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Fetally-injected drugs for immobilization and analgesia do not modify fetal brain development in a rabbit model

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Bulleted statements:

A. What's already known about this topic?

- During fetal surgery, fetuses receive medication (atropine-fentanyl-curare) to prevent fetal pain, movement and bradycardia.
- Maternal anesthesia can cause impaired brain development
- There are no reports on neurodevelopmental effects of direct fetal anesthesia

B. What does this study add?

- This is the first study investigating the effects of fetal medication
- Many centers still rely on the transplacental transfer of maternal anesthesia to achieve fetal analgesia. Based on these findings, a direct fetal injection with medication would be preferred.

Ethics approval: These experiments were approved on 18/04/2018 by the Ethics Committee for Animal Experimentation of the Faculty of Medicine of the Katholieke Universiteit Leuven (LA1210191) (P078/2017).

Data sharing: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Keywords: Fetal anesthesia, fetal analgesia, fetal immobilization, fetal surgery, brain development, neurobehavior, rabbit

Abstract

Objective: During fetal surgery, fetuses receive medication (atropine-fentanyl-curare) to prevent fetal pain, movement and bradycardia. Although essential there has been no detailed review of potential side effects. Herein we aimed to assess the effects of this medication cocktail on fetal brain development in a rabbit model.

Methods: Pregnant does underwent laparotomy at 28d of gestation. Two pups of each horn were randomized to an ultrasound guided injection with medication (atropine-cisatracurium-fentanyl, as clinically used) or saline (sham). The third pup was used as control. At term, does were delivered by cesarean. Outcome measures were neonatal biometry, neuromotoric functioning and neuro-histology (neuron density, synaptic density and proliferation).

Results: Maternal vital parameters remained stable during surgery. Fetal heart rates did not differ before and after injection, and were comparable for the three groups. At birth, neonatal body weights and brain-to-body weight ratios were also comparable. Both motor and sensory neurobehavioral scores were comparable. There were no differences in neuron density or proliferation. Sham pups, had a lower synaptic density in the hippocampus as compared to the medication group, however there was no difference in the other brain areas.

Conclusion: In the rabbit model, fetal medication does not appear to lead to short-term neurocognitive effects.

Introduction

Fetal surgery has rapidly progressed during the last decades, both in terms of indications for interventions as well as advances in operative techniques. Selected fetuses now benefit from prenatal surgery with improved short- and long-term outcomes ¹⁻³. The time point in gestation for these procedures to be performed, depends on the indication, though typically gestational age (GA) ranges from 16 weeks to the time point at which ex utero management is preferred.

It remains uncertain and debated from what gestational age and to which degree a fetus is capable of experiencing pain. Therefore it is conservative to presume that fetuses are capable of experiencing pain from the second half of pregnancy^{4,5}. Moreover, neonatal repetitive noxious stimuli alter normal neurodevelopment in rat pups ⁶. Therefore, providing adequate fetal analgesia is important ⁷. Fetal analgesia is frequently combined with a muscle relaxant to prevent sudden fetal movements which could be harmful during the procedure. To counteract possible bradycardia caused by fetal pain or during fetal laryngoscopy, some centers add atropine to the fetal medication. Therefore, fetuses are being administered a combination of fentanyl, a muscle relaxant and atropine either by intravenous (IV) (umbilical or hepatic vein) or intramuscular (IM) injection.

Multiple animal studies demonstrated that lengthy or repetitive use of maternal anesthesia can cause impaired brain development ⁸. These observations led to an FDA safety communication for the use of general anesthetics in pregnant women ⁹. We previously demonstrated in the rabbit model that a single two-hour maternal surgery caused transient negative neurobehavior and brain histopathological changes in the fetus ¹⁰. The use of maternal anesthetic agents in that study was associated with a lower neuron density, lower synaptic density and decreased the proliferation rates in multiple brain regions. The pups of exposed does had a worse neurobehavioral outcome at birth. However unlike maternal anesthetic medication given during surgery, which has been well investigated, the neurodevelopmental effects of drugs directly administered to the fetus have not yet been investigated. Due to the many confounding factors and because of ethical reasons, it is (nearly) impossible to investigate the effect of fetal medication in humans. Therefore we translated this to our rabbit

neurodevelopment model and herein we aimed to measure the effect of fetal medication during surgery under general anesthesia.

