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In Utero Exposure to Intralipid Emulsion during Gestation does not lead to Behaviourally Abnormal Juvenile Rats

Ming Xiong, Pratap Nadavaluru, Vincent Reformato, Jing Li and Jean Daniel Eloy*

Rutgers New Jersey Medical School, Newark, NJ, USA

***Corresponding author:** Jean Daniel Eloy

eloyje@njms.rutgers.edu

Rutgers New Jersey Medical School, Newark, NJ, USA.

Tel: 973-972-0470

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Abstract

Increasing evidences suggest that propofol could have adverse effects on the developing brain by triggering apoptotic neurodegeneration, which is noted in rodents and non-human primates. Intralipid emulsion is a vehicle to carry propofol in propofol formulation. This emulsion may produce a significant lipid load and by itself has previously been found to affect NMDA receptor activity. The effect of intralipid itself on the developing brain has not been investigated; however, we designed an experiment to examine whether intralipid itself can cause apoptotic neurodegeneration in the fetus and cause learning and memory impairment in the offspring when administered to pregnant rats. With IACUC approval, 24 pregnant Sprague-Dawley rats were used in this study. Normal saline (control) or 20% intralipid emulsion (the vehicle of propofol) were infused to pregnant rats (gestational day 20) via a tail vein catheter for 1 h. The final volume for saline and intralipid emulsion was around 2.0 ml. Brain tissues of fetal rats were harvested at 6 h later and subjected to Western blot to assess cleaved caspase-3 levels. Separately, another two groups of pregnant rats were allowed to deliver via normal vaginal delivery after infusion. Locomotor activity of the offspring on postnatal day (P28) were monitored using TruScan 2.0, spatial working memory was assessed from P28 using an 8-arm radial maze. The number of total errors, number of correct choices to first error, and time to complete the maze was recorded and analysed using a repeated measure Analysis of Variance (ANOVA), with $P<0.05$ being considered statistically significant.

Keywords: Neurodegeneration; Apoptosis; Intralipid exposure; Propofol; Anesthetics

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Introduction

Nowadays general anesthesia administration prenatally while performing wide surgical procedures is common. The overall incidence of non-obstetric surgeries during pregnancy is 1 in 50 to 1 in 100, with trauma, appendicitis, and cholecystitis being the most frequent causes [1]. The second trimester is the optimal time for elective non obstetric surgeries as the risk of preterm labor is lowest. Neurodegeneration and wide spread apoptosis following exposure to anesthetics has been clearly established in developing animals, and a few studies demonstrate cognitive impairment in adult animals after neonatal anesthetic exposure [2].

New evidences suggest that general anesthetic agents like Propofol, Isoflurane, have the potential to cause neurotoxicity to young developing brains [3-8]. Intralipid is the vehicle that carries propofol in propofol formulation. This emulsion may produce a significant lipid load and by itself has previously been found to affect NMDA receptor activity [9]. In a Miles et al. study, medium chain triglycerides are shown to have significant dose-related central nervous system toxicity in rats [10]. No other study has yet to investigate the effect of intralipid itself on the developing brain.

We designed an experiment to examine whether prenatal intralipid administration can cause apoptotic neurodegeneration in the fetus as well as any impairment of spontaneous locomotor activity, learning and memory deficit in the offspring. To test this hypothesis, a group of pregnant rats on gestational day 20(G20) are infused Intralipid. Apoptotic activity in fetal brain was assessed

by western blot technique. Spontaneous locomotor activity and, long-term memory and learning in P28 pups were assessed by Truscan technology and radial arm maze respectively.

Materials and Methods

Animals

Approval for all experiments was obtained from the IACUC of Rutgers-New Jersey Medical School. Pregnant Sprague-Dawley (SD) rats (Taconi Farms, Germantown, NY, USA) and their offspring were sheltered in standard polypropylene cages in a temperature and humidity regulated room and exposed to a 12 h light-dark cycle with access to standard rat chow and water ad lib. The pregnant SD rats were acclimated to the approved housing facility for 3 days before targeted treatment on gestational day 20 (G20).

