OBSTETRICS

Repeated isoflurane exposure and neuroapoptosis in the midgestation fetal sheep brain

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BACKGROUND: Advances in surgery and technology have resulted in increased in-utero procedures. However, the effect of anesthesia on the fetal brain is not fully known. The inhalational anesthetic agent, isoflurane, other gamma amino butyric acid agonists (benzodiazepines, barbiturates, propofol, other inhalation anesthetics), and N-methyl D aspartate antagonists, eg, ketamine, have been shown to induce neuroapoptosis. The ovine model has been used extensively to study maternal-fetal physiologic interactions and to investigate different surgical interventions on the fetus.

OBJECTIVE: The purpose of this study was to determine effects of different doses and duration of isoflurane on neuroapoptosis in midgestation fetal sheep. We hypothesized that repeated anesthetic exposure and high concentrations of isoflurane would result in increased neuroapoptosis.

STUDY DESIGN: Time-dated, pregnant sheep at 70 days gestation (term 145 days) received either isoflurane 2% × 1 hour, 4% × 3 hours, or 2% × 1 hour every other day for 3 exposures (repeated exposure group). Euthanasia occurred following anesthetic exposure and fetal brains were processed. Neuroapoptosis was detected by immunohistochemistry using anticaspase-3 antibodies. Fetuses unexposed to anesthesia served as controls. Another midgestation group with repeated 2% isoflurane exposure was examined at day 130 (long-term group) and neuronal cell density compared to age-matched controls. Representative sections of the brain were analyzed using Aperio Digital imaging (Leica Microsystems Inc, Buffalo Grove, IL). Data, reported by number of neurons per cubic millimeter of brain tissue are presented as means and SEM. Data were analyzed using the Mann-Whitney *U* and Kruskal-Wallis tests as appropriate.

RESULTS: A total of 34 fetuses were studied. There was no significant difference in neuroapoptosis observed in fetuses exposed to 2% isoflurane for 1 hour or 4% isoflurane for 3 hours. Increased neuroapoptosis was observed in the frontal cortex following repeated 2% isoflurane exposure compared to controls ($1.57 \pm 0.22 \times 10^6/mm^3$ vs $1.01 \pm 0.44 \times 10^6/mm^3$, $P = .02$). Fetuses at 70 days gestation with repeated exposure demonstrated decreased frontal cortex neurons at day 130 when compared to age-matched controls ($2.42 \pm 0.3 \times 10^5/mm^3$ vs $7.32 \pm 0.4 \times 10^5/mm^3$, $P = .02$). No significant difference in neuroapoptosis was observed between the repeated exposure group and controls in the hippocampus, cerebellum, or basal ganglia.

CONCLUSION: Repeated isoflurane exposure in midgestation sheep resulted in increased frontal cortex neuroapoptosis. This persisted into late gestation as decreased neuronal cell density. While animal studies should be extrapolated to human beings with caution, our findings suggest that the number of anesthetic/sedative exposures should be considered when contemplating the risks and benefits of fetal intervention as certain fetal therapies may need to be repeated.

Key words: anesthesia, brain injury, fetal, isoflurane, ovine

Introduction

Advances in surgery and technology allow for a variety of fetal interventions despite current concerns about the detrimental effects of anesthesia on the developing brain. These concerns are very pertinent for in-utero procedures. Currently, little is known about the long-term effects of anesthesia on the fetus.[1]

The inhalational anesthetic agent, isoflurane, with other gamma amino butyric acid (GABA) agonists (benzodiazepines, barbiturates, propofol, other inhalation anesthetics) and N-methyl D aspartate antagonists, eg, ketamine, have been shown to induce neuroapoptosis.[2-4] Isoflurane potentiates inhibition through its GABA-mimetic actions[5] and causes caspase-3 activation following action on inositol triphosphate receptors.[6,7] Neuroapoptosis after different anesthetic exposures has been studied in different animal models.[8-10] However, many of these studies have involved small animals, very high anesthetic concentrations, or an anesthetic duration longer than that in routine anesthetic practice.[8-13]

A small animal model study demonstrated that high-dose (3%) isoflurane exposure can cause neuroapoptosis and learning impairment compared to lower isoflurane concentrations (1.3%).[14] Large animal studies in nonhuman primates have also shown evidence of neuroapoptosis in various brain regions, notably the frontal, temporal cortices and hippocampus following postnatal exposure to anesthetics.[13,15,16] Recently, exposure of fetal nonhuman primates to inhaled anesthetics also demonstrated detrimental effects on oligodendrocytes and the cerebellum.[10,13]

The ovine model has been used extensively to study maternal-fetal physiologic interactions and to investigate different surgical interventions on the fetus.[17-20] Prior to now, the effect of anesthesia dose and duration on neuroapoptosis has not been studied in this model. Our study was designed to assess the effect of different doses and duration of isoflurane anesthesia on the development of neuroapoptosis in the large ovine animal model during the midgestation period, a time that correlates with the period human fetuses are

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TABLE 1
Number of fetuses and anesthetic exposure

	Total no. of fetuses
Day 70 control	5
Day 70 2% isoflurane × 1 h	6
Day 70 4% isoflurane × 3 h	6
Day 70 2% isoflurane × 1 h QOD ×3	6
Day 70 2% isoflurane × 1 h QOD ×3 to day 130	6
Day 130 control	5

QOD, every other day.

