for each treatment group and each counted brain area are presented in figure 3 and table 2. No positive immunostained cells were found in the Purkinje cell layer for any treatment group. One-way ANOVA showed a significant difference in Cleaved caspase-3–positive cells ($P < 0.05$). *Post hoc* analysis (Tukey–Kramer) showed that the NTIso group had a significant increase in Cleaved caspase-3–positive cells

Table 2. Cleaved Caspase-3 Cell-counting Results

	N	Cortex	Putamen	Hippocampus	Thalamus	White Matter
HTXeFe	7	4.00 (1.83–10.50)	0.00 (0.00–0.50)	0.33 (0.17–0.50)	2.50 (2.00–4.67)	2.00 (0.17–3.17)
NTXeFe	6	3.83 (0.96–7.54)	0.08 (0.00–0.49)	0.00 (0.00–0.21)	1.08 (0.62–1.83)	0.67 (0.17–1.33)
NTFe	6	1.42 (0.95–1.83)	0.00 (0.00–0.12)	0.08 (0.00–0.45)	1.08 (0.41–2.33)	0.33 (0.17–1.04)
NTCTR	7	1.33 (0.67–1.50)	0.17 (0.00–0.17)	0.00 (0.00–0.33)	1.50 (0.50–2.00)	0.50 (0.33–1.00)
NTIso	5	78.00 (37.50–226)	0.25 (0.00–0.87)	0.75 (0.37–1.12)	1.50 (0.62–6.25)	3.00 (1.75–6.50)

Total cell count for each brain region in one hemisphere for Cleaved caspase-3–positive cells. Results are presented as median (interquartile range) number of Cleaved caspase-3–positive cells. There was a significant increase in Cleaved caspase-3–positive cells in the NTIso group for cortex ($P < 0.001$), hippocampus ($P = 0.005$), and white matter ($P = 0.010$) in the two-group comparison with the other treatment groups. The NTIso group was subsequently added and is separated from the other groups.

HTXeFe = 24-h ventilation with 50% xenon and iv fentanyl at hypothermia; iv = intravenous; NTCTR = juvenile controls at normothermia; NTFe = 24-h iv fentanyl at normothermia; NTIso = 24-h ventilation with isoflurane at normothermia; NTXeFe = 24-h ventilation with 50% xenon and iv fentanyl at normothermia.

Table 3. TUNEL Cell-counting Results

	N	Cortex	Putamen	Hippocampus	Thalamus	White Matter
HTXeFe	7	27.50 (21.75–34.50)	0.75 (0.50–2.00)	1.00 (0.50–1.75)	6.00 (4.00–9.00)	14.25 (13.75–15.75)
NTXeFe	6	14.88 (7.95–30.06)	0.46 (0.00–1.12)	0.38 (0.00–0.81)	2.88 (1.45–6.87)	5.88 (4.39–6.75)
NTFe	6	13.38 (10.20–20.00)	0.25 (0.18–0.81)	1.08 (0.75–1.50)	6.75 (5.06–10.00)	9.50 (5.94–13.68)
NTCTR	7	9.00 (7.00–18.17)	0.00 (0.00–1.33)	1.00 (0.75–1.50)	6.67 (4.50–10.83)	7.50 (4.67–10.50)
NTIso	5	183.00 (124.12–223.00)	11.25 (4.87–28.37)	5.00 (4.00–8.50)	36.25 (24.75–59.37)	32.50 (19.75–68.25)

Total cell count for each brain region in one hemisphere for TUNEL-positive cells. Results are presented as median number (interquartile range) of TUNEL-positive cells. There was a significant increase in TUNEL-positive cells in the NTIso group for all brain regions in the two-group comparison with the other treatment groups (cortex [$P < 0.001$], putamen [$P = 0.003$], hippocampus [$P < 0.001$], thalamus [$P < 0.001$], and white matter [$P = 0.001$]). The NTIso group was subsequently added and is separated from the other groups.

HTXeFe = 24-h ventilation with 50% xenon and iv fentanyl at hypothermia; iv = intravenous; NTCTR = juvenile controls at normothermia; NTFe = 24-h iv fentanyl at normothermia; NTIso = 24-h ventilation with isoflurane at normothermia; NTXeFe = 24-h ventilation with 50% xenon and iv fentanyl at normothermia; TUNEL = terminal deoxynucleotidyl transferase–mediated deoxyuridine-triphosphate nick-end labelling.

