HHS Public Access

Author manuscript

Alcohol. Author manuscript; available in PMC 2016 November 01.

Published in final edited form as:

Alcohol. 2015 November ; 49(7): 647–656. doi:10.1016/j.alcohol.2015.08.005.

The Impact of Micronutrient Supplementation in Alcohol-Exposed Pregnancies on Information Processing Skills in Ukrainian Infants

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Abstract

Objectives—The potential of micronutrients to ameliorate the impact of prenatal alcohol exposure was explored in a clinical trial conducted in Ukraine. Cardiac orienting responses during a habituation/dishabituation learning paradigm were obtained from 6–12-month-olds to assess neurophysiological encoding and memory of environmental events.

Materials and methods—Women who differed in prenatal alcohol use were recruited during pregnancy and assigned to a group (no study-provided supplements, multivitamin/mineral supplement, or multivitamin/mineral supplement plus choline supplement). An infant habituation/dishabituation paradigm was used to assess outcomes in the offspring. Ten trials were used for the habituation and five for the dishabituation condition. Heart rate was collected for 30 sec prior to stimulus onset and then 12 sec post-stimulus onset. Difference values (ΔHR) were computed for

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

the first three trials of each condition and aggregated for analysis. Gestational blood samples were collected to assess maternal nutritional status and changes as a function of the intervention.

Results—Choline supplementation resulted in a greater ΔHR on the visual habituation (Wald Chi-Square (1, 149) = 10.9, $p < .001$, eta-squared = .043) trials for all infants and for the infants with no prenatal alcohol exposure on the dishabituation (Wald Chi-Square (1, 139) = 6.1, $p < .013$, eta-squared = .065) trials. The latency of the response was reduced in both conditions (Habituation: Wald Chi-Square (1, 150) = 9.0, $p < .003$, eta-squared = .056; Dishabituation: Wald Chi-Square (1, 137) = 4.9, $p < .027$, eta-squared = .032) for all infants whose mothers received choline supplementation. Change in gestational choline level was positively related ($r = .19$) to ΔHR during habituation trials, and levels of one choline metabolite, dimethylglycine (DMG), predicted ΔHR during habituation trials ($r = .23$) and latency of responses ($r = -.20$). A trend was found between DMG and ΔHR on the dishabituation trials ($r = .19$) and latency of the response ($r = -.18$). Multivitamin/mineral or multivitamin/mineral plus choline supplementation did not significantly affect cardiac orienting responses to the auditory stimuli.

Conclusion—Choline supplementation when administered together with routinely recommended multivitamin/mineral prenatal supplements during pregnancy may provide a beneficial impact to basic learning mechanisms involved in encoding and memory of environmental events in alcohol-exposed pregnancies as well as non- or low alcohol-exposed pregnancies. Changes in nutrient status of the mother suggested that this process may be mediated by the breakdown of choline to betaine and then to DMG. One mechanism by which choline supplementation may positively affect brain development is through prevention of fetal alcohol-related depletion of DMG, a metabolic nutrient that can protect against overproduction of glycine, during critical periods of neurogenesis.

Keywords

prenatal alcohol; micronutrient supplementation; choline; cardiac orienting; infants

Introduction

The negative impact of prenatal alcohol exposure (PAE) on neurodevelopmental functioning has been investigated in both human and animal models for over 40 years (Thomas, Warren, & Hewitt, 2010) but it is only recently that factors that modify the teratogenic impact of PAE have been the focus of research (Guerri et al., 2005; Peadon, Rhys-Jones, Bower, & Elliott, 2009). Such factors include changes to postnatal environmental circumstances (Guerri et al., 2005; Thomas, Sather, & Whinery, 2008), cognitive behavioral interventions (Adnams et al., 2007; Bertrand, 2009; Coles, Kable, & Taddeo, 2009; Coles, Strickland, Padgett, & Bellmoff, 2007; Kable, Coles, & Taddeo, 2007; Loomes, Rasmussen, Pei, Manji, & Andrew, 2008; O'Connor et al., 2006, 2012; Olson & Montague, 2011), pharmacological agents (Incerti, Vink, Roberson, Benassou, et al., 2010; Incerti, Vink, Roberson, Wood, et al., 2010; Marche, Danel, & Bordet, 2011; Zhou, Fang, & Goodlett, 2008), and nutritional interventions (Naseer, Lee, & Kim, 2010; Ojeda et al., 2009; Serrano, Han, Brinez, & Linask, 2010; Summers, Rofe, & Coyle, 2009; Thomas, Abou, & Dominguez, 2009).

Maternal nutritional status has been posited as one potential moderator of the teratogenic impact of PAE (Keen et al., 2003, 2010). Women who drink heavily in pregnancy often have poor nutritional status (Guerrini, Thomson, & Gurling, 2007) and poor diets alone have been linked to adverse pregnancy outcomes (Keen, Bendich, & Willhite, 1993; Walker et al., 2011). Maternal drinking during the periconceptual period (Weiss & Chambers, 2013) has also been found to be dose-related to a reduction in multivitamin supplement use. Even among women who maintain adequate diets, moderate and heavy alcohol consumption can interfere with micronutrient absorption (Carter, Jacobson, Molteno, & Jacobson, 2007; Church, Jen, Pellizzon, & Holmes, 1998).

