Original Research

Fatty acid ethyl esters in meconium: A biomarker of fetal alcohol exposure and effect

Charlie T Cheng, Enrique M Ostrea Jr, Joseph NB Alviedo, Felix P Banadera and Ronald L Thomas

Hutzel Women's Hospital and Children's Hospital of Michigan, Detroit, MI 48201, USA
Corresponding author: Enrique M Ostrea. Email: enrique.ostrea@gmail.com

Impact statement

Alcoholism in pregnant women is associated with mental retardation and developmental problems in their offspring. Thus, identification and potential intervention of the affected infants are important. Although fatty acid ethyl esters (FAEE) is a known biomarker of prenatal exposure to alcohol in infants, information on FAEE as biomarker of effect is limited and more studies are needed to show this relationship. Using a rat model, we demonstrated that FAEE in meconium of the pups is a useful biomarker not only of prenatal alcohol exposure, but also of its adverse effect on fetal body and brain weights. Thus, in newborn infants prenatally exposed to alcohol, FAEE may provide useful information on alcohol exposure and the potential risk in the infant to adverse growth and development.

Abstract

To determine if meconium fatty acid ethyl esters (FAEE) in rat pups is a good biomarker of prenatal exposure and effect to alcohol, three groups of pregnant rats were studied: one control (pair fed) and two treatment groups given 25% alcohol at 2.2 or 5.5 $g^{-1}\,kg^{-1}\,d^{-1}$. The pups were delivered on day 20 and, for each dam, were separated into a male and female group. The body, brain, intestines, and placenta of the pups were obtained, weighed, and stored at −20°C. The pups' intestines (as surrogate of meconium) from each group were pooled, and meconium was analyzed by gas chromatography/mass spectroscopy for FAEE. The meconium showed the following FAEE: ethyl palmitate, ethyl stearate, and ethyl linolenate and were only found in the alcohol-treated group and with high specificity but low sensitivity. Mean body weight of the pups was lower in the treatment groups compared to the control groups. Ethyl palmitate concentration correlated negatively to the pups' mean body and brain weights. Therefore, ethyl palmitate, stearate, and linolenate, in meconium of rat pups prenatally exposed to alcohol, are useful biomarkers of prenatal alcohol exposure, with ethyl palmitate a good biomarker of adverse effect on the pups' body and brain weight.

Keywords: Meconium, rat pups, gas chromatography/mass spectroscopy, fatty acid ethyl esters, fetal alcohol, biomarkers

Experimental Biology and Medicine **2021; 246: 380–386. DOI: 10.1177/1535370220971562**

Introduction

Alcoholism in pregnant women is a major health problem since it can lead to serious birth defect and developmental disorders in her offspring, e.g. fetal alcohol spectrum disorder and fetal alcohol syndrome (FAS). Although the severity of the disorder is directly related to the amount of alcohol consumed by the mother, threshold studies have shown that even small doses of alcohol consumed prenatally can have some adverse effect on the mental development of the child.[1,2]

The adverse effects of ethanol may be due to its direct toxicity or to one of its oxidative and non-oxidative metabolites.[3] Acetaldehyde is the main oxidative metabolite of alcohol and FAEE, ethyl glucuronide, ethyl sulfate, and phosphatidylethanol are the non-oxidative metabolites which remain in the blood and other tissues longer due to their prolonged half-life and allows for retrospective assessment of ethanol intake.[4]

Annually, about 130,000 fetuses are prenatally exposed to high levels of alcohol in the United States of America.[5] The Centers for Disease Control (CDC) has identified fetal alcohol exposure to be 0.2 to 1.5 per 100 live births and FAS as 0.3 per 1000 children of ages 7 to 9 years.[6] Despite widespread public education, the percentage of alcohol consumption by pregnant women in the United States of America has steadily increased across three study periods (2006 to 2010, 2011 to 2013, and 2015 to 2017).[7] In the 2015 to 2017 CDC survey, binge drinking was nearly 4% of pregnant women within the prior 30 days and nearly 12% reported any alcohol consumption.[7] Thus, a biomarker of alcoholism in pregnant women is needed to help identify affected newborns and their risk to adverse growth

and development which could benefit from early intervention.