We opted for the rabbit model because similar to humans, the rabbit is a perinatal brain developer ¹¹ and many evaluations have been standardized to investigate perinatal insults ¹⁰.

Material and Methods

Time-mated rabbits (hybrid of Vlaamse Reus and New-Zealand White) were housed in individual cages at 21°C, 42% humidity with a 12h day-night cycle and free access to water and food. Animals were treated according to current guidelines for animal well-being, and experiments were approved by the Ethics Committee for Animal Experimentation of the Faculty of Medicine (P078/2017).

Anesthetic and surgical procedures (Figure 1)

At gestational day 28, pregnant does were anesthetized with sevoflurane as previously described¹⁰.

A median laparotomy (5cm) was made to partially expose the uterus and allow micro-ultrasound Doppler (Vevo 2100 Imaging System, Fujifilm VisualSonics Inc., Toronto, ON, Canada). With minimal manipulation, the two ovarian-end pups of each horn were randomized to receive an ultrasound guided intrahepatic injection either with 0.1mL fetal medication or 0.1mL saline (sham). The third ovarian end pups from each horn were used as controls. Fetal medication was standardized to fentanyl 0.2µg, cisatracurium 10µg and atropine 0.2µg. This was based on clinical equivalent concentrations (fentanyl 12µg/kg, cisatracurium 0.4 mg/kg and atropine 13 µg/kg) and available average weights of pups of similar gestational age from a previous study¹²). The fetal heart rate was measured with ultrasound before and 10 minutes after injection. Maternal anesthesia was standardized to 1h of sevoflurane maintenance.

Once awake, animals were returned to their cage for recovery. During the remainder of the pregnancy, rabbits were daily monitored using the Rabbit Grimace Scale. With this scale rabbits are observed for a couple of minutes and five action units are scored: orbital tightening, cheek flattening, nostril shape, whisker change & position and ear shape & position¹³. Each item is scored as either 0 (not present), 1 (moderately present) or 2 (obviously present).

Delivery

Caesarean was performed at postconceptional day 31 (term). After sedation with IM ketamine (15 mg/kg, ketamine Nimatek; Eurovet Animal Health BV, Bladel, The Netherlands) and medetomidine (25 mg/kg, Domintor, Orion Pharma, Aartselaar, Belgium), the doe was placed in the supine position, and

2% lidocaine local anesthesia was injected to allow median laparotomy. The bicornuate uterus was exposed and all pups were extracted through hysterotomy. Following delivery, the doe was euthanized with a mixture of 200 mg embutramide, 50 mg mebezonium, and 5 mg tetracain hydrochloride (IV bolus of 1 mL T61; Intervet International BV, Boxmeer, The Netherlands).

After birth the pups were dried, stimulated and kept in an incubator (TLC-50 Advance, Brinsea Products, Weston Super Mare, UK) set on 32°C and 55% humidity. Litters were separately housed in plastic boxes (20 x 25 cm) and pups were numbered according to their position in utero. Pups were gavage fed twice daily with Milk Replacer (FoxValley 30/50, Lakemoor Illinois, US) supplemented with probiotics and colostrum (Col-o-cat, Sanobest, 's Hertogenbosch, Netherlands) at 150mL/kg/feeding. At each feeding, the bladder was emptied by genital stimulation.

Neurobehavioral assessments

On postnatal day one, all pups underwent neurobehavioral assessment, as used in previous studies^{10,12}. All tests were performed out of the incubator, and for each pup individually. For neuromotor evaluation, an open field test was performed on a designated 30 x 30 cm area divided in quadrants over a time period of 90 seconds. Each animal was left free to ambulate. The following items were scored: locomotion; motor activity of head, fore and hind limbs; limb tone. The sum of these scores represented the total motor score, that was calculated out of a maximum of 26 points. Sensory testing included: soft sensation, sucking and swallowing, head turning, surface righting reflex, odor aversion and pain sensation. The maximum sensory score attainable was 20 points.

All assessments (performed by blinded investigators) were recorded and videos were scored *a posteriori* by two observers blinded to group assignment.