Multivitamin use in pregnancy has been associated with significant improvements in pregnancy outcomes, including lengthening gestation, increasing birth weight and length, reducing miscarriages, and preventing oral clefts (Glenville, 2006). Information on the long-term impact to the development of the offspring has been more limited. Among women from Tanzania who were HIV positive, multivitamin supplementation resulted in improvements in toddler motor functioning but not cognitive development (McGrath et al., 2006). In a U.S. study based on parent ratings of preschool children, multivitamin use in pregnancy was associated with a decreased risk for delays in language and personal/social functioning but was not found to impact overall development (Wehby & Murray, 2008). In another study, multivitamin use was associated with improvements in verbal abilities on standardized tests but was not related to changes in perceptual, quantitative, memory, motor, and executive functioning in 4-year-olds (Julvez et al., 2009).

The potential of micronutrient supplementation to ameliorate the negative impact of PAE is unknown as no previous human studies have attempted to administer micronutrient supplements during alcohol-exposed pregnancies, and studies using animal models have limited the intervention to only a few discrete minerals or micronutrients (El Banna, Picciano, & Simon, 1983; Rufer et al., 2012; Summers et al., 2009; Thomas et al., 2009). Among these, the use of choline supplementation appears to be the most promising. Using animal models, improvements in behavioral flexibility (Thomas, Idrus, Monk, & Dominguez, 2010), reduced activity (Thomas, Garrison, & O'Neill, 2004), and improved performance on a variety of learning tasks has been found in response to choline supplementation, including tasks of spontaneous alternation (Thomas, Biane, O'Bryan, O'Neill, & Dominguez, 2007; Thomas, Garrison, et al., 2004), discrimination learning (Thomas, La Fiette, Quinn, & Riley, 2000), and trace eyeblink conditioning (Thomas & Tran, 2012). Choline supplementation has also been found to reduce the impact of PAE on the density of muscarinic M(1) receptors in the dorsal hippocampus (Monk, Leslie, & Thomas, 2012) and to reduce hyper-methylation associated with PAE in the hippocampus and prefrontal cortex (Otero, Thomas, Saski, Xia, & Kelly, 2012). Gestational choline supplementation also prevented PAE modulation of histone and DNA methylation in hypothalamic proopiomelanocortin neurons (Bekdash, Zhang, & Sarkar, 2013). In contrast, choline supplementation was not found to alter the impact of PAE on motor coordination deficits (Thomas, O'Neill, & Dominguez, 2004), delayed eyeblink response (Thomas & Tran, 2012), or olfactory habituation (Hunt, Jacobson, & Kim, 2014).

The impact of choline levels and dietary supplementation among humans with PAE is not yet known but has been posited as a potentially helpful agent (Ballard, Sun, & Ko, 2012), particularly for women who may have an inadequate intake of choline in their everyday diets (Zeisel, 2011). The impact of gestational choline in the absence of PAE on child neurodevelopmental outcomes has been ambiguous. Choline was not found to be related to 5-year cognitive status in a population of children in Alabama with a history of poverty and poor nutrition (Signore, Ueland, Troendle, & Mills, 2008). An observational study conducted in the Seychelles also found no relationship between choline concentration and neurodevelopmental outcome in children at 5 years of age but found levels of betaine, a metabolite of choline, were positively associated with language functioning (Strain et al., 2013). In other prospective studies of healthy pregnant women, positive associations were found between infant cognitive functioning as assessed using the Bayley Scales of Infant and Toddler Development, 3rd edition (Bayley, 2006) and maternal plasma free choline and betaine levels collected at 16 weeks of gestation (Wu, Dyer, King, Richardson, & Innis, 2012), and second-trimester intake of choline was associated with modest improvements in visual memory of offspring at 7 years of age (Boeke et al., 2013).

To evaluate the impact of choline supplementation in pregnancy, a double-blind clinical trial was conducted in the United States with healthy pregnant women. In this study, choline supplementation was not found to improve global development, number of words spoken, short-term visuospatial memory, or long-term episodic memory in infants under a year of age (Cheatham et al., 2012). The authors noted that no efforts were made to control dietary intake of choline and its metabolites, and further explorations revealed that gestational betaine levels were negatively related to short-term visuo-spatial memory and global development at 12 months of age.

To assess the impact of micronutrient supplementation on the cognitive functioning of offspring with a history of PAE, a task that assesses early indices of alcohol-related neurodevelopmental damage is needed that also minimizes other environmental factors associated with maternal drinking, such as lower socioeconomic status, environmental chaos (i.e., poor stability in caregivers or the home environment), and exposure to violence, that may adversely impact development (Blair & Raver, 2012). One such index of early cognitive functioning assesses the neurophysiological encoding and memory of environmental events using cardiac orienting responses (Kable & Coles, 2004). Cardiac orienting responses (ORs) to stimuli are characterized by a specific pattern of heart rate deceleration (Graham & Jackson, 1970) in the presence of novel or interesting stimuli and are triggered by mechanisms that enable the heart to gate oxygen away from the periphery to the central nervous system, allowing for higher-level information processing and learning about environmental events. The neural circuitry that supports ORs is present in all mammals and can be reliably elicited within the first few months of life in humans (Sokolov, Spinks, Näätänen, & Lyytinen, 2002).