Intralipid administration

Pregnant rats were randomly assigned to receive 2 ml of either Intralipid or normal saline intravenous infusion over 1 h duration via tail vein catheter on day 20 of pregnancy. Intralipid 20% intravenous fat emulsion was obtained from Baxter (Baxter Health Corporation, Dearfield, IL, USA). The dams were restrained to facilitate insertion of 24 gauge IV catheter in the lateral tail vein on embryonic day 20 (E20). After successful intravenous access, the catheter was secured in place with HY-Tape (Allegro Medical Supplies Inc., Bolingbrook, IL, USA) and attached to a T-connector extension set (Baxter Healthcare Corporation, Deerfield, IL, USA) for the continuous target control infusion of intralipid or normal saline. To avoid stress to pregnant mother and maintain adequate IV infusion, dams were placed in infusion cages (Harvard Apparatus, Holliston, MA, USA) allowing the rats to move freely. The dams' activity was closely observed to ensure their tolerance to either intralipid or normal saline infusion. Application of soft rodent cervical collars to the dams was performed to prevent biting and removal of the IV catheter during the infusion.

After infusion, pregnant rats were randomly divided into two groups. Group 1 pregnant rat's fetuses were obtained via cesarean section(C/S) at 6 h after intralipid or normal saline infusion for apoptosis study. Group 2 pregnant rats underwent normal spontaneous vaginal delivery (NSVD). Group 2 pregnant rat's offspring were used in performing behavioural and locomotion studies.

Western blot analysis for cleaved caspase-3 in the fetal brain

Western blot analysis was used to measure activated caspae-3 levels in fetal brain tissues obtained from pregnant rats treated with either intralipid or normal saline. Group 1 pregnant rats were C-sectioned under chloral hydrate anesthesia (400 mg/kg, IP) after 6 h post infusion of either intralipid or normal saline. The fetuses were then euthanized with chloral hydrate, and perfused intracardially with normal saline. Two fetal brains from each pregnant rat were collected and stored at -80°C for western blot analysis.

Western blot analysis was also performed on half cerebral hemispheres by using the methods as described in previous studies [11]. Protein concentration was determined by the BCA method with bovine serum albumin as the standard. Briefly, protein samples (50 µg) were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membranes were blocked by non-fat dry milk buffer for 1.5 h and then incubated overnight at 4°C with primary antibody against cleaved caspase-3 (17-19 kDa) resulting from cleavage at aspartate position 175 (1:1,000; cell signaling technology, Danvers, MA, USA). The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and developed with ECL kit. Equal protein loading was confirmed by GAPDH levels, detected by hybridization with 1:2,000 dilution of anti-GAPDH antibody (1:2,000; sigma Aldrich, St. Louis, MO, USA).

The results were expressed as a relative density to GAPDH. The optical densities of bands were quantitatively analysed by using Image J version 1.38 (NIH, Bethesda, MD). We quantified the western blot in two steps. First we used GAPDH levels to normalize (e.g., determining the ratio of cleaved caspase-3 to GAPDH amount) protein levels and control for loading differences in the total protein amount. In the next step, we presented changes in protein levels in intralipid group as percentage of those in the saline control group.

Animal behavioural study: eight-arm radial maze

We used the standard eight-arm radial maze (Med associates Inc., St. Albsan, Vermont, USA) in an isolated dark room between 1:00 pm and 3:00 pm to assess learning and memory of offspring rats on P28. A dustless precision pellet (Bio-Serv, Frenchtown, NJ) was used as reinforcer. To maintain 85% of their baseline weight, all rats were food restricted. The rats gained about 5 grams body weight per week. As described in Liu and Bikely [12] control(n= 7 from pregnant rats treated with saline) and intralipid(n=12 from pregnant rats treated with intralipid) group's offspring rats received seven sessions of habituation (one session per day) on postnatal days 28 to 34 before training in the radial 8-arm maze. During habituation reinforcers are scattered randomly along the arms and central platform. During each session rats were placed onto the central platform and allowed to adapt to the maze for 2 min, then the doors were automatically opened, and the rats were allowed to explore the arms for 10 min. The session ended when 10 min had elapsed. On the last two days of habituation (day 6 and 7), one reinforcer was placed at the end of each arm. After habituation, all rats were tested on the 8-arm radial maze for 5 consecutive days (one session per day). Each testing session had a single reinforcer at the end of each arm. Each testing session ran until (a) all eight arms had been chosen, (b) 5 min had elapsed since the start of the test, or (c) 2 min had elapsed since the rat's last choice [12,13]. The following data were recorded in each session (a) the number of errors(entering a previously visited arm) (b) the number of correct choices prior to first error, and (c) the total time to complete entering all eight arms. The maze was wiped clean with 75% alcohol between rats.