FAEE is formed from catalysis of ethanol by FAEE synthase and acyl-coenzyme A:ethanol O-acyltransferase.[8] Among individuals intoxicated with alcohol at the time of death, FAEE has been found in their plasma and various organs such as the liver, pancreas, heart, and adipose tissue.[9] Animal studies have shown that chronic and subchronic alcohol intake significantly inhibited ALDH (hepatic alcohol dehydrogenase) activity. In humans, ALDH activity has been reduced in alcoholics with severe liver disease and predisposes ethanol to be metabolized nonoxidatively to FAEE in various organs.[10] A study of simultaneously extracted FAEE and ethyl-glucuronide from meconium was both associated with good sensitivity and reproducibility.[11] These studies demonstrated a good correlation between ethyl glucuronide in meconium to maternal alcohol consumption and neonatal outcome.

A relationship also exists between FAEE serum concentration and blood alcohol concentration,[12] and a higher concentration of FAEE has been shown in patients with chronic compared to acute alcohol abuse.[13]

Meconium is the infant's initial stool after birth and has been formed at around the 12th week of gestation when fetal swallowing starts. Meconium is a good matrix for the analysis of licit and illicit drugs.[14] Mac *et al.* were the first to report in an abstract and subsequently cited by Burd and Hofer on high levels of FAEE in meconium in 15 infants prenatally exposed to alcohol compared to 10 control.[15] This finding was subsequently confirmed by other investigators in human and animal studies.[16–22] FAEE does not cross the human placenta and so its presence in meconium indicates the effect of fetal exposure to ethanol.[19] Quantification of FAEE in meconium has been shown to correlate to the amount of maternal alcohol intake.[23–29] FAEE has been found in other matrices, e.g. hair, urine, placenta, nail, neonatal and maternal blood.[30–34] Studies have shown improved correlation between blood FAEE and alcohol concentrations with the inclusion of triglyceride concentration.[13] These studies have further advanced FAEE as a viable biomarker of prenatal alcohol exposure.

However, as a biomarker of adverse effect, the correlation of meconium FAEE to effect on body organs has not been adequately studied except in two animal studies. In one study in guinea pigs, an inverse relationship between meconium FAEE concentration and body and brain weights in the offspring was demonstrated.[35] In another study, ethanol-induced toxic effect in various organs was shown in the sheep.[36] The aim of our study is to further demonstrate in alcoholic, pregnant rats that FAEE is a useful biomarker of both fetal alcohol exposure and effect, the latter by correlating FAEE to the pups' body, placental, and brain weights. There are several advantages in using an animal model. The dosing of alcohol can be controlled for to simulate occasional, moderate, or heavy alcoholic drinker in humans. Similarly, confounding variables such as race, ethnicity, nutrition, and concomitant use of other drugs can be controlled for.

Materials and methods

Three groups of timed-pregnant Sprague Dawley rats were obtained on gestation day 4 from the Charles River Laboratories (Portage, MI). There were one control and two treatment groups with nine pregnant rats in each group. The control group was pair fed with standard rat chow and allowed the same amount of food that was consumed by the high alcohol treatment group and gastric tube fed with additional water at a volume that was equal to the alcohol volume given to the high dose (HD) treatment group. The dams were weighed on the first day of treatment to determine their initial alcohol dose. The treatment groups were given, by gavage, either as a low dose (LD) of 25% alcohol at $2.2\,g^{-1}\,kg^{-1}\,d^{-1}$ or a HD at $5.5\,g^{-1}\,kg^{-1}\,d^{-1}$. The subsequent alcohol dose for each dam was given at the same, initial dose throughout the study period to provide uniformity in the amount of alcohol given. No subsequent weights of the dam were therefore taken. The alcohol levels were not determined, but based on our previous study the peak blood alcohol level in Sprague Dawley rats at 4 h for 5 and $6\,g^{-1}\,kg^{-1}\,d^{-1}$ doses were 325 ± 75 and 372 ± 74 mg%, respectively. For the $2\,g^{-1}\,kg^{-1}\,d^{-1}$ the peak blood alcohol level at 0.5 h was 91 ± 31mg%.[37]

Alcohol treatment was started on gestational day 8 and administered daily until gestational day 19. Gavage feeding was used so that the appropriate alcohol dose could be given and binge exposure simulated. The dams were sacrificed and delivered on gestational day 20 to allow the timely delivery of all pups. The dams were sacrificed by initial CO_2 narcosis and then decapitation. At birth, the pups from each dam were separated by sex into a male and female group and the body, brain, intestines, and placenta of the pups were obtained in each group, weighed and stored at $-20^{\circ}C$. In each dam, the intestines of the pups (as surrogate for meconium) were pooled into one sample based on sex and analyzed for FAEE.

