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Isoflurane exposure for three hours triggers apoptotic cell death in neonatal macaque brain

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Abstract

Background: Retrospective clinical studies suggest there is a risk for neurodevelopmental impairment following early childhood exposure to anaesthesia. In the developing animal brain, including those of non-human primates (NHPs), anaesthetics induce apoptotic cell death. We previously reported that a 5 h isoflurane (ISO) exposure in infant NHPs increases apoptosis 13-fold compared with control animals. However, the majority of paediatric surgeries requiring anaesthesia are of shorter durations. We examined whether 3 h ISO exposure similarly increases neuroapoptosis in the NHP developing brain.
Methods: Six-day-old NHP infants (*Macaca mulatta*) were exposed to 3 h of a surgical plane of ISO (n=6) or to room air (n=5). Following exposure, NHP brains were screened for neuronal and oligodendrocyte apoptosis using activated caspase-3 immunolabelling and unbiased stereology.
Results: ISO treatment increased apoptosis (neurones + oligodendrocyte) to greater than four times that in the control group [mean density of apoptotic profiles: 57 (SD 22) mm^{-3} *vs* 14 (SD 5.2) mm^{-3}, respectively]. Oligodendrocyte apoptosis was evenly distributed throughout the white matter whereas neuroapoptosis occurred primarily in the cortex (all regions), caudate, putamen and thalamus.
Conclusions: A 3 h exposure to ISO is sufficient to induce widespread neurotoxicity in the developing primate brain. These results are relevant for clinical medicine, as many surgical and diagnostic procedures in children require anaesthesia durations similar to those modelled here. Further research is necessary to identify long-term neurobehavioural consequences of 3 h ISO exposure.

Key words: anaesthesia; apoptosis; growth and development; isoflurane

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Editor's key points

- Previous studies in non-human primates show that isoflurane exposure for 5 h produces widespread apoptotic cell death in neurones and glia.
- The effects of a shorter 3 h exposure, which is more relevant to paediatric anaesthesia, on apoptosis in neonatal macaque brain was analysed.
- The shorter isoflurane exposure also produced widespread apoptosis; further studies are required to test for long-term neurobehavioural sequelae.

Exposure to general anaesthetics can cause structural injury[1–11] and/or long-term neurodevelopmental impairment (NDI)[2 4 10–14] in several animal species, including non-human primates (NHPs).[5–9 12–14] Currently, anaesthesia exposure in NHP infants is known to produce widespread apoptosis of two cell types: neurones and oligodendrocytes (oligos).[5–9 15 16] Loss of either or both cell types might contribute to long-term NDI because the integrity of neurones is dependent on an intact myelin sheath, which is produced exclusively by oligos.

Retrospective clinical studies have found early childhood anaesthesia exposure is associated with an increased risk for NDI with a stronger association following repeated or more prolonged anaesthesia.[17–21] However, whereas similar studies identified increased risk for NDI after a short anaesthetic exposure (1–4 h),[22 23] other such studies produced ambiguous results[24] or observed no increased risk,[25] which was echoed most recently in two publications reporting data from prospective clinical trials.[26 27] Therefore, it is important to determine whether a single, short exposure triggers brain injury in an experimental model that more closely resembles the human condition.

We previously reported significant neuronal and oligodendrocyte apoptosis following 5 h of isoflurane (ISO) in the NHP;[6 15] however, this may have limited clinical relevance because the majority of anaesthetic exposures are of shorter duration. We recently assessed that at Doernbecher Children's Hospital (Oregon Health & Science University, OR, USA), about 30% of infant anaesthetic exposures lasted 3 h or longer[28] (institutional electronic medical records data). At Texas Children's Hospital, about 10% of paediatric patients under 3 years of age undergo anesthesia for more than 3 h.[29] This suggests that several hundred thousand of the 1.5 million American infants who undergo anaesthesia every year[30] are exposed to anaesthesia for periods of 3 h or longer. Accordingly, we designed the present study to determine whether a 3 h exposure to ISO causes apoptotic cell death in the neonatal NHP brain.

Methods

All animal protocols received approval by the Institutional Animal Care and Use Committee of the Oregon National Primate Research Center, Oregon Health & Science University, and were conducted in full accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. All procedures were performed according to the same methods and standards that are employed in a human paediatric surgical setting.

General anaesthesia

Six-day-old infant rhesus macaques received ISO anaesthesia for 3 h (n=6; 2 females and 4 males). ISO was administered as described,[6] at a tightly regulated concentration to maintain a surgical plane of anaesthesia (no movement and not more than 10% increase in heart rate or blood pressure in response to a profound mosquito-clamp pinch at hand and foot; checked every 30 min). During ISO anaesthesia, animals were mechanically ventilated via a tracheal tube (FiO_2=0.30), and their physiological status was extensively monitored and maintained as described.[6] After anaesthetic exposure, animals were recovered for 3 h (n=4) or 5 h (n=2) in an intensive care unit (ICU) system (Snyder ICU cage; Snyder MFG, Centennial, CO, USA), were visually monitored and were fed milk formula as tolerated. At the end of the observation period, animals were immediately euthanized as described below and their brains were prepared for histopathological analysis. Other animals received no anaesthesia and served as controls (n=5; 3 females and 2 males).[6] They underwent a similar procedure, including insertion of an i.v. catheter, physiological measurements and a period of handling to simulate the environment that the other animals experienced prior to induction of anaesthesia. Control animals were then monitored in the ICU cage until final measurements and euthanasia 8 h after time zero.

Histopathological analysis

All subjects were euthanized by methods approved by the American Veterinary Medical Association. In brief, animals were anaesthetized with ketamine, followed by high-dose pentobarbital to induce a deep surgical plane of anaesthesia, and were exsanguinated by incision of the right cardiac atrium. Paraformaldehyde (4% in phosphate buffered saline) fixative was then perfused via the left ventricle to prepare the brain for histopathological analysis. A battery of previously described histological procedures[6–9] was applied to characterize cell death, identify the cell types affected and evaluate the pattern of injury.

Quantitative assessment

For quantitative analysis, coronal 70 μm-thick sections (cut by vibratome) were cut across the entire rostro-caudal extent of the brain. From these sections, in an unbiased manner, sections at 4 mm intervals (approximately 15 sections per brain) were selected for antigen retrieval and immunohistochemical (IHC) staining with antibodies to activated caspase-3 (AC3), a well-established marker for detection of brain cells undergoing apoptosis.[6–9] The control group consisted of stored brain sections used in a prior study[6] that was re-immunolabelled for AC3. To make sure the stored tissue does not lose AC3 antigenicity over time, counts were performed on both these newly stained control sections and previously stained sections from the same animals archived from the prior study. Re-using anaesthesia-naive NHP subjects from a prior study is justified in that it is not ethically defensible to sacrifice additional NHP infants to re-establish that naive animals display low levels of physiological apoptosis.

An experienced neurohistologist who was blinded to the experimental conditions counted all cellular profiles that stained positive for AC3 using a computer-assisted Microbrightfield Stereo-Investigator system (Microbrightfield, Inc., Williston, VT, USA) to record the location and number of dying cells and the quantitative dimensions of the counting field. It was possible to distinguish apoptotic neurones from apoptotic oligos,[6–8] because the AC3 stain displays the full cell body and processes of both cell types, and they are morphologically dissimilar (see Figs. 1, 2 and 3).