ORs have been found in response to the onset of both visual and auditory stimuli (Berg, Berg, & Graham, 1971; Brown, Leavitt, & Graham, 1977; Lewis, Kagan, Campbell, & Kalafat, 1966) and measures during the first year of life have been linked to later cognitive functioning at 18 months (O'Connor, 1980) and 5 years (O'Connor, Cohen, & Parmelee,

1984). Characteristics of the OR response, in particular the sustained deceleration or the trough of the OR, reflect the degree of neurophysiological encoding and sustained interest to the stimuli (Lansink, Mintz, & Richards, 2000; Richards, 1995) and have been sensitive to effects of PAE in human (Kable & Coles, 2004) and animal models (Hunt & Phillips, 2004; Morasch & Hunt, 2009).

To evaluate the impact of multivitamin/mineral and choline supplementation, a clinical trial of the impact of prenatal micronutrient supplementation was conducted with a prospective cohort of moderate/heavy drinking pregnant women and low/unexposed comparison women in Ukraine. Estimates of the prevalence of alcohol use among pregnant women in Ukraine (Bakhireva et al., 2011; Chambers et al., 2014) and the rates of Fetal Alcohol Syndrome have been high (European Surveillance of Congenital Anomalies [EUROCAT], 2014). Women and their offspring were assigned to one of three intervention groups: 1) standard of care (no multivitamin/mineral [MVM] supplements provided by the study, but standard recommendation to take prenatal vitamins), 2) MVM supplements provided by the study, or 3) MVM supplements plus choline (750 mg) supplements provided by the study (MVM +choline). To evaluate early neurodevelopmental outcome associated with the intervention, ORs to information processing tasks assessing encoding and memory functioning were administered within the first year of life. Both the MVM and choline supplements were anticipated to improve the encoding and memory skills of infants with a history of PAE. In addition, these supplements were anticipated to have beneficial effects for those without a history of PAE, and the magnitude of change was anticipated to differ from those with a history of PAE. Finally, changes in choline levels and levels of metabolites of choline as a function of the nutrient supplementation intervention were anticipated to be related to infant encoding and memory skills.

Materials and methods

Participants for this study were recruited at their first prenatal visit to a diagnostic medical center located in Rivne, Ukraine (Rivne Regional Medical Diagnostic Center), which is a referral center for routine prenatal ultrasound for pregnant women. This study involves a subset of participants who were enrolled in a multi-site clinical trial carried out in Ukraine as part of the Collaborative Initiative on Fetal Alcohol Spectrum Disorders (CIFASD), which is an international consortium of basic science and clinical investigations funded by the National Institute of Alcohol Abuse and Alcoholism (NIAAA). The study protocol was approved by institutional review boards at the Lviv National Medical University in Ukraine and the University of California San Diego, La Jolla, California, and informed written consent was provided by all participants.

Recruitment and procedures

Three hundred and seventy-two pregnant women were enrolled into the intervention study based on their alcohol intake (moderate/heavy drinking vs. comparison) between April of 2008 and August of 2012 at this site. Of these, 361 pregnancies ended with a live born but 4 of these were excluded because of being multiparous pregnancies. At the first prenatal appointment, a trained nurse interviewer screened all women for alcohol use during

pregnancy. All women who reported alcohol use were provided with information on the risks of alcohol consumption during pregnancy. Women who reported at least weekly binge-drinking episodes (5+drinks), at least five episodes of 3–4 standard drinks, or at least 10 episodes of 1–2 standard drinks either in the month around conception or in the most recent month of pregnancy, were invited to participate in the study. Those who agreed read and signed the informed consent. A comparison woman was immediately sought for each enrolled alcohol-exposed woman. The next woman meeting comparison subject screening criteria (defined as no binge episodes, minimal or no alcohol in the month around conception, and no continued drinking in pregnancy) was asked to participate, enrolled, and interviewed. Participants were then randomly assigned to an intervention group using a random numbers table. Further details regarding recruitment are available elsewhere (Chambers et al., 2014).

The MVM and choline supplements were provided after randomization and throughout the study at interim visits with the interviewers. Although women in Ukraine are advised to take MVM supplements, use during pregnancy is relatively low. Half of the women enrolled were assigned to receive daily MVM (an over-the-counter prenatal vitamin/mineral supplement marketed under the brand name Theravit) from the study and the other half to the standard of care, which was to recommend MVM usage. Those assigned to the standard of care condition would have had to purchase the supplements on their own. In addition, half of those who received MVM were assigned to receive a daily dose of choline (750 mg). The MVM and choline gel caps were United States' products that were registered and purchased in Ukraine.