FAEE analysis

Meconium (0.5 g) was analyzed for FAEE by positive chemical ionization gas chromatography/mass spectroscopy according to previously published method.[22] The GC/MS was an Agilent GC6890/MS5973N with positive chemical ionization in selected ion monitoring mode. Ethyl heptadecanoate was used as the internal standard. To determine the limit of detection (LOD), matrix-spiked calibrators were used at a concentration range of 0.05 to $8\,\mu g\,g^{-1}$ of the FAEE. The mean (SD) recovery rate of the FAEE was $101.0\% \pm 4.6\%$ and the mean (SD) interassay and intraassay coefficients of variation were $12.1\% \pm 5.3\%$ and $4.6\% \pm 1.1\%$, respectively. The LOD for the individual FAEEs was determined using the empirical approach with decreasing concentrations of matrix-spiked calibrators.[38,39] The LODs were $0.05\,\mu g\,g^{-1}$ for ethyl laurate, ethyl myristate, ethyl palmitate, ethyl linoleate, and ethyl oleate; $0.10\,\mu g\,g^{-1}$ for ethyl α-linolenate and ethyl stearate; $0.20\,\mu g\,g^{-1}$ for ethyl AA, and $1\,\mu g\,g^{-1}$ for ethyl DHA. FAEE were considered as "negative or zero concentration" if the actual concentration was below the LOD.

Statistical analysis

Descriptive analysis was initially performed on continuous and non-continuous variables. We employed nested MEM (Mixed Effect Models) to analyze each dependent variable collected given that the pups are nested within their respective litters (dams) and not independent observations. Analysis of this design with a General Linear Model would not be appropriate since it requires the data to be independent observations. The MEM analysis adjusted the error terms for the nesting. Dose group was held fixed in the model, and number of pups by litter was built as a random, nested effect in the model. Parameter estimates for fixed effects were reported, with their SE or standard error, calculated with a Type III sum of squares selection. Main pair-wise comparisons were examined using a Bonferroni correction. Bivariate correlation of the different variables to FAEE was analyzed using Spearman's rho. A $p < 0.05$ was considered as the level of statistical significance.

Results

One dam in the HD group died. A total of 52 pooled male and female pups were studied: 18 pooled male and female pups in the pair fed (n = 9 dams), 18 pooled male and female pups in the low-dose (n = 9 dams) and 16 pooled male and female pups in high-dose groups (n = 8 dams).

By nested Mixed effect model analysis, as alcohol dose was increased, there was a significant decrease in mean body weight in the pups in the alcohol compared to the control group (1.91 versus 2.17 g, $p = 0.007$) (Table 1). When grouped according to sex, a similar trend of lower birth weight was noted in the alcohol-exposed male (1.95 versus 2.22, $p = 0.220$) and female (1.89 versus 2.12, $p = 0.079$) groups at increasing alcohol dose, compared to control. However, the difference was not significant probably due to a small sample size (N = 9) in each group. In contrast, the mean placental and brain weights were not different in the control versus treatment groups.

The frequency and mean (SD) FAEE concentrations in meconium ($\mu g\, g^{-1}$ meconium) are shown in Table 2. Ethyl palmitate (11.5%), ethyl linolenate (19.2%), and ethyl stearate (3.8%) were found only in the alcohol-exposed pups

Table 2. Frequency and mean (SD) concentrations ($\mu g\, g^{-1}$ meconium) of FAEE in meconium of the alcohol-exposed pups.

FAEE ($\mu g\, g^{-1}$)	N	% positive	Mean	Std. deviation
Ethyl laurate	52	0	0	0
Ethyl myristate	52	0	0	0
Ethyl palmitate	52	11.5	0.048	0.134
Ethyl linoleate	52	0	0.000	0.000
Ethyl oleate	52	0	0.000	0.000
Ethyl linolenate	52	19.2	0.068	0.156
Ethyl stearate	52	3.8	0.069	0.354
Ethyl AHA	52	0	0.000	0.000
Ethyl DHA	52	0	0.000	0.000

No FAEE was detected in the control (pair fed) group.
A comparison of the frequency and mean (SD) concentrations ($\mu g\, g^{-1}$ meconium) of FAEE in meconium of the alcohol-exposed pups.
AHA: arachidonate; DHA: docosahexanoate; FAEE: fatty acid ethyl esters.

