A potential role for neurosteroid anesthesia in protection from anesthetic neurotoxicity.

Michael C. Montana, MD, PhD; Brant S. Swiney, BS; Amber Li; Alex S. Evers, MD; Kevin K. Noguchi, PhD

Washington University in St.Louis School of Medicine

Children's Hospital • St. Louis — BJC HealthCare

Washington University in St.Louis Physicians

Abstract

Introduction: General anesthesia in children carries low risk of acute morbidity or mortality. However, data from animals suggest that anesthetics in clinical use may be neurotoxic to the developing brain. Anesthetic neurotoxicity has not been rigorously assessed for older, off-market anesthetics, including neurosteroids. The neurosteroid anesthetic alfaxalalone was the major component of the intravenous anesthetic Althesin, but it was withdrawn from the market due to anaphylactic reactions attributed to its excipient, Cremophor. However, a less toxic excipient, beta-cyclodextrin, has enabled the reformulation of alfaxalone, and it is currently FDA approved as the veterinary anesthetic Alfaxan. Interestingly, neurosteroids inhibit neuronal apoptosis, one of the key steps implicated in anesthetic neurotoxicity. Thus, neurosteroid anesthetics may be non-apoptogenic. The goal of this study is to assess the extent to which the anesthetic neurosteroid alfaxalone causes neuronal apoptosis in mice.

Methods: P7 male and female C57BL/6 mice were injected intraperitoneally with either hydroxypropyl-beta-cyclodextrin, 30 mg/kg alfaxalone (5α-Pregnan-3α-ol-11,20-dione), or 175 mg/kg propofol (2,6-Diisopropylphenol). Both anesthetics were dissolved in cyclodextrin. The dose of alfaxalone and propofol were chosen from preliminary studies performed to identify doses that resulted in loss of righting reflex for approximately 60 minutes. A separate cohort of mice was injected with either normal saline or cyclodextrin to assess any effects of cyclodextrin alone on apoptosis. Stereological counts of the brain were obtained from 75 um midline sagittal sections stained for activated caspase-3 (AC3). Immunostaining was normalized to cyclodextrin treated mice, as background AC3 levels are expected to be non-zero due to the developmental age of the mice.

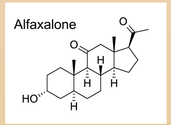
Results: No significant differences were seen in AC3 immunostaining between animals injected with saline vs. cyclodextrin (6 mice per group). A one-way between subject's ANOVA was conducted to compare the effect of the anesthetics on normalized AC3 counts. There was a significant main effects difference [$F(2,28) = 3.815$, $p = 0.0343$]. Post hoc comparisons using Bonferroni's Multiple Comparison Test indicated that the mean normalized score for propofol injected mice (n=12) (M = 2.2, SD = 1.3) was significantly different ($p < 0.05$) than cyclodextrin injected mice (n=7). Alfaxalone injected mice (n=12) (M = 1.6, SD = 0.8) did not differ significantly from cyclodextrin mice. However, contrary to results obtained in preliminary studies, the duration of loss of righting reflex was significantly different between the two experimental groups (propofol 150 +/- 74 minutes vs. alfaxalone 89 +/1 41 minutes, p = 0.02).

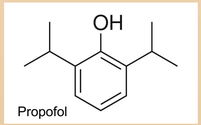
Discussion: These data suggest that a short duration anesthetic with alfaxalone does not significantly increase the degree of neuronal apoptosis seen in neonatal mice when compared to vehicle injected mice. However, experiments to assess the effects of longer duration anesthetics, and repeated administration of alfaxalone need to be performed. The possibility for alfaxalone administered in the neonatal period to alter biologically relevant behaviors in adulthood should also be assessed.

Alfaxalone & Methods

Both alfaxlaone and propofol are poorly water soluble. Alfaxalone is prepared for veterinary use as an intravenous anesthetic and solubilized in 8% 2-Hydroxypropyl-beta-cyclodextrin.

Propofol is also prepared in a commercially available intravenous formulation of an oil-water emulsion of soybean oil, glycerol, and egg lecithin.





When administered intravenously in cats and dogs, both alfaxalone and propofol induce anesthesia in approximately 60 seconds. The duration of anesthesia for a single bolus dose of both drugs is between 10 to 30 minutes (species dependent).

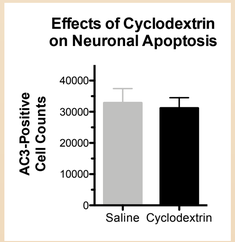
While pharmaceutical grade propofol is prepared in an oil-water emulsion, 2,6-Diisopropylphenol (propofol) will form an injectable suspension when mixed with beta-cyclodextrin. We sought to compare the effects of alfaxalone vs. propofol on neuronal apoptosis when each drug was dissolved in the same excipient (beta-cyclodextrin). Prior to assessing this we first assessed the effects of 8% cyclodextrin (vehicle) on activated caspase 3 staining as compared to saline. The methodology used in this experiment was used for all subsequent experiments.

P7 C57Bl/6 mice were injected i.p. with either saline or cyclodextrin (n= 6 per group). Six hours following injection mice were sacrificed, and perfused with paraformaldehyde. Brains were harvested and 75 micron sagittal sections were cut serially on a vibratome. Immunohistochemistry to assess for neuronal apoptosis was performed for activated caspase-3 (AC3). Whole brain sagittal sections were assessed for AC3 positive neurons using an unbiased stereology approach.