Spontaneous locomotor activity

The P28 offspring rats also were subjected to test spontaneous locomotor activity in a clear Plexiglas chamber (16 × 16 × 16 inches) with an automated infrared activity monitoring system from Coulbourn Instruments (Truscan photo beam Activity Monitors, Whitehall, PA). The animal was placed in the box and allowed to move spontaneously for 5 min. The distance travelled, mean speed, central duration and peripheral zone duration were recorded automatically using TruScan 2.0 software (Coulbourn Instruments, Whitehall, PA) at 1 min intervals for 5 min.

Statistical analysis

Data from eight-arm radial maze was subjected to a two-way repeated measures analysis of variance (RM ANOVA), and was followed by *Tukey's post hoc* analysis when a significant overall main effect was found (P<0.05). One way ANOVA was used in obtaining spontaneous locomotor activity and Western blot results. Statistical significance was declared at P<0.05.

Results

Cleaved caspase-3 levels in fetal rats

Cleaved caspase-3 and GAPDH immunoblotting showed that intralipid group induced visible bands were same when compared to control group. Quantification of the western blot showed that the intralipid treatment slightly decreased cleaved caspase-3 levels in brain tissues of fetal rats as compared with the control condition **(Figure 1)**, but there was no significant statistical difference. Considered together, these results suggested that Intralipid infused to pregnant rats did not affect the cleaved caspase-3 levels in the brain tissues of fetal rats.

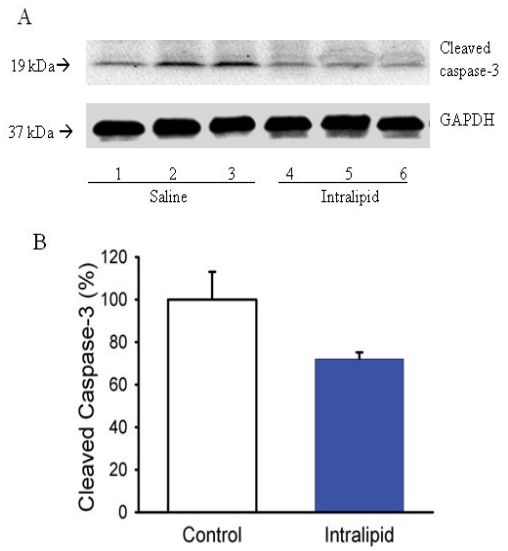

Figure 1 Prenatal intralipid exposure did not seem to affect the levels of cleaved caspase-3 in the brain tissues of fetal rats. Intralipid or saline control (IV infusion, 1 h) was administered to pregnant rats on gestational day 20. Fetus was removed via C-section at 6 h post infusion. Whole cerebral hemispheres of fetal rats were harvested for Western blot. (A) Representative bands of cleaved caspase-3 by western blot analysis. (B) Quantification of the Western blot showed that there was no statistically significant difference in the amounts of cleaved caspase-3 in the rat's brain tissues between the intralipid treatment and control condition (*P*=0.10). Data from six offspring per group are expressed as means ± S.E.M.

Eight-arm radial maze

The radial eight-arm maze task is used to measure specific aspects of spatial working and reference memory in rodents [12,13]. It assesses the ability of animals to develop an optimal strategy for exploring their environment and obtaining food with minimal effort. Juvenile rats (P28) from both control and intralipid groups were selected randomly for the radial eight-arm maze task.

Juvenile rats exposed to intralipid *in utero* (n=12) were no different than age-matched controls (n=7) in either number of errors made, total errors, time to complete the maze task or correct choices prior to first error in radial arm maze test **(Figure 2)**. Data were analysed with repeated-measures ANOVA, with intralipid group as a between-subject factor and day of testing as the within subject factor. Data are expressed as mean ± S.E.M. P values.