Table 1. Mean weight (g) of body, placenta, and brain in pups based on alcohol dose and gender.

Alcohol dose	Control (Pair fed) (0 g kg^{-1})	Low dose (2.2 g kg^{-1})	High dose (5.5 g kg^{-1})	p
A. Male and Female pups				
N	18	18	16	
1. Body weight (g)				
Mean (SE)	2.17 (0.06)	2.08 (0.06)	1.91 (0.06)	0.007
2. Placenta weight (g)				
Mean (SE)	0.49 (0.01)	0.48 (0.01)	0.49 (0.01)	0.800
3. Brain weight (g)				
Mean (SE)	0.14 (0.01)	0.14 (0.01)	0.13 (0.01)	0.118
B. Male pups				
N	9	9	8	
1. Body weight (g)				
Mean (SE)	2.22 (0.09)	2.11 (0.09)	1.95 (0.09)	0.220
2. Placenta weight (g)				
Mean (SE)	0.50 (0.02)	0.49 (0.02)	0.49 (0.02)	0.898
3. Brain weight (g)				
Mean (SE)	0.14 (0.01)	0.14 (0.01)	0.13 (0.01)	0.196
C. Female pups				
N	9	9	8	
1. Body weight (g)				
Mean (SE)	2.12 (0.07)	2.07 (0.07)	1.89 (0.07)	0.079
2. Placenta weight (g)				
Mean (SE)	0.47 (0.01)	0.47 (0.01)	0.48 (0.01)	0.827
3. Brain weight (g)				
Mean (SE)	0.13 (0.01)	0.13 (0.01	0.13 (0.01)	0.509

Comparison of fixed effects by mixed effects models.
The mean (SE) body, placenta, and brain weights (g) of the pups were compared based on their alcohol dose (low and high) and gender.

and none in the control group. The other FAEE were not detected in either group. Calculation of the mean (SD) concentrations of the FAEE in Table 2 included zero concentrations which accounts for the high standard deviation. When only the mean of positive samples was calculated, the mean (SD) concentrations was for ethyl palmitate = $0.414 \pm 0.041\,\mu g\,g^{-1}$, for ethyl linolenate = $0.352 \pm 0.166\,\mu g\,g^{-1}$, and for ethyl stearate = $1.785 \pm 0.530\,\mu g\,g^{-1}$.

Based on alcohol dose and gender (Table 3), there was a trend toward an increase in concentrations of ethyl linolenate, as alcohol dose increased, in both the combined gender and male pups. This trend was not observed in the female pups. In Table 4, the sensitivity of ethyl palmitate for detecting alcohol exposure was 17%, 29% for ethyl linolenate, and 5% for ethyl stearate. On the other hand, their specificity and positive predictive values were 100%.

Table 5 shows the correlation between the concentrations of specific meconium FAEE and the mean body, placenta, and brain weight of the rat pups. There was a negative correlation of ethyl palmitate concentration ($ug\,g^{-1}$) to mean body weight (g) in the combined male/female pups (rho = −0.486, p = 0.001), in the individual male pups (rho = −0.461, p = 0.018), and the female pups (rho = −0.463, p = 0.017). Ethyl palmitate also correlated to the brain weight in the combined male and female (rho = −0.426, p = 0.002), male (rho = −0.381, p = 0.055) and female (−0.471, p = 0.015) groups. Ethyl linolenate correlated negatively in the male pups to mean body weight (rho = −0.379; p = −0.056) and brain weight (rho = −0.374, p = 0.061) but the difference did not achieve statistical significance. When the concentrations of the three FAEEs were combined (cumulative FAEE), there was a negative correlation of cumulative FAEE to the mean body weight (rho = −0.588, p = 0.017) in the male/female pups and male pups (rho = −0.867, p = 0.001) and to the mean brain weight (rho = −0.927, p = 0.000) in the male pups.

Discussion

Prenatal alcohol is a teratogen and can cause adverse outcome in the newborn infant. Our study showed a decrease in the pups' body weight with an increased dose of alcohol (Table 1) and consistent with other studies. For example, in rat pups, Abel reported that prenatal alcohol at HDs produced a significant decrease in the birth weight of rat pups.[40] In rhesus monkeys, exposure of the pregnant monkey to alcohol and stress resulted in a significant

Table 4. The sensitivity, specificity, PPV, and NPV of ethyl palmitate, ethyl linolenate, and ethyl stearate in detecting alcohol exposure.