Mothers were interviewed at enrollment, again in the third trimester at approximately 32 weeks of gestation, and again post-partum using a structured questionnaire to obtain information regarding the mothers' demographic characteristics, lifestyle, and substance use in pregnancy, including maternal alcohol and tobacco consumption and paternal alcohol use. Day by-day alcohol quantity and type consumed in the week around conception and in the 2 weeks before enrollment was assessed using a timeline follow-back procedure. Absolute ounces of alcohol per day (ozAA/day) and per drinking day (ozAA/drinking day) were computed from the mother's report of the amount and frequency of alcohol intake for each of the two time points. The latter was used to capture episodic or binge drinking behaviors. In addition, maternal responses to items from the TWEAK (Chan, Pristach, Welte, & Russell, 1993), a measure of heavy alcohol consumption, were scored for both the mother and father. Blood samples were collected from the mother at enrollment and at the third-trimester visit, and shipped to the University of California Davis for evaluation of nutrient content. Choline, betaine, and dimethylglycine (DMG) separation, detection, and quantification was performed using a UPLC-Micro triplequad MS/MS (Waters, Micromass) based on modification of the methods of Holm (Holm, Ueland, Kvalheim, & Lien, 2003) and Innis (Innis & Hasman, 2006). Plasma homocysteine, folate, and vitamin B12 concentrations were analyzed using a chemiluminescent immunoassay system (Immulite® 1000, Siemens, PA). Compliance with taking the supplements was assessed by maternal report of frequency of use.

First year follow-up assessment procedures

Mothers and infants were seen again at the diagnostic center for assessment prior to the infant's first birthday. During their visit, a medical interview and developmental testing were conducted. Two hundred and eleven children returned for a follow-up visit but 29 were excluded from this analysis because of being either younger than 4 months of age or older than 11 months of age at the time of assessment. An additional 14 participants were excluded who were recruited because of study staff's concerns regarding potential PAE but a denial from the mother regarding prenatal alcohol intake.

Infant measures

Visual and auditory stimuli were presented using a fixed-trial habituation/dishabituation paradigm to elicit ORs. The habituation trials involved repeatedly presenting a stimulus to assess the initial encoding of the stimulus and the dishabituation trials involved presenting a similar but different stimulus to determine if the infant can differentiate the novel stimulus. The dishabituation trials allow for an assessment of memory of the initial stimulus. During the information processing tasks, infants were placed into an age-appropriate child seat and mothers were allowed to observe the testing but were instructed to be non-responsive to their infants during the testing procedure. All stimuli were digitized and presented via stimulus presentation software available from the James Long Company. The auditory stimuli consisted of a standard stimulus of alternating 400-Hz and 1,000-Hz pure tones presented contiguously for 2 sec each with a 5-msec controlled linear rise and fall time for each tone and a novel stimulus consisting of alternating 700-Hz and 1000-Hz pure tones presented in a similar fashion. The visual stimuli consisted of chromatic Caucasian faces of a baby (standard stimulus) and a woman (novel stimulus). The standard stimulus was presented for a total of 12 sec followed by an inter-stimulus interval of 12 sec until 10 repetitions were completed. The novel stimulus was then presented under similar conditions (12 sec with 12-sec ISI) for five trials. Total duration of the habituation/dishabituation procedure was approximately 12 min for each stimulus type.

Cardiac responses to the stimuli were monitored throughout the session using an EKG amplifier connected to a data acquisition computer that was triggered by the stimulus presentation software (James Long Company). A 30-sec baseline period was collected prior to initial stimulus onset. Average HR during the interval between 3–7 sec post-stimulus onset was used for analysis. This interval, often referred to as the trough of the OR, typically includes the peak deceleration in heart rate response to the stimulation, and provides an index of sustained attention to the stimuli with more deceleration in HR indicating greater interest. In addition to analyzing the average magnitude of the response, the latency of the OR was also determined. This was defined as the post-stimulus second when HR reaches more than 2 bpm of deceleration from the average baseline level of HR. For infants that did not have an OR response on a given trial, the maximum second for the OR response of 9 was assigned to the trial. Finally, infants' state after each presentation of stimulus was rated. Physiological data obtained while infants were in either state 1-Deep Sleep or State 7-Vigorous Crying was excluded from analysis. The first three trials of the habituation and dishabituation trials were used for analysis as significant diminution of the OR occurs by the fourth trial of exposure (Kable & Coles, 2004; O'Connor, 1980). Change in HR was

computed by subtracting the average HR during the trough period from the average HR during the baseline period for each stimulus condition (auditory or visual). The change in HR and latency of the response was then aggregated across the first three trials for each stimulus condition (auditory or visual) and learning condition (habituation or dishabituation) for group comparisons.

Data analysis

Maternal interview and neurophysiological data were collected and entered on site in Ukraine and transmitted to the University of California San Diego, La Jolla, California and Emory University, Atlanta, Georgia. Micronutrient concentrations in blood samples were measured at the University of California Davis and data were sent to the University of California San Diego and Emory University. Change in choline and its metabolites, betaine and dimethylglycine (DMG), and other nutrients involved in methyl donor reactions (e.g., vitamin B12, folate, and homocysteine) were computed by subtracting baseline concentrations from levels obtained at the second visit. Change in nutrient concentration values were then related to the neurophysiological outcomes.