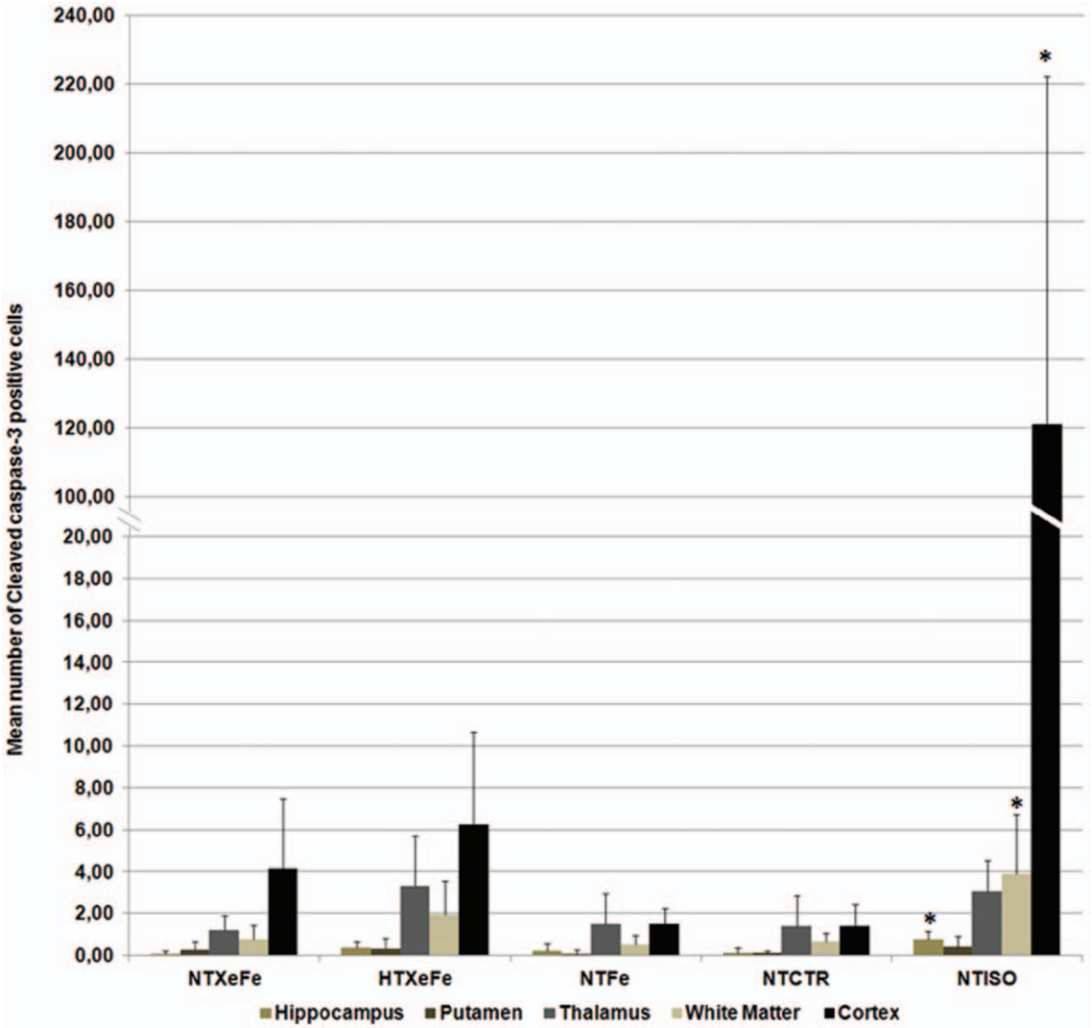

Fig. 3. Mean number of Cleaved caspase-3–positive cells (+SD) per counted brain region. There was a significant increase in Cleaved caspase-3–positive cells in the NTIso group (positive control group) in cortex ($P < 0.001$), hippocampus ($P = 0.005$), and white matter ($P = 0.010$). *Asterisks* indicate a significance level of $P < 0.05$. HTXeFe = 24-h ventilation with 50% xenon and iv fentanyl at hypothermia; NTCTR = juvenile controls at normothermia; NTFe = 24-h iv fentanyl at normothermia; NTIso = 24-h ventilation with isoflurane at normothermia; NTXeFe = 24-h ventilation with 50% xenon and iv fentanyl at normothermia.