In summary, juvenile rats exposed prenatally to intralipid emulsion were not different than controls on radial arm maze performance, suggesting intralipid emulsion administered to pregnant animals produces no lasting impairment in spatial working memory in offspring rats.

Spontaneous locomotor activity

There were no differences in either the distance travelled, central duration, peripheral zone duration or the mean speed between offspring rats exposed to intralipid (n=19 pups) *in utero* and age-matched controls (n=14 pups) **(Figure 3)**. Data are expressed as mean ± S.E.M. In summary, the above results suggested that locomotor activity was not affected in offspring rats by prenatal intravenous intralipid administration.

Discussion

In this study, intravenous intralipid was infused to pregnant (G20) rats to ascertain intralipid effects on fetal brain and subsequently any alterations in the neurological behaviour of offspring rats (P28). Our results suggest that prenatal intravenous infusion may not affect (a) neuronal apoptosis in fetal brain, (b) spontaneous locomotor activity in offspring (P28) and, (c) learning and memory in offspring rats (P28).

In anaesthesiology, some of the significant considerations of intralipid are (A) a vehicle to carry propofol in propofol emulsion and (B) an antidote of Local anesthetic toxicity [14,15]. Propofol is a common intravenous anesthetic agent used in clinical practice. A number of studies suggest that propofol, may cause neurotoxicity to young developing brains [3-8]. Daily propofol intraperitoneal (IP) injection in 7 day old rats induced a wide spread apoptosis with significantly increased caspase-3 activation and behavioural

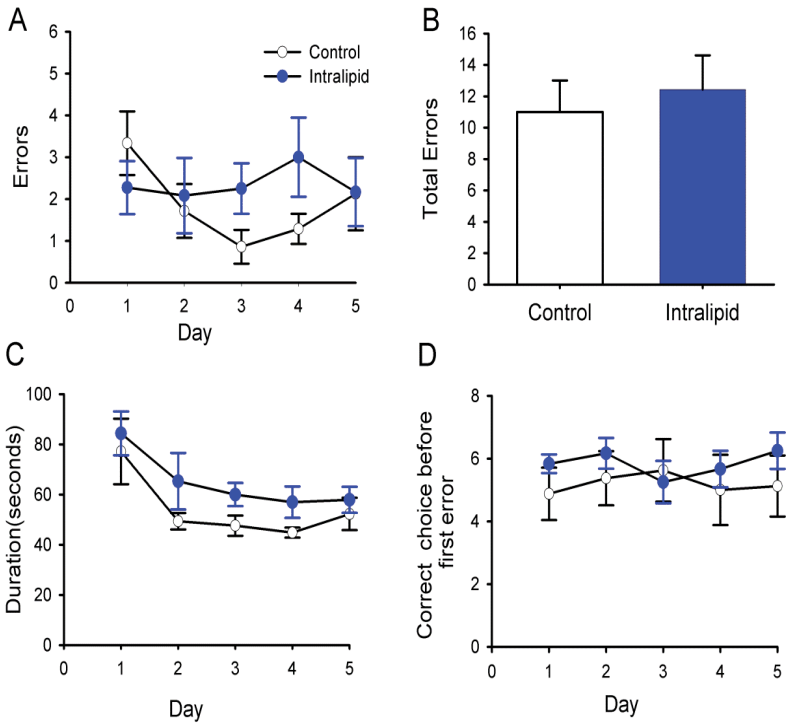

Figure 2 The offspring rat's performance on the radial arm maze. Juvenile rats exposed to intralipid *in utero* (n=12) were no different than age-matched controls (n=7) in either number of errors made (A), total errors (B), time to complete the maze task (C) or correct choices prior to first error (D) in radial maze test. Data were analyzed with repeated-measures ANOVA, with intralipid group as a between-subject factor and day of testing as the within subject factor. Data are expressed as mean ± S.E.M.