Neuropathological assessments

After neurobehavioral testing rabbits were anesthetized with IM ketamine (35 mg/kg) and xylazin (6 mg/kg) and were perfused transcardially with 0.9% saline and heparin (100IU/mL) followed by perfusion fixation with 4% formaldehyde. The brain was removed to be immersion fixed in 4%

formaldehyde. Whole brain volumes, including cerebellum and brainstem, were determined using the fluid displacement method. After 72 hours, brains were paraffin embedded and subsequently serially sectioned at 4µm from anterior to posterior, starting from the prefrontal cortex. Sets of 3 coronal sections were taken every 100 µm, at two levels for further staining and analysis. The first level started at the start of the medial septal nucleus and the second level at the beginning of the hippocampal formation. Primary antibodies used in these experiments included: mouse monoclonal anti-NeuN antibody (MAB377, Millipore, Billerica, MA, USA); mouse monoclonal anti-human Ki67 (M724001-2; Agilent, Diegem, Belgium); mouse anti-synaptophysin monoclonal antibody (Sy38, ab8049; Abcam, Cambridge, UK). The secondary antibody was Alexa Fluor® 488 goat anti-mouse conjugate (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Sections were counterstained with Hoechst 33342 (Sigma-Aldrich, Bornem, Belgium).

Imaging Acquisition and Quantification

Histological slides were digitized using the Zeiss AxioScan Z1 imaging platform (AxioScan® Slide Scanner, Carl Zeiss MicroImaging GmbH, Munich, Germany), using a 20× Plan Apochromat objective coupled to a 3 Chip CCD Camera (Hamamatsu Photonics, Japan). All focusing and field of view assembly was handled by Carl Zeiss Zen software (Carl Zeiss MicroImaging GmbH, Munich, Germany), which is integrated with the AxioScan device.

For neuronal densities, regions of interest (ROI) were selected on three consecutive slides. In each ROI, five different 100x100µm squares were selected at low magnification so individual cells were not visible to avoid bias. In these squares individual neurons were manually counted.

For quantification of the Ki-67 positive cells quantification profiles on the digitized whole slide images were done using QuPath software ¹⁴ by utilizing the fast cell counting and positive cell detection functions. Herein the whole region of interest was annotated, quantification was done, and positive cell counts were expressed as percentage of positive cells per total cells detected.

For the mean fluorescence intensity, quantification of the synaptophysin stain (Sy38) open source Fiji software (ImageJ) (<http://fiji.sc/Fiji>) ¹⁵ was used.

Statistics

The study was powered to detect a 15% difference in neuron density (based on findings in a previous experiment on maternal surgery during pregnancy). Data was analyzed using R (Foundation for Statistical Computing, Vienna, Austria). Data distribution was checked and confirmed for normality by the Shapiro Wilk test and normality plots. Comparisons of the outcomes between the treatment groups was carried out with Analysis of Variance (ANOVA) assuming equal variances. We used linear mixed models to account for the potential clustering effect in our data. Post hoc comparisons were done with Students t-test with Bonferroni-correction for multiple testing. A p-value <0.05 was considered significant. All data are presented as means with standard deviation and individual data points are plotted.

Results

Eight pregnant does underwent surgery and had live born pups. During the first surgery, maternal vital signs remained stable and were within the normal range except for two rabbits that had hypotension throughout surgery¹⁶. Immediately after intubation, the does had a slightly lower pH (7.32 ± 0.08 ; $p=0.02$) compared to normal, however, pCO_2 and lactate levels remained within their normal range. At the end of surgery, pH values were comparable and within the normal range (7.40 ± 0.04 ; $p=0.6$) (Table 1).

Fetal heart rates of the three groups were comparable before injection (192 ± 47 control; 189 ± 40 sham; 182 ± 49 medication; $p=0.4$). Following injection, the average heart rate did not change, both in the sham group ($p=0.5$) as in medication group ($p=0.2$) (Supplement Figure 1). Also there were no differences between the three groups at the second time point.

Survival till birth was comparable (79% control; 80% sham; 63% medication $p=0.5$). Therefore, we eventually harvested 10 control, 12 sham and 9 medicated pups, with comparable body, brain weights and brain-to-body weight ratios (Supplement Figure 1).

The total motor scores ($p=0.4$) and total sensory scores ($p=0.2$) were not different, even not for any of the sub-scores (data not shown) (Figure 2).