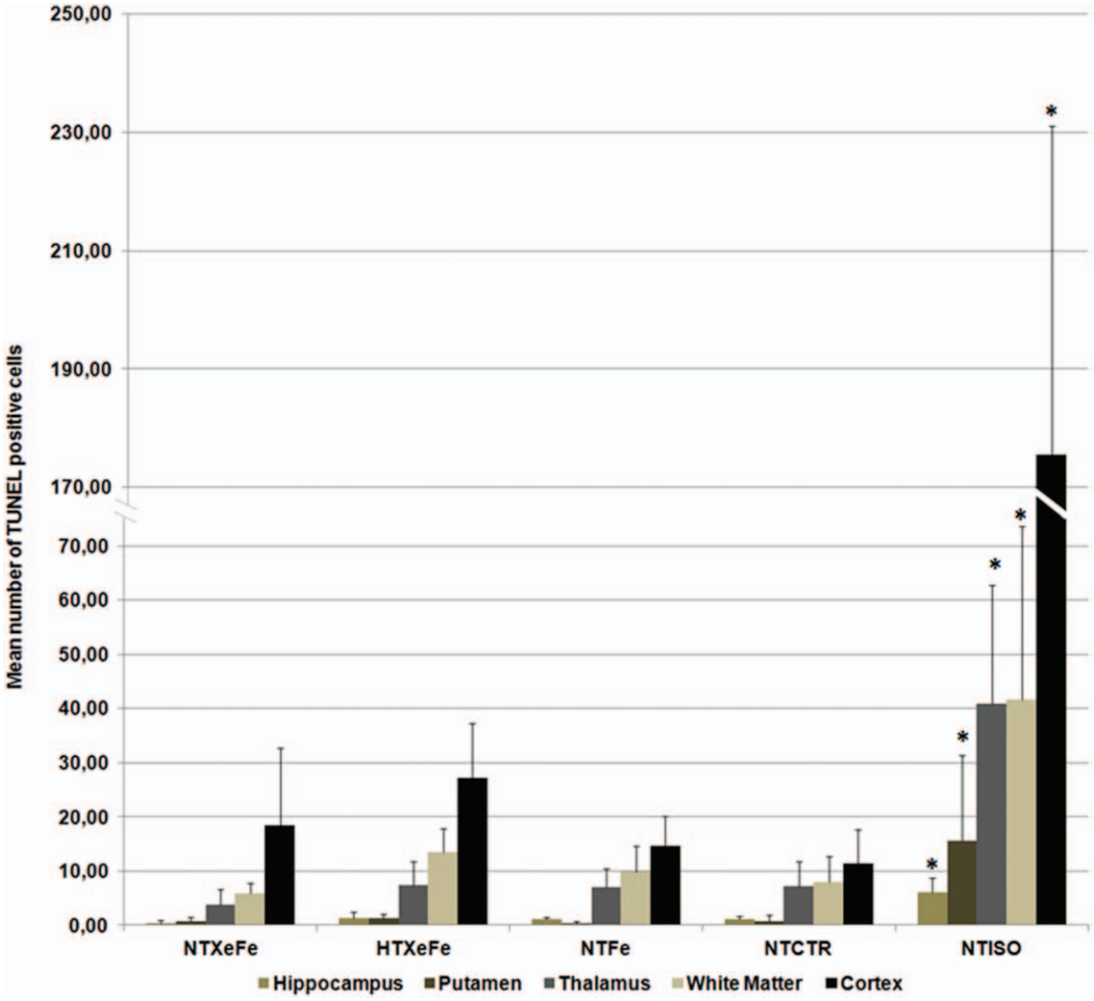

Fig. 4. Mean number of TUNEL-positive cells (+SD) per counted brain region. There was a significant increase in TUNEL-positive cells in the NTIso group (positive control group) in all brain regions (cortex [$P < 0.001$], putamen [$P = 0.003$], hippocampus [$P < 0.001$], thalamus [$P < 0.001$], and white matter [$P = 0.001$]). *Asterisks* indicate a significance level of $P < 0.05$. HTXeFe = 24-h ventilation with 50% xenon and iv fentanyl at hypothermia; NTCTR = juvenile controls at normothermia; NTFe = 24-h iv fentanyl at normothermia; NTIso = 24-h ventilation with isoflurane at normothermia; NTXeFe = 24-h ventilation with 50% xenon and iv fentanyl at normothermia; TUNEL = terminal deoxynucleotidyl transferase–mediated deoxyuridine-triphosphate nick-end labeling.

in the cortex ($P < 0.001$), the hippocampus ($P = 0.005$), and the white matter ($P = 0.010$). In addition, there was a significant difference in the one-way ANOVA for terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling positive cells, and *post hoc* analysis (Tukey–Kramer) showed that animals breathing isoflurane for 24 h had a significant increase in terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling positive cells in all assessed brain regions (cortex [$P < 0.001$], putamen [$P = 0.003$], hippocampus [$P < 0.001$], thalamus [$P < 0.001$], and white matter [$P = 0.001$]; fig. 4 and table 3). For both immunostaining methods, the percentage of injured cells in all treatment groups except group (5) was less than 0.1%, representing the normal developmental level in a newborn pig brain.[38]

We compared the number of apoptotic cells in each of the five brain regions within the four different treatment groups, except the positive control group which was not randomized, in a multivariate regression model. The results from all the ten regressions showed that there were no indications of any effect neither of fentanyl, of 24-h ventilation with 50% xenon, nor of sex on apoptosis. There was a slight indication that being cooled as well as being very young increased the number of caspase-3–positive cells, but if corrected for multiple testing only 3 of the 20 regression coefficients for cooling and age were statistically significant at the 5% level.

Figure 5 shows representative pictures of apoptotic cells (hematoxylin and eosin, Cleaved caspase-3, and terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling) from different brain regions and different treatment groups.

Discussion

This study showed that neither breathing 50% xenon combined with iv fentanyl nor fentanyl sedation alone for 24 h