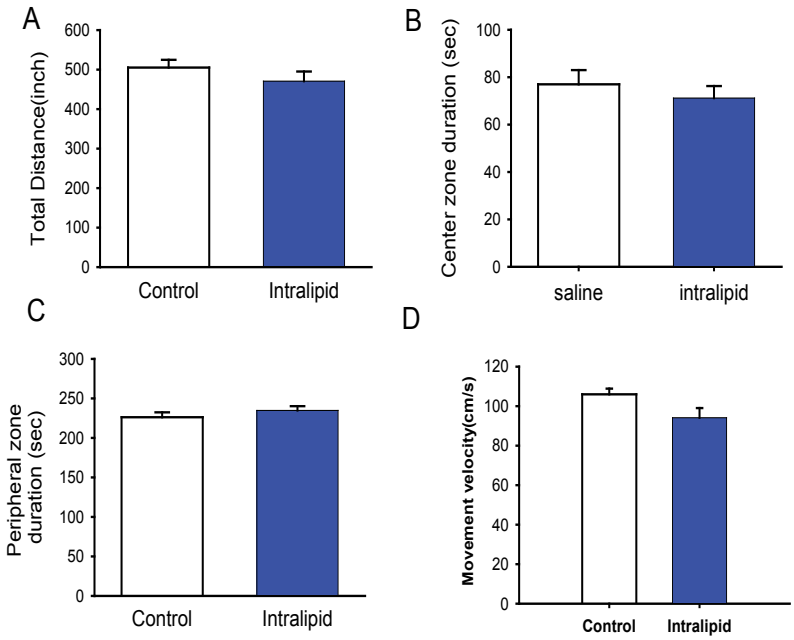

Figure 3 Spontaneous locomotor activity of the offspring rats on P28. There were no differences in in either total travelled distance (A), center zone duration (B), peripheral zone duration (C) or movement velocity (D) between juvenile rats exposed to intralipid *in utero* (n=19) and age-matched controls (n=14). Data are expressed as mean ± S.E.M.

changes such as reduced learning capacity were detected in 1 month old propofol exposed new-born rats [6]. Furthermore, prenatal propofol exposure in a rhesus monkey model induced wide spread apoptosis in the fetal brains [8]. In addition, propofol even at sub-anesthetic doses causes widespread neuroapoptosis in the neonate and leads to persistent decline in the dendritic growth of cultured GABA neurons [5,16]. In contrast, two studies demonstrate that propofol may be neuroprotective to cultured hippocampal neurons from fetal or post natal day 2 (P2) rats [17,18]. Intralipid is an essential component of propofol emulsion (Diprivan) being a vehicle to carry the propofol, but no study has investigated the effects of intralipid itself on the fetal brain. In Weigt et al. study lipid itself effects NMDA receptor activity [9]. In another study, Miles JM et al. suggested that medium chain triglycerides may cause neurotoxicity in dogs [10]. It is a known fact in current clinical practice that the use of intralipid is an effective antidote to Local anesthetics like mepivacaine, prilocaine, levobupivacaine induced neuro and cardiac toxicity [14,15]. Intra-cerebroventricular intralipid application reduced the extracellular glutamate accumulation during the peri-ischemic period indicating neuroprotective effects [19]. Considering the possible action of intralipid on brain from above studies, we uniquely designed our current study by intravenously administering intralipid prenatally to rats to demonstrate any neurological effects of intralipid in offspring rats. In our study, intralipid treatment slightly decreased cleaved caspase-3 levels in the brain tissues of fetal rats as compared with control condition. Even though there was no significant statistical difference, intralipid induced decreased levels of cleaved caspase-3 can't be underestimated.

The dose of intralipid we used was about 2 ml over 1 h duration (40 µg/kg/min). According to a study on rats, the estimated LD 50 within 48 h after a rapid, high volume intravenous infusion of a 20% soy-based oil emulsion in rats was 67.7 ± 10.7 ml/kg [20]. There were no reported deaths of either pregnant or their offspring rats post intralipid infusion.

The limitations of our present study were:

1. We didn't measure the vital signs as it was not feasible to apply available monitors to the rats but we did close monitoring of rats during infusion by observer. The rats were tolerant of 1 h infusion of both normal saline and intralipid with minimal abnormal behaviour.
2. Unable to measure intralipid blood levels.
3. No receptor level study was done.
4. Focused only on intralipid not on other lipid emulsions.

Conclusion

Intralipid emulsion administered to pregnant animals does not seem to cause apoptosis in the fetus or persistent learning impairment in the juvenile offspring rats. Further animal studies has to be done to study intralipid as a potential neuroprotective agent in reversing neurotoxicity to fetal brain induced by prenatal administration of general anesthetic agents.

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