Cardiac data from some participants were lost due to movement artifacts or bad cardiac traces. On the habituation trials, data were available on 152 participants for the auditory task and 166 for the visual task. Additional data were lost due to fatigue or attrition within the tasks, resulting in 137 participants for the auditory and 149 for the visual dishabituation tasks. There were no significant differences in baseline level of heart rate but this was used as a covariate in subsequent analyses of HR to control for individual differences that might affect changes in HR in response to the procedures (Manning & DuBois, 1962). Infant's adjusted gestational age at the assessment, which involves adjusting for the infants who were born ≤39 weeks gestation by subtracting the number of weeks of less than 40 from the infant's chronological age, was also included as a covariate in the model as a result of maturational effects of the OR over the first year of life.

Group effects were then explored using generalized linear models on each of the outcome variables. Potential covariates were assessed using a forward linear regression procedure with a probability of .10 selected for inclusion in the model to minimize the multi-collinearity of the variables. Variables evaluated were mother's and father's age, maternal education, family socioeconomic status incorporating maternal and paternal education and occupation (Hollingshead, 2011), parity, maternal pre-pregnancy body mass index, gestational age at enrollment in the study, gestational age at pregnancy recognition, prenatal folic acid supplement use, prenatal iodine supplement use, prenatal multivitamin/mineral use prior to study enrollment, maternal drinking at conception and in the 2 weeks prior to enrollment, and an index of the father's drinking habits (TWEAK). The child's sex and height, weight, and head circumference percentile at birth were also entered into the model. An incomplete factorial design using the identified covariates retained in the regression model was then used to explore group effects with the following factors: prenatal alcohol exposure (yes vs. no), MVM treatment (yes vs. no), and MVM+choline treatment (yes vs. no). Finally, OR outcomes were correlated with changes in gestational choline concentrations and its metabolites.

Results

Group characteristics of sample

Family and infant characteristics by group status are presented in Table 1, including mean group differences and statistics to assess group differences. The table contains columns representing the values for those that received MVM treatment or not relative to their alcohol status. In addition, the table breaks out the mean values of those who received MVM treatment by whether or not they received additional choline supplementation. These values are presented in the gray-shaded columns. The babies in the PAE group were born lighter on average than those born to women who did not drink alcohol just prior to or during pregnancy and were more likely to be girls. The mothers in the PAE group were of lower socioeconomic status and reported receiving fewer years of education and having fewer previous live-born children. The fathers of those in the PAE group were also older. The mothers in the PAE group had significantly more alcohol and cigarette consumption both in preconception and in the most recent 2-week period at the time of enrollment. The MVM group had heavier babies who were longer and had larger head circumference compared to the No MVM group. The group who received choline supplementation had fathers who were significantly older, mothers who were less educated, and babies with smaller birth weights relative to those who received MVM supplementation but did not receive choline.

Self-report of supplementation use

MVM use prior to study enrollment was reported by 14.0% of the women. Of the women assigned to the No MVM group, 28.4% of the women reported early MVM use but only one maintained use through the pregnancy. Single vitamin/mineral usage was more common, with 49.4% of those assigned to the No MVM group, 60.0% of the MVM group, and 52.8% of the MVM+choline group reporting self-initiated single supplement use. The most common single vitamin/minerals used were folate and iodine, with 40.7% of those in the No MVM group, 20.3% of the MVM group, and 23.7% of the MVM+choline group reporting folate use and 8.6% of the No MVM group, 8.0% of the MVM group, and 0% of the MVM +choline group reporting iodine use. At the third-trimester visit, participants were interviewed regarding compliance with the use of the MVM and MVM+choline supplements if they were assigned to a group that was given the supplements. Of those assigned to the MVM group, 97.6% reported taking the MVM on a daily or almost daily basis with only two participants reporting taking the MVM less frequently. One reported taking the MVM supplement a few times a week and the other reported once or less than once a week. For those assigned to the MVM+choline group, all participants reported taking the choline capsules on a daily or almost daily basis.

Neurophysiological outcomes

Table 2 contains the percentage of ORs and the unadjusted means and standard deviations of the average change in HR and the latencies of the OR by stimulus condition and group status as indicated in Table 1. As there were no significant effects for MVM or MVM+choline supplements on the auditory trials, only analyses pertinent to the visual trials will be further discussed. On the Visual Habituation trials, after including baseline HR, age at assessment, maternal education, prenatal iodine exposure, AA/Day at conception, AA/Day in the 2

weeks prior to enrollment, and child's gender (Regression Model: $F(7,160) = 8.84$, $p < .000$; RSQR = .279), a significant effect of choline (Wald Chi-Square (1 ,149) = 10.9, $p < .001$, eta-squared = .043) was found on the change in HR (ΔHR). *Post hoc* comparisons indicted that the ΔHR was greater for those who received choline supplementation than for those who did not (least square means/standard of error: choline (3.48/1.06) and no choline (−0.02/0.58)). A trend for a MVM effect (Wald Chi-Square (1, 149) = 3.6, $p < .057$, eta-squared = .005) was also found (least square means/standard of error: MVM [1.18/0.69] and no MVM [1.08/0.72]).