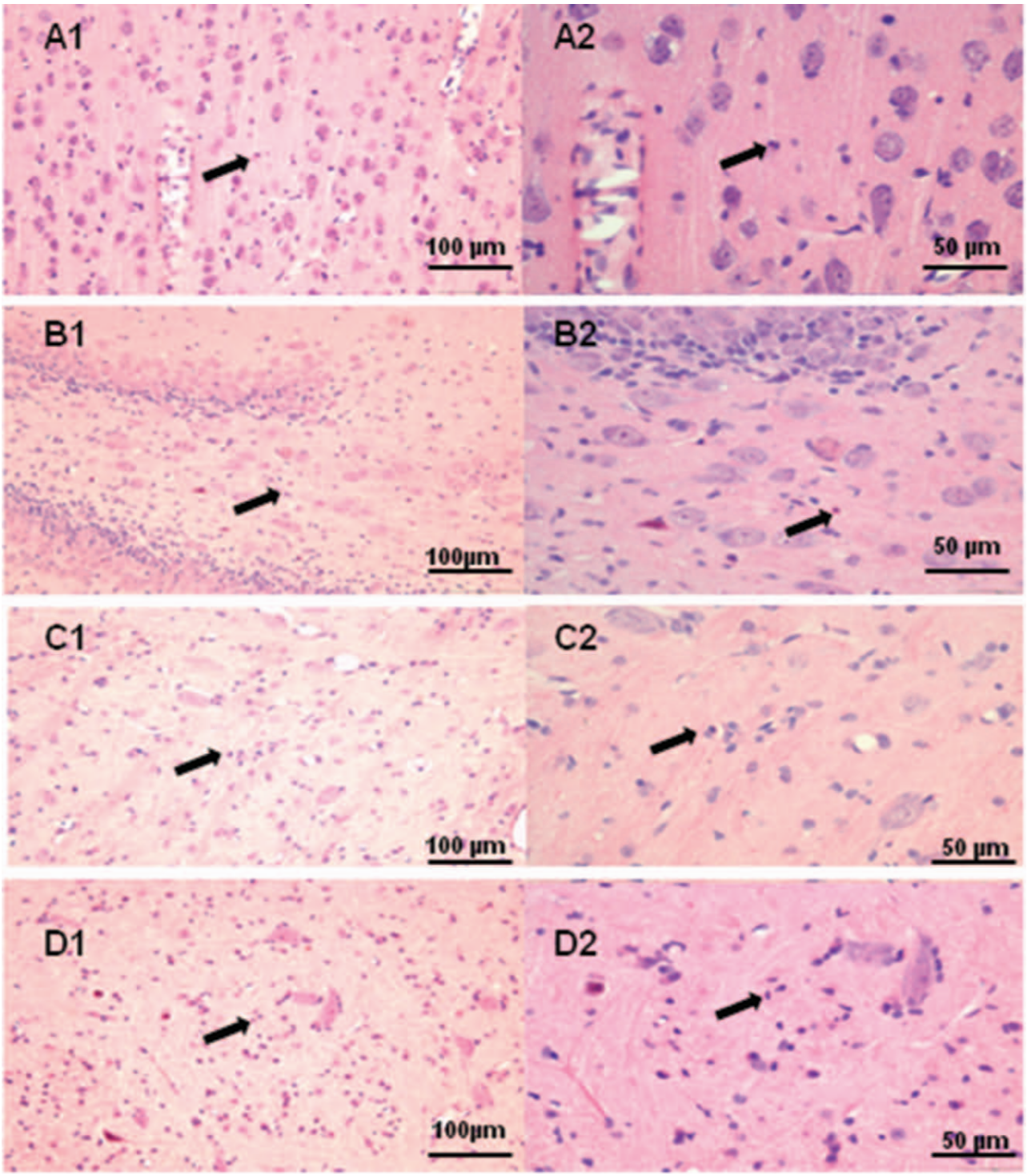

Fig. 5. Photomicrographs showing regional histopathological (*A–D*) and immunohistochemical (*E–F*) features. Hematoxylin and eosin–stained pictures from cerebral cortex (*A1* to *A2*). CA4 area in the hippocampus (*B1* to *B2*), putamen (*C1* to *C2*), and pons (*D1* to *D2*). Cleaved caspase-3–stained pictures (*E1* to *E2*) and TUNEL-stained pictures (*F1* to *F2*) from cerebral cortex. *G1* shows a representative cortex from a juvenile control pig and *G2* a representative cortex of our positive control group. *Arrows* in *A–D* and *G* point to apoptotic neurons with homogenous eosinophilic cytoplasm and pyknotic nuclei. *Arrows* in *E* to *F* point to Cleaved caspase-3 and TUNEL-positive cells. A *scale bar* for each picture is shown in the lower right. TUNEL = terminal deoxynucleotidyl transferase–mediated deoxyuridine-triphosphate nick-end labeling.

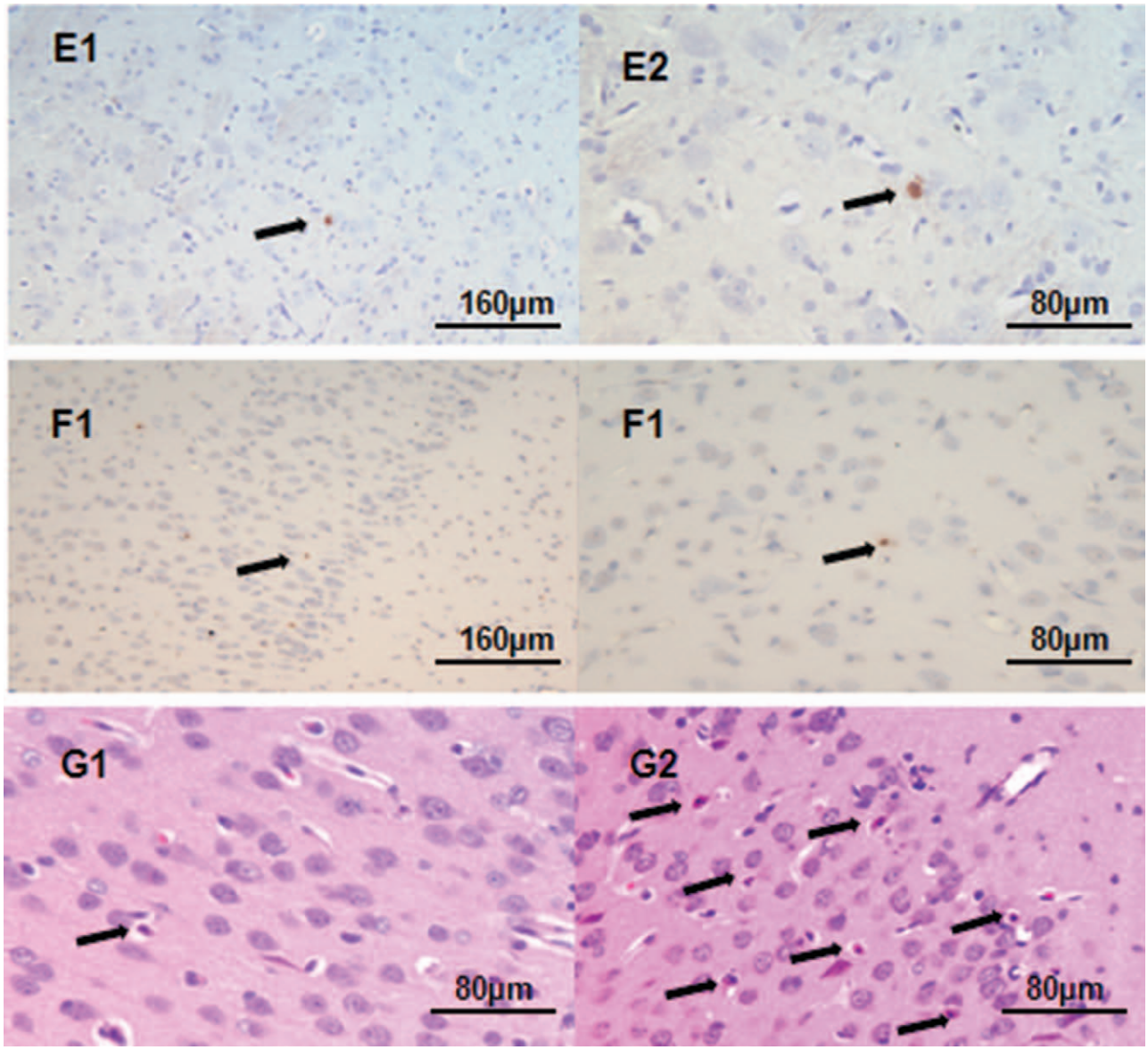

Fig. 5. (Continued)

induces neuroapoptosis in newborn pig brains. In addition, we showed that the newborn pig is a feasible animal model to examine anesthesia-induced neurotoxicity, and also that 24-h ventilation with 2% isoflurane induced a large increase in apoptosis.