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exposed to anesthesia for in-utero procedures. We hypothesized that repeated anesthetic exposure and high concentrations of isoflurane would result in increased neuroapoptosis.

Materials and Methods

Animals

Approval from Baylor College of Medicine Institutional Animal Care and Use Committee (AN-6149) was obtained prior to performing this study. Timedated pregnant sheep at 70 days gestation (G70) (term 145 days) were studied. A total of 24 fetuses at G70 were exposed to predetermined doses and duration of isoflurane (2% isoflurane for 1 hour, n = 6; 2% isoflurane × 1 hour every other day [QOD] × 3, n = 6; and 4% isoflurane × 3 hours, n = 6). Concentrations of isoflurane were determined based on doses that induce uterine relaxation, required for fetal intervention.[21,22] Animals were sacrificed and brain tissue harvested immediately following anesthetic exposure. A separate group of G70 fetuses (n = 6) were exposed to 2% isoflurane × 1 hour QOD × 3 episodes with sacrifice and brain tissue harvest 60 days following exposure, ie, at 130 days' gestation (G130). Ten fetuses not exposed to isoflurane served as gestational age-matched controls (G70, n = 5; and G130, n = 5) (Table 1).

Experimental procedures

Pregnant ewes received intramuscular doses of Telazol (Zoetis Inc., Kalamazoo, MI) (0.07 mg/kg) and atropine (2.5 mg/kg) for sedation and antisialorrhea preoperatively. Inhalation induction of ewes occurred with 5% isoflurane in oxygen (for approximately 5 minutes) followed by direct laryngoscopy and endotracheal intubation (size no. 10; Mallinckrodt, Hazelwood, MO). After placement of an orogastric tube to prevent aspiration, mechanical ventilation was commenced and the assigned isoflurane dose was administered. Oxygen saturation and temperature were continuously monitored using tongue or ear and rectal probes, respectively.

The ewe was positioned supine with left lateral tilt to limit aortocaval compression and intravenous access was obtained in the left external jugular vein for infusion of intravenous fluids. A right internal carotid artery cut-down was performed in sterile fashion for continuous invasive blood pressure monitoring and arterial blood gas measurements (Spacelabs Monitor Ultraview SL, Garnerville, NY; and VetScan iSTAT 1 handheld analyzer with CG-8+ cartridges, Abaxis Inc, Union City, CA, respectively) where applicable. Arterial blood gas samples were obtained at catheter insertion and every hour during mechanical ventilation, and utilized to guide ventilator settings. Heart rate, oxygen saturation, end-tidal carbon dioxide, and temperature were recorded every 10 minutes throughout anesthetic exposure. A Bair Hugger blanket (Arizant Healthcare Incorporated, Eden Prairie, MN) was used to maintain normothermia. Upon completion of the assigned anesthetic exposure, ewes were euthanized; necropsy performed on the fetuses and fetal brain tissue was subsequently prepared for histopathological analysis.

The brain tissue of G70 fetuses exposed to either a single low dose of isoflurane (2%), single high dose of isoflurane (4%), or repeated doses of 2% isoflurane QOD for a total of 3 exposures were compared to each other and to age-matched controls. A separate group of G70 fetuses that received repeated doses of 2% isoflurane for 1 hour QOD for a total of 3 exposures were not sacrificed until G130 to determine delayed effects of anesthesia. The brain tissue of this group was compared to G130 control brains.

Anesthetic exposure has been associated with learning and cognitive function deficits.[23-25] The neocortex and archicortex of the brain, prototypes of which are the frontal lobe and hippocampus (dentate gyrus, end plate, and hippocampal pyramidal layer), areas of the brain responsible for short- and long-term memory as well as cognitive learning,[26,27] were therefore the focus of this study. Sections were obtained from similar regions in all animals. In addition, we analyzed the cerebellum due to recent observations of anesthesia-induced neuroapoptosis in the cerebellum.[13] The caudate nucleus was analyzed as a prototype of the diencephalon (basal ganglia and thalamus).

Histopathology

Representative histologic sections from all brain regions were systematically examined. We obtained 5 μm of paraffin-embedded coronal brain sections and processed them for hematoxylin-eosin staining and immunohistochemistry. Slides were screened and analyzed by a neuropathologist (A.M.A) blinded to the treatment groups. We used Aperio Digital imaging (Leica Microsystems Inc, Buffalo Grove, IL) to quantitatively analyze representative brain sections.

Method of detection of neuroapoptosis

Apoptotic neurons were detected by immunohistochemistry using activated caspase-3-specific antibodies (anti-human/mouse Caspase 3 Active, catalog no. AF835; R&D Systems, Minneapolis, MN). In brief, 1:200 dilutions of primary antibody were incubated at 37°C for 1 hour followed by incubation for 24-48 hours. The biotinylated secondary antibody step was performed at room temperature with 30-minutes incubation followed by ABC reagent (catalog no. PK-6100; Vector, Burlingame, CA), DAB

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