For latency on the Visual Habituation trials, a significant effect of choline (Wald Chi-Square (1, 150) = 9.0, $p < .003$, eta-squared = .056) was found after controlling for baseline HR, age at assessment, gestational age at pregnancy recognition, prenatal iodine exposure, and child's gender (Regression Model: $F(5,162) = 2.7$, $p < .022$; RSQR = .077). *Post hoc* comparisons indicated that the latency was less for those who received choline supplementation than for those who did not (least square means/standard of error: choline (4.57/0.33) and no choline (5.50/0.18)). A significant MVM effect was also found (Wald Chi-Square (1, 150) = 4.8, $p < .029$, eta-squared = .008) but the *post hoc* comparisons were not significant (least square means/standard of error: MVM (5.23/0.22) and no MVM (5.10/0.22)). The direction of the effects suggested that those who received MVM were slower rather than faster.

For magnitude of ΔHR on the Visual Dishabituation trials, a significant alcohol by choline effect (Wald Chi-Square (1,139) = 6.1, $p < .013$, eta-squared = .065) was found after controlling for baseline HR, gestational age at assessment, and maternal education (Regression Model: $F(3,164) = 18.7$, $p < .000$; RSQR = .255). The direction of the interaction suggested that the no-alcohol exposure group who received choline supplementation had greater HR change than those in the other groups (least square means/standard of error: No Alc+choline [4.16/2.49]; No Alc/no choline [−1.18/1.30]; Alc+choline [−3.87/2.52]; Alc/no choline [−0.29/1.41]). There was also a trend for an alcohol exposure effect with those in the alcohol-exposed group demonstrating less ΔHR (Wald Chi-Square [1, 139] = 2.9, $p < .087$, eta-squared = .004; least square means/standard of error: Alcohol [−1.48/1.27] No Alcohol [0.60/1.20]).

For the latency on the Visual Dishabituation trials, a significant effect of choline (Wald Chi-Square (1, 137) = 4.9, $p < .027$, eta-squared = .032) was found after controlling for baseline HR, gestational age at assessment, family SES, folate usage, and iodine usage (Regression Model: $F[5,162] = 7.2$, $p < .000$). *Post hoc* comparisons were non-significant ($p < 0.13$), but the direction of the means suggested that those who received choline supplementation were faster than for those who did not (least square means/standard of error: choline [5.21/0.41] and no choline [5.92/0.22]). A significant MVM effect (Wald Chi-Square [1, 137] = 4.7, $p < .031$; eta-squared = .013) was also found but the *post hoc* comparisons were also not significant (least square means/standard of error: MVM [5.81/0.27] and no choline [5.43/0.28]). The direction of the effects suggested that those who received MVM were slower rather than faster.

Finally, the change in choline and its metabolites, betaine and DMG, and other nutrients involved in methyl donor reactions such as vitamin B12, folate, and homocysteine were correlated with visual outcome measures in both PAE and contrast groups combined. There were no significant relationships between changes in levels of homocysteine, vitamin B12, or folate and the visual outcome measures. Change in choline levels from enrollment to the third-trimester prenatal assessment was positively related ($r = .19$, $p < .05$) to ΔHR during the habituation task but not to ΔHR in the dishabituation trials or latency of response in either condition. Similarly, changes in betaine levels trended toward a positive relationship ($r = .18$, $p < .07$) with the ΔHR during the habituation task but not to ΔHR in the dishabituation trials or latency of the response in either condition. In contrast, changes in DMG levels predicted ΔHR during the habituation task ($r = .23$, $p < .02$) and the latency of the habituation response ($r = -.20$, $p < .04$). Trends were found for the changes in DMG levels predicting ΔHR during the dishabituation task ($r = .19$, $p < .06$) and latency of the dishabituation response ($r = -.18$, $p < .08$). The results are presented in Table 3.

Discussion

This study evaluated the impact of MVM and choline supplementation to women in pregnancy that differed in their PAE histories. MVM treatment was associated with longer gestations and changes in the physical features of the infant at birth, including being heavier, longer, and having larger head circumference, which is consistent with previous research on the positive impact of MVM on pregnancy outcomes in other populations (Glenville, 2006). In contrast to our initial hypothesis, MVM supplementation alone was not found to affect neurophysiological encoding and memory, and PAE history had no impact on the effectiveness of the MVM supplement. Although there were some significant MVM group differences, these were only marginal and did not hold up to *post hoc* comparisons. In addition, the observed means on the latency of the ORs were actually in the opposite direction. Caution should be used in interpreting these negative results of MVM supplementation, because the model we utilized for the analysis covaried out pre-enrollment use of supplements and it is possible that early, first-trimester use is needed to have an impact on the outcomes assessed in this study. In addition, a recent publication from this project found that MVM supplementation positively affected infant developmental outcome scores using the Bayley Scales of Infant Development, 2nd edition (Coles et al., 2015). Previous research on MVM supplementation in other samples found positive impact on early motor (McGrath et al., 2006) and language skills (Julvez et al., 2009; Wehby & Murray, 2008), suggesting that MVM supplementation may differentially affect areas of neurodevelopmental outcome.