The effect of anesthetics on the newborn brain is of major clinical and research interest. The most commonly used anesthetic drugs have been shown to induce apoptosis particularly in the newborn brain.[28] In perinatal hypoxic-ischemic brain injury, cell death by necrosis and apoptosis is predominantly caused by cellular energy failure.[39,40] In contrast, anesthesia-induced neuronal cell death seems to be apoptotic and involves several caspase-dependent pathways.[30] Caspase-3 activation, by the intrinsic mitochondria-dependent pathway or extrinsic by death-receptor activation or neurotrophic factor activation, results in DNA fragmentation and apoptosis.[41] Anesthetic drugs mainly trigger apoptosis by acting as *N*-methyl-D-aspartate receptor antagonists and/or γ-aminobutyric acid receptor agonists. Ikonomidou *et al.*[42] first reported that MK-801 and Ketamine, both are potent *N*-methyl-D-aspartate receptor antagonists, lead to widespread apoptotic neurodegeneration in the developing rat brain. This has also been shown by many other groups in the following years in newborn mammalian models.[6,32] Xenon mainly acts by the inhibition of the *N*-methyl-D-aspartate receptor,[43] and unlike other *N*-methyl-D-aspartate receptor antagonists, xenon does not induce neuroapoptosis, but instead is exceptionally neuroprotective.[21,23] As reported by Ma *et al.*,[22] xenon protects against isoflurane-induced neuroapoptosis

but does not induce neuroapoptosis itself in P7 rat pups. In contrast, Cattano *et al.*[29] reported that xenon has paradoxical properties triggering apoptosis when administered alone, whereas being neuroprotective when administered in combination with isoflurane in P7 mice.

When assessing the neuroapoptotic effect of anesthetics, it is very important that the dosages tested in animal research are comparable with that used in humans. It is also important to use the same additional drugs such as opiates or type of inotropes in the design. In addition, one of the major complicating factors is the variation in brain maturation between animal species.[5] In the past, it has been feasible to examine brain development and synaptogenesis in different animal species.[2,42,44,45] In rodents, brain development occurs mostly postnatally,[2,6,30] whereas in pigs and humans brain development occurs both before and after birth.[6] Rizzi *et al.* assessed whether vulnerability to anesthesia neurotoxicity depends on when synaptogenesis occurs. They found that the most important determinant seems to be the timing of the exposure to anesthesia relative to the time point in synaptogenesis.[6] They stated that the brain regions that are at the peak of synaptogenesis are most vulnerable, but that the precise timing of regional brain development and the exact peak of synaptogenesis for each brain region have not been well established.[6] In our study, we focused on the potential neuroapoptotic effect of xenon on a newborn pig brain, which has been shown to be comparable in maturation with a term newborn brain.[36,46] Compared with rodents, there is not much evidence for anesthesia-induced neurotoxicity in the newborn pig brain. Rizzi *et al.*[6] found a significant increase in caspase-3–positive cells in 5-day-old pigs, after 4 h of breathing 0.55% isoflurane with 75% nitrous oxide. Schubert *et al.*[47] found a significant increase in terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling positive cells in growth-restricted newborn pigs (<24 h) breathing 10 h of 1.6% isoflurane with 70% nitrous oxide. In a previous study, we did not find increased apoptosis after 24 h of iv anesthesia (propofol + fentanyl), in the parietal cortex of the newborn pig.[32] Both propofol and fentanyl have been shown to induce neuroapoptosis in rodents and cell cultures.[48–50] Parietal cortex synaptogenesis peaks at day 5 in newborn pigs. In the previous article,[32] pigs were 24-h old when exposed to iv anesthesia for ~30 h. The delay in examination of cell death as compared with anesthetic exposure may explain why there was no increase in apoptosis. Another potential explanation is that the combination of propofol with fentanyl does not induce apoptosis.

In our current study, we assessed anesthesia-induced neuroapoptosis caused by xenon in different brain regions with different peak times of synaptogenesis and consequently different vulnerabilities to xenon. The pigs also received fentanyl sedation in much higher dosages than in our previous study where the animals received only propofol. This was required to augment the 50% xenon which alone would have not produced sufficient sedation to allow tolerance of a tracheal tube and mechanical ventilation. Therefore, the fentanyl dosages varied within the first three treatment groups because the mechanically ventilated pigs required higher fentanyl dosages than the self-breathing pigs. Hence, this fentanyl dosage is very likely to induce increased apoptosis in rodent brains.[51] High-dose fentanyl alone or in combination with xenon did not increase apoptosis in our pigs. This is in keeping with other authors using similar high dosages of fentanyl.[6] However, we found a large increase in apoptotic cells in pig brains breathing 2% isoflurane for 24 h. We chose 2% isoflurane, as we have previously shown that this is corresponding to 1 minimum alveolar concentration in newborn pigs.[52] Median arterial blood pressure was higher than 40 mmHg in all pigs of all the five groups throughout the 24-h treatment period, providing an adequate cerebral blood flow in newborn pigs.[34–37] We also found that lactate values did not differ among the treatment groups, indicating an adequate organ perfusion.

Our findings validated the newborn pig as a feasible animal model to examine anesthesia-induced neurotoxicity. However, the duration of isoflurane exposure might also play an important role, as pigs in groups (1) to (3) were also exposed to 2% isoflurane during preparation and baseline data recording (≤90 min).