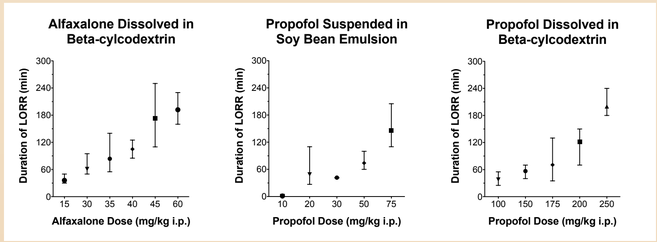
Next we established alfaxalone and propofol dose response curves for loss of righting reflex (LORR). Veterinary grade alfaxalone (Jurox; NSW, Australia) was injected i.p. into P7 C57Bl/6 mice. Propofol dose response curves were established for both a suspension of 2,6-Diisopropylphenol (Sigma; St. Louis, MO) in beta-cyclodex and pharmaceutical grade propofol (Fresenius; Lake Zurich, Il)..

Finally, cylcodextrin, alfaxalone, or propofol was injected i.p. into C57Bl/6 littermate mice. Doses of alfaxalone and propofol were chosen based on those expected to induce LORR for approximately one hour. For all AC3 immunohistochemistry experiments data are presented as mean +/- standard deviation.

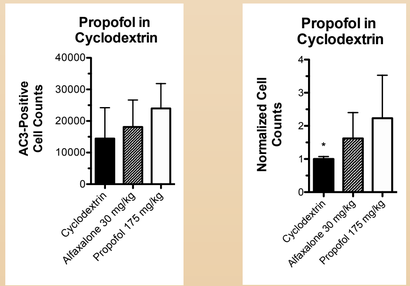
Dose Response Curves & Activated Caspase 3 Staining



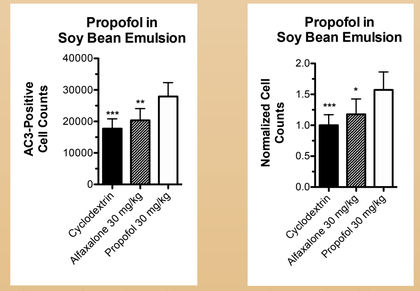
Effects of Saline vs. Cyclodextrin on AC3-Positive Cells. No LORR was observed.



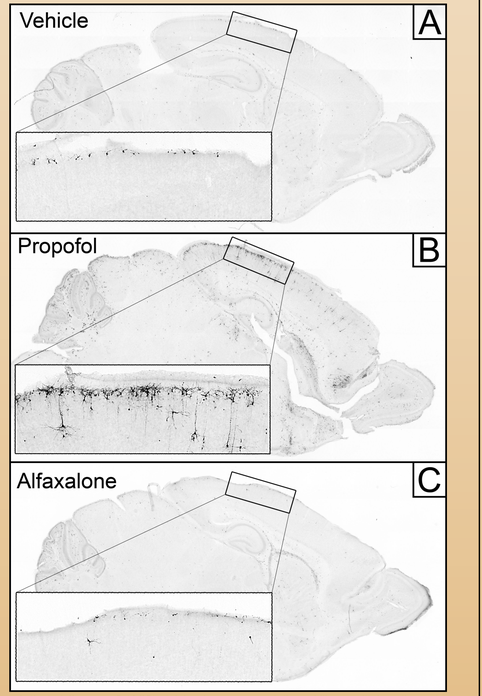
Duration of LORR as a function of dose for 6 different doses of alfaxalone and 5 doses of propofol. 175 mg/kg propofol suspended in Soy-Bean Emulsion was lethal within 20 minutes to 3/3 animals administered that dose. When propofol in soy bean emulsion was used, a lower dose was required to achieve similar duration of LORR as compared to propofol dissolved in cyclodextrin. Data are presented as mean and range. n = 4 – 9 mice per group.



AC3-Positive Cell Counts in mice injected with either cyclodextrin (non-sedating), 30 mg/kg alfaxalone (in cyclodextrin), or 175 mg/kg propofol (in cyclodextrin). No significant differences were seen between groups (left figure). To control for variable baseline apoptosis between litters, AC3 positive cell counts were normalized to the average number of cyclodextrin positive neurons within a given litter (right panel). There were significantly more AC3 positive cells in mice given propofol compared to mice given cyclodextrin.



When propofol was dissolved in soy bean emulsion a significant difference was seen between both cyclodextrin and propofol, and alfaxalone and propofol injected mice. No significant difference was seen between cyclodextrin and alfaxalone injected mice.



Activated Caspase 3 (AC3) staining from representative midline sagittal sections of vehicle (A), propofol in cyclodextrin (B), and alfaxalone (C) treated mice. Magnified areas show cortical staining.

Discussion & Conclusions

- Intraperitoneal Alfaxalone and Propofol Injections Induced LORR in P7 Mice.
- Duration of Propofol Induced LORR was Influenced by both Dose and Excipient.
- Doses of Afaxalone that Result in Approximately 60 Minutes of LORR Do Not Significantly Increase AC3 Staining.
- Doses of Propofol that Results in Approximately 60 Minutes of LORR Significantly Increase AC3 Staining.

Limitations & Future Directions

- These Data are Only Relevant to a Single Short Duration Anesthetic, Which Has Not Been Implicated in Neurotoxicity in Humans.
- Future Studies Will Assess the Effects of Longer Durations and Multi-Day Dosing of Both Propofol and Alfaxalone on AC3 Immunostaining
- Future Studies Will Assess the Effects of These Dosing Regimens on Behaviors Related to Learning and Memory in Adult Mice Administered Alfaxalone and Propofol in the Neonatal Period