MVM combined with choline supplementation was found to positively affect neurophysiological encoding and memory of visual stimuli, in that the magnitude of the change in HR was greater and the speed of the response was faster, suggesting that the combination of MVM and choline supplementation resulted in significant improvements in basic attentional regulation systems in the first year of life. Using animal models of prenatal alcohol exposure, reduced activity, improved development, and improved performance on learning tasks have been found in response to choline supplementation (Ryan, Williams, & Thomas, 2008; Thomas et al., 2000, 2007, 2009; Thomas, Garrison, et al., 2004). Although

an interaction between prenatal alcohol and choline supplementation was found in an animal model of supplementation (Thomas et al., 2000), the results found in this study did not indicate that choline supplementation differentially affected infants with a positive PAE history. Instead, the results suggested that choline positively affected all participants with the exception of the magnitude of the response in the memory condition where infants without a PAE history seemed to benefit more than did those with a PAE history.

Although Zeisel has suggested that choline supplementation is likely to be most effective in a population with choline deficiency (Zeisel, 2011), the extent of choline deficiency in this sample was not assessed. There is not a defined level of choline deficiency based on blood levels, and dietary intake was not assessed in this study, so only comparison of levels within the study can be made. Relative to the question of whether or not those who are low in choline show the greatest improvements, there were no cases greater than 2 standard deviations from the mean of the sample at pre-test and only 9.3% of the sample met criteria for 1 standard deviation below the mean at baseline. Of these 9.3%, only two of these received choline intervention, making it difficult to draw conclusions regarding whether or not those who were low in choline, at least relative to the sample, had the greatest impact of the choline supplementation.

Compliance in this study was assessed by maternal self-report and by examining changes in blood plasma levels of micronutrients during the pregnancy and the relationships between these changes on the encoding and memory outcomes. Of the women assigned to the MVM group, 97.6% reported taking the supplement on a daily or almost daily basis with only two participants reporting taking the supplement less. One participant indicated that she had taken the supplement a few times a week and one participant indicated once a week or less. All mothers who were given choline supplements reported taking the capsules on a daily or almost daily basis. The lack of a pill count to confirm maternal report of micronutrient and choline use is a limitation of this study and is recommended in future investigations.

The design used in this study was an incomplete factorial design because of not having a group that received only choline supplementation without a MVM supplement. The results of this study, therefore, cannot clarify whether or not the same outcomes would be obtained if choline were given in the absence of multivitamins/minerals or what the interaction effect, if any, is of the combination of the MVM and choline. Previous research on choline supplementation alone in an animal model was not found to alter alcohol-related disruption to ORs in an olfactory habituation paradigm (Hunt et al., 2014), suggesting the interaction between the MVM and choline may be important to explore in further research. However, in terms of clinical relevance, we were able to examine the effect of choline in addition to the recommended standard of care of MVM supplementation in pregnancy compared to MVM supplementation alone.

Despite animal model evidence that choline supplementation results in improved behavioral and learning outcomes (McCann, Hudes, & Ames, 2006), the evidence of the impact of maternal levels of choline on offspring development in humans has been equivocal. Choline levels were not found to be related to 5-year cognitive status in a population of children in Alabama (Signore et al., 2008), but second-trimester levels of maternal plasma choline and

betaine, a metabolite of choline, were found to be predictive of infant cognitive status at 18 months of age in a sample of Canadian women (Wu et al., 2012). Choline levels and its metabolites were also not found to be predictive of 5-year developmental outcome in the Seychelles Child Development Nutrition Study (Strain et al., 2013) with the exception of betaine, a metabolite of choline, that predicted language functioning. The combination of results from these studies suggests that the driving component of choline's impact on development seen in the animal studies may be further along the metabolic pathway.

In this study, changes in plasma choline across pregnancy were positively related to the changes in HR during the visual stimuli habituation trials but were not related to the speed of the response or to either indices of the dishabituation trial. A trend was found for a similar relationship between betaine and **Δ**HR in the habituation condition. Changes in DMG were also positively associated with **Δ**HR and negatively associated with the speed of the OR during the visual stimuli habituation trials. Trends for similar relationships were found for DMG on the dishabituation trials, suggesting DMG may play an important role in explaining the impact of choline supplementation. Choline is metabolized to betaine, which can participate in remethylation of homocysteine to form methionine, with subsequent formation of DMG. DMG can be further catabolized to glycine. No significant relationships were found for changes in homocysteine, folate, or vitamin B12, which are involved in alternative metabolic pathways associated with choline.