The absolute number of Cleaved caspase-3 and terminal deoxynucleotidyl transferase–mediated dUTP nick-end–labeling positive cells among the treatment groups was very low in individual regions. By counting the whole hemisphere of every brain in all the treatment groups, we were able to quantify the number of Cleaved caspase-3 and terminal deoxynucleotidyl transferase–mediated dUTP nick-end–labeling positive cells in different brain regions. It is important to recognize that apoptotic cell death is a normal and necessary phenomenon in developing mammalian brains.[5] The high maturational number of neurons seen during fetal and early neonatal life is reduced to normal values by apoptosis, leading to the elimination of surplus neurons.[4,53] This phenomenon mostly occurs antenatally. This process establishes the normal structure of the central nervous system, and disruption of the physiological apoptotic cell death mechanism may lead to abnormal brain development.[54] As stated by Istaphanous and Loepke[1], it remains unclear whether anesthetics accelerate apoptosis of cells destined to die by this physiological apoptosis, or whether it induces apoptosis of cells not otherwise destined to die.

We found that the number of apoptotic cells was somewhat increased if the pigs were cooled. This effect of cooling might represent cellular stress induced by hypothermia. As shown before, stress induced by awake hypothermia counteracts with its neuroprotective effect.[55] As the pigs in our study did not have a hypoxic-ischemic insult and were only sedated, not fully anesthetized, it suggests that hypothermia by itself might be stressful, inducing apoptosis.

Xenon is a rare and expensive gas, not often used in clinical practice even though it was introduced in anesthesia more

than 60 yr ago.[56] We have previously published our closed-circuit recycling neonatal xenon delivery system that has been shown to decrease the cost of xenon usage and makes xenon applicable for clinical use even for long periods.[31] We and others have previously shown that xenon is neuroprotective after hypoxic-ischemic injury,[17,20] and we found that xenon adds equal neuroprotection to hypothermia.

These findings have led to the recent clinical investigation of xenon as an add-on treatment for newborns suffering from hypoxic-ischemic encephalopathy being treated with therapeutic hypothermia to reduce brain injury. We have carried out the first human clinical feasibility study and showed that breathing 50% xenon while being cooled is practical without significant side effects in newborns after perinatal asphyxia.††

In our current study, all the treatment groups received additional sedation with fentanyl. This was to reduce stress in the animals, to mimic the clinical scenario of therapeutic hypothermia, and to show any proapoptotic interaction between xenon and a background opioid sedation. Morphine is the first-line opioid used to achieve stress reduction in cooled newborns. However, in our previous studies, we did not achieve an adequate sedative effect with morphine in pigs. For this reason, fentanyl was used in the current and previous studies. The dosage used to achieve a sedative effect in healthy pigs is much higher than that used in newborns, and even with a dose ten times the human dose, we did not see increased apoptosis neither when given alone nor in combination with xenon.

There are some limitations to our study. First, the article reporting the potency of xenon to trigger apoptosis used a xenon concentration of 70% for 4 h in 7-day-old mice.[29] We selected a xenon concentration of 50% administered for 24 h to healthy newborn pigs, as in all our previous neuroprotection work because 50% is the highest clinically practical fraction to administer because it allows the option of moderately increasing the oxygen fraction in those neonates who require this. One could argue that our xenon concentration was too low to increase apoptosis. However, it was applied for 24 h. The 7-day-old mouse is surely more immature than the newborn pig; however, neurogenesis is complete in both species. Second, even though median arterial blood pressure was kept above 40 mmHg during the 24-h treatment period, hypotension might be a confounding factor for increased neuroapoptosis. As our study was performed in healthy newborn pigs and not after hypoxia-ischemia, we found that lactate values did not differ among the treatment groups. However, we did not perform a dose-time-response for each pig at each time point, as this was not feasible. Low blood pressure indicates reduced cerebral blood flow which might result in increased neuroapoptosis.

In conclusion, our study shows that neither breathing 50% xenon combined with iv fentanyl nor high- or low-dose fentanyl alone induce neuroapoptosis when administered for 24 h in healthy newborn pigs. As the combination of xenon and therapeutic hypothermia is feasible in newborns suffering from hypoxic-ischemic encephalopathy and has shown additional neuroprotection in preclinical studies, this study supports the implementation of a clinical neonatal neuroprotection trial and may have implications for the choice of anesthesia in the developing brain.

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References

1. Istaphanous GK, Loepke AW: General anesthetics and the developing brain. Curr Opin Anaesthesiol 2009; 22:368–73
2. Jevtovic-Todorovic V, Hartman RE, Izumi Y, Benshoff ND, Dikranian K, Zorumski CF, Olney JW, Wozniak DF: Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. J Neurosci 2003; 23:876–82
3. Loepke AW, Soriano SG: An assessment of the effects of general anesthetics on developing brain structure and neurocognitive function. Anesth Analg 2008; 106:1681–707
4. Rakic S, Zecevic N: Programmed cell death in the developing human telencephalon. Eur J Neurosci 2000; 12:2721–34
5. Northington FJ, Chavez-Valdez R, Martin LJ: Neuronal cell death in neonatal hypoxia-ischemia. Ann Neurol 2011; 69:743–58
6. Rizzi S, Ori C, Jevtovic-Todorovic V: Timing *versus* duration: Determinants of anesthesia-induced developmental apoptosis in the young mammalian brain. Ann N Y Acad Sci 2010; 1199:43–51
7. Reddy SV: Effect of general anesthetics on the developing brain. J Anaesthesiol Clin Pharmacol 2012; 28:6–10
8. Olney JW, Young C, Wozniak DF, Ikonomidou C, Jevtovic-Todorovic V: Anesthesia-induced developmental neuroapoptosis. Does it happen in humans? Anesthesiology 2004; 101:273–5
9. Todd MM: Anesthetic neurotoxicity: The collision between laboratory neuroscience and clinical medicine. Anesthesiology 2004; 101:272–3
10. Soriano SG, Loepke AW: Let's not throw the baby out with the bath water: Potential neurotoxicity of anesthetic drugs in infants and children. J Neurosurg Anesthesiol 2005; 17: 207–9
11. Stratmann G: Review article: Neurotoxicity of anesthetic drugs in the developing brain. Anesth Analg 2011; 113:1170–9
12. Fatemi A, Wilson MA, Johnston MV: Hypoxic-ischemic encephalopathy in the term infant. Clin Perinatol 2009; 36:835–58, vii
13. Chiang MC, Ashraf QM, Ara J, Mishra OP, Delivoria-Papadopoulos M: Mechanism of caspase-3 activation during hypoxia in the cerebral cortex of newborn piglets. Neurosci Lett 2007; 421:67–71
14. Perlman JM, Wyllie J, Kattwinkel J, Atkins DL, Chameides L, Goldsmith JP, Guinsburg R, Hazinski MF, Morley C, Richmond S, Simon WM, Singhal N, Szyld E, Tamura M, Velaphi S; Neonatal Resuscitation Chapter Collaborators: Part 11: Neonatal resuscitation: 2010 International Consensus on Cardiopulmonary Resuscitation and Emergency Cardiovascular Care Science With Treatment Recommendations. Circulation 2010; 122(16 suppl 2):S516–38