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Ethyl palmitate	17	100	100	39
Ethyl linolenate	29	100	100	42
Ethyl stearate	5	100	100	36

NPV: negative predictive value; PPV: positive predictive value.

Table 3. FAEE concentrations of ethyl palmitate, ethyl stearate, and ethyl linolenate in meconium of rat pups based on alcohol dose and gender.

	Control (pair fed)	Low dose alcohol	High dose alcohol	p
	(0 g kg^{-1}	(2.2 g kg^{-1})	(5.5 g kg^{-1})	
A. Male and female pups				
N	18	18	16	
Ethyl palmitate ($\mu g\,g^{-1}$)				
Mean (SE)	0	0.048 (0.028)	0.101 (0.029)	0.224
Ethyl stearate ($\mu g\,g^{-1}$)				
Mean (SE)	0	0.121 (0.076)	0.092 (0.080)	0.794
Ethyl linolenate ($\mu g\,g^{-1}$)				
Mean (SE)	0	0.088(0.038)	0.106 (0.038)	0.731
B. Male pups				
N	9	9	8	
Ethyl palmitate ($\mu g\,g^{-1}$)				
Mean (SE)	0	0.062 (0.041)	0.055 (0.044)	0908
Ethyl stearate ($\mu g\,g^{-1}$)				
Mean (SE)	0	0	0	ns
Ethyl linolenate ($\mu g\,g^{-1}$)				
Mean (SE)	0	0.155 (0.061)	0.193 (0.065)	0.670
C. Female pups				
N	9	9	8	
Ethyl palmitate ($\mu g\,g^{-1}$)				
Mean (SE)	0	0.044 (0.039)	0.146 (0.041)	0.082
Ethyl stearate ($\mu g\,g^{-1}$)				
Mean (SE)	0	0.239 (0.146)	0.176 (0.155)	0.771
Ethyl linolenate ($\mu g\,g^{-1}$)				
Mean (SE)	0	0.027 (0.014)	0	0.198

Comparison of fixed effects by mixed effect models.
A comparison of FAEE concentrations of ethyl palmitate, ethyl stearate, and ethyl linolenate in meconium of rat pups based on low or high dose alcohol and gender of the pups.
FAEE: fatty acid ethyl esters; ns: not calculated.

Table 5. Correlation of FAEE concentration in meconium ($\mu g\,g^{-1}$) with mean body, placenta, and brain weights (g) in pups (Spearman rho).

		Mean body weight (g)	Mean placental weight (g)	Mean brain weight (g)
A. Male and female pups				
Ethyl palmitate ($\mu g\,g^{-1}$)	Correlation coefficient	–0.486	–0.187	–0.426
	Significance (two-tailed)	0.001	–0.184	–0.002
Ethyl linolenate ($\mu g\,g^{-1}$)	Correlation coefficient	–0.096	–0.021	–0.218
	Significance (two-tailed)	–0.497	–0.887	–0.121
Ethyl stearate ($\mu g\,g^{-1}$)	Correlation coefficient	0.025	0.101	0.002
	Significance (two-tailed)	0.86	–0.476	–0.988
Cumulative FAEE ($\mu g\,g^{-1}$)	Correlation coefficient	–0.588	–0.268	–0.450
(Ethyl palmitate, linolenate, and stearate)	Significance (two-tailed)	0.017	0.316	0.080
B. Male pups				
Ethyl palmitate ($\mu g\,g^{-1}$)	Correlation coefficient	–0.461	–0.351	–0.381
	Significance (two-tailed)	–0.018	–0.081	–0.055
Ethyl linolenate ($\mu g\,g^{-1}$)	Correlation coefficient	–0.379	–0.295	–0.374
	Significance (two-tailed)	–0.056	–0.143	–0.061
Ethyl stearate ($\mu g\,g^{-1}$)	Correlation coefficient	0	0	0
	Significance (two-tailed)	0	0	0
Cumulative FAEE ($\mu g\,g^{-1}$)	Correlation coefficient	–0.867	–0.588	–0.927
(Ethyl palmitate, linolenate, and stearate)	Significance (two-tailed)	0.001	0.074	0.000
C. Female pups				
Ethyl palmitate ($\mu g\,g^{-1}$)	Correlation coefficient	–0.463	0.003	–0.471
	Significance (two-tailed)	0.017	0.989	0.015
Ethyl linolenate ($\mu g\,g^{-1}$)	Correlation coefficient	0.200	0.281	–0.147
	Significance (two-tailed)	0.327	0.166	0.475
Ethyl stearate ($\mu g\,g^{-1}$)	Correlation coefficient	0.092	0.253	0.005
Cumulative FAEE ($\mu g\,g^{-1}$)	Significance (two-tailed)	0.653	0.212	0.981
(Ethyl palmitate, linolenate, and stearate)	Correlation coefficient	0.086	–0.143	0.543
	Significance (two-tailed)	0.872	0.787	0.266