Neuron densities were comparable for all studied brain regions (Figure 3). There were neither any differences in proliferation rates (Supplement Figure 2). There was a slightly higher synaptic density in the hippocampus of animals injected with medication as compared to the two other groups (1641 ± 437 control; 1695 ± 330 sham; 2324 ± 314 medication; $p=0.004$). In the other regions no differences could be detected between the groups (Figure 4).

Discussion

This is to our knowledge the first study exploring the effects of widely used fetal injected atropine-fentanyl-curare on fetal brain development. Whereas in a previous experiment in the same model, we demonstrated measurable neonatal, yet transient functional and morphological neurologic changes following general anesthesia, herein, no effect of direct fetal injected fentanyl, cisatracurium and atropine was demonstrated. There was only a higher synaptic density in the hippocampus of pups injected with the fetal medication cocktail, without measurable functional impact in the early neonatal period.

To study the effects of the fetal medication cocktail, we resorted to the rabbit model. The rabbit model is a very suitable model to investigate the impact of potentially noxious events on fetal brain development. Rabbits are so-called perinatal brain developers, which means that their brain mainly develops in the third trimester and neonatal period, as is the case in humans¹¹. We injected fetal medication on gestational day 28 which, in terms of brain development, corresponds to gestational week 24-28 in humans, the period when fetal surgery is typically performed¹⁷. The early third trimester also marks the beginning of the brain growth spurt both in rabbits as well as humans, which makes the brain most sensitive to external disruptive factors^{18,19}.

The mix we used consisted of fentanyl, cisatracurium and atropine in *clinically* equivalent doses²⁰. Atropine is used by several fetal surgery centers to prevent fetal bradycardia which can be triggered by fetal pain or e.g. by laryngoscopy during Fetoscopic Endoluminal Tracheal Occlusion (FETO) procedures^{21,22}. Whether the use of atropine is essential is unknown. To our knowledge there is nothing reported on the use and postnatal effects of atropine administered to fetuses. Also, in this model, it is uncertain whether one can properly study the fetal effects, as up to 70% of New Zealand white rabbits have an esterase which immediately degrades atropine, before it has the time to exert any effects²³. This may not be impossible, as we did not observe the (expected) increase in fetal heart rate. We could not demonstrate differences between control fetuses or fetuses injected with saline or with fetal medication in terms of growth, neurobehavioral assessment or in neuron density.

There is no literature on the brain effects of opioids directly given to the fetus. Fisk et al. demonstrated that fentanyl attenuates fetal stress during needling procedures but they did not investigate brain changes²⁴. Maternally administered opioids and its metabolites cross the placenta, hence exert effects on the fetus. Both clinical and preclinical studies demonstrated that *sustained* maternal opioids administration adversely impacts fetal brain development, leading to lower neuroproliferation rate, lower neuron density and functional impairment²⁵⁻²⁸. Whereas these studies investigated chronic maternal opioid administration, single maternal exposure studies are scarce. Rizzi et al. administered a single dose of fentanyl to pregnant guinea pigs for anesthetic purposes and did not observe any effect. Though the guinea pig may not be the ideal model to study perinatal brain development, those observations are in line with ours, i.e. no difference in neuron density following a single administration^{11,29}.

The effect of cistracurium or other curariform products on the developing brain is also poorly studied. Fodale et al. suggested a potential neuroprotective effect of *laudanoline* on the fetal brain based on preclinical data in rats and in vitro studies³⁰. Laudanosine is a metabolite of cisatracurium and a nicotine acetylcholine subtype $\alpha 4\beta 2$ receptor agonist³¹. Activation of this receptor has been shown to provide neuroprotection against NMDA (N-methyl-D-aspartate) excitotoxicity in a neonatal rat model³². In our previous experiment we hypothesized that excitotoxicity was a potential mechanism underlying adverse neurologic effects. Therefore the protective effect of laudanoline against the excitotoxicity of general anesthesia could explain the higher synaptic density measured in the hippocampus. However in that case we would also expect to see a higher proliferation rate and higher neuron density, yet we only observed a higher synaptic density in the hippocampus. Therefore it is also possible that this was an incidental finding.