†† Thoresen M, Liu X, Tooley J, Chakkarapani E, Dingley J: First human use of 50% xenon inhalation during hypothermia for neonatal hypoxic-ischemic encephalopathy: The "Coolxenon" Feasibility Study. Platform Presentation, Pediatric Academic Society Meeting 2011, April 30 to May 3, 2011, Denver, Colorado.

15. Azzopardi D, Brocklehurst P, Edwards D, Halliday H, Levene M, Thoresen M, Whitelaw A; TOBY Study Group: The TOBY Study. Whole body hypothermia for the treatment of perinatal asphyxial encephalopathy: A randomised controlled trial. BMC Pediatr 2008; 8:17
16. Gluckman PD, Wyatt JS, Azzopardi D, Ballard R, Edwards AD, Ferriero DM, Polin RA, Robertson CM, Thoresen M, Whitelaw A, Gunn AJ: Selective head cooling with mild systemic hypothermia after neonatal encephalopathy: Multicentre randomised trial. Lancet 2005; 365:663–70
17. Jacobs SE, Morley CJ, Inder TE, Stewart MJ, Smith KR, McNamara PJ, Wright IM, Kirpalani HM, Darlow BA, Doyle LW; Infant Cooling Evaluation Collaboration: Whole-body hypothermia for term and near-term newborns with hypoxic-ischemic encephalopathy: A randomized controlled trial. Arch Pediatr Adolesc Med 2011; 165:692–700
18. Shankaran S, Laptook AR, Ehrenkranz RA, Tyson JE, McDonald SA, Donovan EF, Fanaroff AA, Poole WK, Wright LL, Higgins RD, Finer NN, Carlo WA, Duara S, Oh W, Cotten CM, Stevenson DK, Stoll BJ, Lemons JA, Guillet R, Jobe AH; National Institute of Child Health and Human Development Neonatal Research Network: Whole-body hypothermia for neonates with hypoxic-ischemic encephalopathy. N Engl J Med 2005; 353:1574–84
19. Cilio MR, Ferriero DM: Synergistic neuroprotective therapies with hypothermia. Semin Fetal Neonatal Med 2010; 15:293–8
20. Chakkarapani E, Dingley J, Liu X, Hoque N, Aquilina K, Porter H, Thoresen M: Xenon enhances hypothermic neuroprotection in asphyxiated newborn pigs. Ann Neurol 2010; 68:330–41
21. Hobbs C, Thoresen M, Tucker A, Aquilina K, Chakkarapani E, Dingley J: Xenon and hypothermia combine additively, offering long-term functional and histopathologic neuroprotection after neonatal hypoxia/ischemia. Stroke 2008; 39:1307–13
22. Ma D, Williamson P, Januszewski A, Nogaro MC, Hossain M, Ong LP, Shu Y, Franks NP, Maze M: Xenon mitigates isoflurane-induced neuronal apoptosis in the developing rodent brain. Anesthesiology 2007; 106:746–53
23. Faulkner S, Bainbridge A, Kato T, Chandrasekaran M, Kapetanakis AB, Hristova M, Liu M, Evans S, De Vita E, Kelen D, Sanders RD, Edwards AD, Maze M, Cady EB, Raivich G, Robertson NJ: Xenon augmented hypothermia reduces early lactate/N-acetylaspartate and cell death in perinatal asphyxia. Ann Neurol 2011; 70:133–50
24. Rossaint R, Reyle-Hahn M, Schulte Am Esch J, Scholz J, Scherpereel P, Vallet B, Giunta F, Del Turco M, Erdmann W, Tenbrinck R, Hammerle AF, Nagele P; Xenon Study Group: Multicenter randomized comparison of the efficacy and safety of xenon and isoflurane in patients undergoing elective surgery. Anesthesiology 2003; 98:6–13
25. Franks NP, Dickinson R, de Sousa SL, Hall AC, Lieb WR: How does xenon produce anaesthesia? Nature 1998; 396:324
26. Dinse A, Föhr KJ, Georgieff M, Beyer C, Bulling A, Weigt HU: Xenon reduces glutamate-, AMPA-, and kainate-induced membrane currents in cortical neurones. Br J Anaesth 2005; 94:479–85
27. Fredriksson A, Pontén E, Gordh T, Eriksson P: Neonatal exposure to a combination of N-methyl-D-aspartate and gamma-aminobutyric acid type A receptor anesthetic agents potentiates apoptotic neurodegeneration and persistent behavioral deficits. Anesthesiology 2007; 107:427–36
28. Mellon RD, Simone AF, Rappaport BA: Use of anesthetic agents in neonates and young children. Anesth Analg 2007; 104:509–20
29. Cattano D, Williamson P, Fukui K, Avidan M, Evers AS, Olney JW, Young C: Potential of xenon to induce or to protect against neuroapoptosis in the developing mouse brain. Can J Anaesth 2008; 55:429–36
30. Yon JH, Daniel-Johnson J, Carter LB, Jevtovic-Todorovic V: Anesthesia induces neuronal cell death in the developing rat brain *via* the intrinsic and extrinsic apoptotic pathways. Neuroscience 2005; 135:815–27
31. Chakkarapani E, Thoresen M, Hobbs CE, Aquilina K, Liu X, Dingley J: A closed-circuit neonatal xenon delivery system: A technical and practical neuroprotection feasibility study in newborn pigs. Anesth Analg 2009; 109:451–60
32. Gressens P, Dingley J, Plaisant F, Porter H, Schwendimann L, Verney C, Tooley J, Thoresen M: Analysis of neuronal, glial, endothelial, axonal and apoptotic markers following moderate therapeutic hypothermia and anesthesia in the developing piglet brain. Brain Pathol 2008; 18:10–20
33. Zhou WH, Shao XM, Zhang XD, Chen C, Huang GY: [Effects of hypothermia on cardiac function in neonates with asphyxia]. Zhonghua Er Ke Za Zhi 2003; 41:460–2
34. Tooley JR, Satas S, Porter H, Silver IA, Thoresen M: Head cooling with mild systemic hypothermia in anesthetized piglets is neuroprotective. Ann Neurol 2003; 53:65–72
35. Tooley J, Satas S, Eagle R, Silver IA, Thoresen M: Significant selective head cooling can be maintained long-term after global hypoxia ischemia in newborn piglets. Pediatrics 2002; 109:643–9
36. Haaland K, Løberg EM, Steen PA, Thoresen M: Posthypoxic hypothermia in newborn piglets. Pediatr Res 1997; 41(4 Pt 1):505–12
37. Thoresen M, Haaland K, Løberg EM, Whitelaw A, Apricena F, Hankø E, Steen PA: A piglet survival model of posthypoxic encephalopathy. Pediatr Res 1996; 40:738–48
38. Rice D, Barone S Jr: Critical periods of vulnerability for the developing nervous system: Evidence from humans and animal models. Environ Health Perspect 2000; 108(Suppl 3):511–33
39. Wyatt JS, Edwards AD, Azzopardi D, Reynolds EO: Magnetic resonance and near infrared spectroscopy for investigation of perinatal hypoxic-ischaemic brain injury. Arch Dis Child 1989; 64(7 Spec No):953–63
40. Yager JY, Brucklacher RM, Vannucci RC: Cerebral energy metabolism during hypoxia-ischemia and early recovery in immature rats. Am J Physiol 1992; 262(3 Pt 2):H672–7
41. Johnston MV, Fatemi A, Wilson MA, Northington F: Treatment advances in neonatal neuroprotection and neurointensive care. Lancet Neurol 2011; 10:372–82
42. Ikonomidou C, Bosch F, Miksa M, Bittigau P, Vöckler J, Dikranian K, Tenkova TI, Stefovska V, Turski L, Olney JW: Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. Science 1999; 283:70–4
43. Wilhelm S, Ma D, Maze M, Franks NP: Effects of xenon on *in vitro* and *in vivo* models of neuronal injury. Anesthesiology 2002; 96:1485–91
44. Slikker W Jr, Zou X, Hotchkiss CE, Divine RL, Sadovova N, Twaddle NC, Doerge DR, Scallet AC, Patterson TA, Hanig JP, Paule MG, Wang C: Ketamine-induced neuronal cell death in the perinatal rhesus monkey. Toxicol Sci 2007; 98:145–58
45. Rizzi S, Carter LB, Ori C, Jevtovic-Todorovic V: Clinical anesthesia causes permanent damage to the fetal guinea pig brain. Brain Pathol 2008; 18:198–210
46. Dobbing J, Sands J: Comparative aspects of the brain growth spurt. Early Hum Dev 1979; 3:79–83
47. Schubert H, Eiselt M, Walter B, Fritz H, Brodhun M, Bauer R: Isoflurane/nitrous oxide anesthesia and stress-induced procedures enhance neuroapoptosis in intrauterine growth-restricted piglets. Intensive Care Med 2012; 38:1205–14
48. Al-Jahdari WS, Saito S, Nakano T, Goto F: Propofol induces growth cone collapse and neurite retractions in chick explant culture. Can J Anaesth 2006; 53:1078–85
49. Tu S, Wang X, Yang F, Chen B, Wu S, He W, Yuan X, Zhang H, Chen P, Wei G: Propofol induces neuronal apoptosis in infant rat brain under hypoxic conditions. Brain Res Bull 2011; 86:29–35