FAEE: fatty acid ethyl esters.

decrease in birth weight among male offspring.[41] In humans, a dose-related association between prenatal alcohol exposure with infant birth weight and length was described.[42,43]

We did not observe a lower mean placental weight with prenatal alcohol exposure. This effect has been reported in the literature that although fetal weights were reduced in prenatal alcohol exposure, the placental weights were increased presumably as a consequence of the feto-protective response of the placenta to safeguard the fetus from the adverse effect of alcohol.[44,45]

Also we did not observe a lower brain weight in the pups (Table 1) in association with prenatal alcohol exposure. Apparently, reduced brain weight may not be evident at birth despite reduction in overall fetal growth although growth differences may be evident later.[46]

Unlike reports in human, FAEEs in our study were detected only in pups born to pregnant rats exposed to alcohol and not in the control group. This demonstrates the advantage of animal versus human model in validating FAEE as a biomarker of alcohol exposure, since in humans, there are other sources of ethanol, besides alcoholic drinks, that could be taken by the pregnant woman which could confound the results.[18] Ethyl linolenate had a sensitivity of 29% in detecting fetal alcohol exposure in our study (Table 4) which is consistent with our findings in infants born to alcoholic mothers.[22] On the other hand, higher sensitivity and specificity were observed by others with ethyl linolenate in humans.[16] Specie difference (rat versus human), longer exposure time to alcohol in human due to longer gestational period, variations in metabolism of ethanol, and difference in methods of detecting FAEE may account for the difference in results.[8,17,47] Finally, the strong negative correlation between ethyl palmitate in meconium and mean body and brain weight in combined or separate genders (Table 5) provides further evidence of ethyl palmitate, as a useful biomarker of effect. Cumulative FAEE concentrations did not add any further advantage to the correlation compared to ethyl palmitate alone.

There are other biomarkers of alcohol exposure in adults which include ethyl glucuronide, ethyl sulfate,[48–50] phosphatidylethanol,[51–53] and carbohydrate deficient transferrin.[50,54,55] The detection of these biomarkers in newborn infants in combination with FAEE provides an opportunity for future study that these metabolites provide as biomarkers of exposure and effect.

The small number of pups in the sex-specific groups is a limitation of this study. The trend of lower mean birth weight in the pups in relation to increased alcohol dose may attain statistical significance with a larger number of pups in the sample.

How relevant is our study to human situation? Although equivalent dosage to blood alcohol levels in rat compared to humans is not available in the literature, at similar blood alcohol levels, similar neurodevelopmental outcomes in domains such as learning, attention, behaviors, and motor performances have been consistently observed in both animals and children.[56]

Conclusions

In a rat model, we have shown that FAEE, specifically ethyl palmitate, ethyl linoleate, and ethyl stearate, were found only in pups prenatally exposed to alcohol with high specificity and a trend toward increasing FAEE concentrations as the alcohol dose was increased. Ethyl palmitate concentration was also negatively related to the body and brain weights of the pups. This study therefore provides evidence that FAEE is a useful biomarker of prenatal alcohol exposure and its adverse effect in the pups. The translational study to newborn infants of FAEE to detect prenatal alcohol exposure and its adverse effect is warranted.

AUTHORS' CONTRIBUTIONS

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript: JBA and FPB conducted the experiment, CTC and EMO wrote the manuscript and RLT did the statistical analysis.

ACKNOWLEDGEMENTS

The authors thank Dawn M Bielawski, Ph.D., for advice in experimental design.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICAL APPROVAL

This study was approved by the IACUC (Institutional Animal Care and Use Committee) of Wayne State University, Detroit, Michigan.

FUNDING

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported in part by grants from the National Institute of Child Health and Human Development (R01HD039428) and US Environmental Protection Agency (RFA 2001-STAR-H1. No. R829395).

ORCID ID

Enrique M Ostrea https://orcid.org/0000-0002-5516-7102

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(Received August 4, 2020, Accepted October 15, 2020)