This study has some limitations. First this experiment includes a maternal laparotomy under general anesthesia, which is not always the case in fetal surgical procedures. To control for this, we used internal controls exposed to the same drugs. Secondly, we administered fetal medication in the liver whereas in humans, it is given either IM or IV. In rabbits, due to the small size of the veins, it is in our

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hands impossible to administer fetal medication IV. Also an IM injection is difficult to control due to the small limb size and the fetal movements. When medication was given intrahepatically, ultrasound imaging enabled us to keep the injected fluid on screen until the uptake was completed. This was in all cases accomplished within 30 sec. Thirdly, since we did not see an effect on day 1, we did not perform long term follow up of the rabbits, which does not exclude long term effects. Lastly, although the rabbit is a suitable model for perinatal insults, the rabbit brain is far less complex than the human brain. In addition, there are differences in equipotency of drugs between adults and children and between humans and rabbits. Although we used a clinical equivalent dose of medication we cannot be 100% certain that this has the same potency in rabbit fetuses.

Our study also has some strengths. First the choice of the rabbit model is a very good model for brain development. The rabbit is a perinatal brain developer, like humans, unlike rodents (postnatal), sheep or even monkeys (prenatal)¹¹. Therefore, brain growth in rabbits mimics the human situation. This allowed us to perform the intervention at a developmental stage which corresponds to the timeframe when fetal surgeries happen. Secondly, the neonatal rabbit model has already frequently been used to study perinatal brain insults³³⁻³⁷. Thirdly, the large litter size allowed us to compare treatments within each mother. Lastly, using the same protocol of maternal anesthesia for all the does, we were able to dissect out the singular effect of fetal injected atropine-fentanyl-curare on brain development, which is hard to analyze in the human setting due to the many confounding factors.

Conclusion

In the rabbit model, additional administration of fetal injected atropine-fentanyl-curare does not lead to measurable short-term neurocognitive effects. Many centers still rely on general maternal anesthesia to achieve fetal analgesia and immobilization³⁸. Based on these findings and our previous findings on maternal anesthesia in the rabbit model, a direct fetal injection with medication would be preferred to achieve fetal analgesia.

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Table 1: Maternal parameters measured during surgery. MAP: Mean Arterial Pressure; HR: Heart Rate; Sat: Saturation; Temp: Temperature. Data are presented as mean $\pm$ SD.* indicate $p<0.05$.

Figure 1: A) drawing of a pregnant doe in the setting of this experiment, rabbits were intubated, received IV and IA line, saturation and temperature monitoring and underwent a median laparotomy that partially exposed the uterus for fetal ultrasound. B) the first two ovarian-end pups in each horn in the bicornuate uterus of the rabbit were randomized to receive ultrasound guided injection with fetal medication (yellow) or saline (black), while the third pup, an untouched littermate served as control. C) Ultrasound image showing the needle injecting the fetal medication in the liver of the pup.

Figure 2: Neurobehavioral outcome on postnatal day 1. A) motor scores. B) sensory scores. Data are presented as individual data points, with mean and SD.

Figure 3: A) Representative image of the hippocampus stained with Hoechst (nuclei) and Neu-N (neurons). B) Neuron density in the 4 investigated regions. Data are presented as individual data points, with mean and SD.

Figure 4: A) Representative image of the hippocampus stained with Hoechst (nuclei) and SY-38 (synapses). B) Integrated density synaptophysin in the 4 investigated regions of interest. Data are presented as individual data points, with mean and SD, * indicates $p<0.05$.

Supplement Figure 1: A) Heart rates of the fetuses in the different groups before and after injection. B) brain-to-body weight ratios at birth. Data are presented as individual data points, with mean and SD.

Supplement Figure 2: A) Representative image of the hippocampus stained with KI-67 (proliferative cells; black) and Mayer's hematoxylin (nuclei; bleu). B) Proliferation rates in the 4 investigated regions. Data are presented as individual data points, with mean and SD.

	Begin procedure	End procedure	Normal value (range)
MAP (mmHg)	40 ± 14	50 ± 15	57 (41-72)
HR (beats/min)	211 ± 36	223 ± 26	237 (177-286)
Sat O ₂ (%)	98 ± 3	97 ± 3	97 (94-100)
Temp (°C)	38.0 ± 0.8	37.5 ± 0.8	38 (37-39)
pH	7.32 ± 0.08 *	7.40 ± 0.04	7.44 (7.38-7.48)
pCO ₂ (mmHg)	33 ± 21	24 ± 9	30.4 (23.8-36.8)
Lactate (mmol/L)	2.4 ± 1.1	2.1 ± 1.3	2.1 (0.7-7.2)