50. Kofke WA, Garman RH, Garman R, Rose ME: Opioid neurotoxicity: Fentanyl-induced exacerbation of cerebral ischemia in rats. Brain Res 1999; 818:326–34
51. Kofke WA, Garman RH, Stiller RL, Rose ME, Garman R: Opioid neurotoxicity: Fentanyl dose-response effects in rats. Anesth Analg 1996; 83:1298–306
52. Satas S, Haaland K, Thoresen M, Steen PA: MAC for halothane and isoflurane during normothermia and hypothermia in the newborn piglet. Acta Anaesthesiol Scand 1996; 40:452–6
53. Oppenheim RW: Cell death during development of the nervous system. Annu Rev Neurosci 1991; 14:453–501
54. Kuida K, Zheng TS, Na S, Kuan C, Yang D, Karasuyama H, Rakic P, Flavell RA: Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature 1996; 384:368–72
55. Thoresen M, Satas S, Løberg EM, Whitelaw A, Acolet D, Lindgren C, Penrice J, Robertson N, Haug E, Steen PA: Twenty-four hours of mild hypothermia in unsedated newborn pigs starting after a severe global hypoxic-ischemic insult is not neuroprotective. Pediatr Res 2001; 50:405–11
56. Cullen SC, Gross EG: The anesthetic properties of xenon in animals and human beings, with additional observations on krypton. Science 1951; 113:580–2