Alcohol consumption has been found to impact maternal DMG levels (Thomas, Otero, Idrus, & Kelly, 2012) and gene expression associated with its metabolite glycine in offspring (Kleiber, Wright, & Singh, 2011), suggesting that this pathway may be important in our understanding of the positive effects of choline seen in animal studies of PAE. Thomas et al. (2012) found transient reductions in plasma DMG in alcohol-exposed dams during intoxication, suggesting that maternal drinking may result in periodic depletion of this basic amino acid involved in neural cell cycle regulation. The use of DMG to assist in the metabolism of alcohol results in an increased production of glycine, which in excess causes disruption to neural development (Avila, Nguyen, & Rigo, 2013). PAE has also been found to disrupt glycine receptor alpha1 subunit (Glra1) gene expression (Kleiber et al., 2011). Although glycine was initially thought to only play a role in the brain stem and spinal cord inhibitory systems, there is greater recognition of its role in regulating synaptic inhibition within the brain (Bowery & Smart, 2006) and in its role in cellular migration during neurogenesis (Avila et al., 2013). These results suggest that one mechanism by which choline supplementation may positively affect brain development is through prevention of the depletion of DMG, and thereby minimizing the overproduction of glycine during critical periods of neurogenesis. Changes in glycine were not assessed in the current study but may provide important information in future studies to assess the mechanisms by which choline supplementation impacts brain development. Using animal models, studies that directly supplement DMG may also help in further evaluating this hypothesis.

Although the results of this study are intriguing, the sample and design of the study limit the conclusions that can be drawn from it. This study does not clarify whether or not choline supplementation alone would have resulted in similar changes. The results also have to be interpreted with caution in that they may not generalize to other populations, as the women

of Rivne have unique characteristics. For example, Ukraine does not engage in folic acid enrichment of their food products, as do many other countries. Although changes in folic acid levels were not related to the obtained outcomes, interactive effects with the MVM and choline supplementation provided are unknown and the generalizability of the results to samples of women where food supplies are enriched with folic acid is not certain. The women in this region of Ukraine also have a unique history of living within an area recognized as being impacted by the meltdown of a nuclear power plant in Chernobyl (Dancause et al., 2010), which may result in differential responsivity to micronutrient supplementation.

An effect of MVM and choline supplementation was also found only on the visual stimuli and not the auditory stimuli, so it is unclear whether the results are limited to one modality or whether attributes of the visual paradigm differed from the auditory paradigm, making it a better discriminator of group differences. ORs to the visual stimuli were found to differ from those of the auditory stimuli in that OR responses were less frequent, generally slower, and characterized by less deceleration during the trough phase of the response than were the ORs to the auditory stimuli. This suggests that the encoding and discrimination of the visual stimuli were more difficult than the auditory stimuli in this particular study, and the enhanced difficultly may have allowed for greater discrimination of effects of supplementation. Differential discrimination was not found in a previous study using these stimuli (Kable & Coles, 2004), but the Ukrainian site had higher levels of ambient lighting in the test room, which may have afforded the infants more visual distractions in the testing environment and, thereby, increased the difficulty level of the task.

The human body's metabolism of nutrients involves complex interactions. A nutrient's optimal dosage is sometimes a delicate range with too much and too little of the nutrient causing potential harm. To further complicate this, the metabolic pathways associated with a given nutrient can have redundant systems, which is adaptive for an organism when a given nutrient is in short supply but can create havoc when trying to investigate the impact of the nutrient in pregnancy because of the complexity of possible interactions that may influence the outcomes. The data from this study do not support a choline deficiency model of PAE effects since, by contrast to the animal model studies, in which a PAE by choline interaction was seen (Thomas et al., 2000), choline supplementation was beneficial to both the alcohol-exposed and the contrast groups in this study. Although the nutritional content of the diet can be carefully controlled in an animal-model study, humans have the capacity to selectively consume a broad array of foods that may alter nutritional status. Unfortunately, dietary intake was not assessed in this study so the extent of differential intake of the micronutrients relative to maternal drinking behavior is unknown and should be assessed in future studies of this nature. In addition, genetic polymorphisms that are associated with variability in choline requirements should be assessed. The results did suggest that a combination of MVM and choline supplementation provided support to systems involved in basic neural development, as indicated by improvements in neurophysiological encoding and memory of visual stimuli that benefited those with and without a PAE history. Replications of these findings are needed in other samples and further explorations of the metabolic pathways associated with choline are needed to gain a better understanding of the basic mechanism of action.

Acknowledgments

This research was funded by support from NIH Research Grant #U01AA014835, funded by the National Institute on Alcohol Abuse and Alcoholism (NIAAA) and the NIH Office of Dietary Supplements (ODS) – Christina Chambers, PI. All or part of this work was done in conjunction with the Collaborative Initiative on Fetal Alcohol Spectrum Disorders (CIFASD), which is funded by grants from the National Institute on Alcohol Abuse and Alcoholism (NIAAA). Additional information about CIFASD can be found at www.cifasd.org. The authors would also like to thank Dr. John Newman, Dr. Heidrun Gross, and Jodi Ensunsa for technical help with the mass spectrometry analysis, and Charles Carlson and Michael Gedestad for the Immulite analyses. Additional support was provided by USDA Agricultural Research Service intramural projects (5306-51530-019-00D). The USDA is an equal opportunity provider and employer. Finally, the authors would like to thank the Ukrainian families who participated in this research and the wonderful staff of the Rivne Oblast Medical Diagnostic Center and the OMNINet Center.

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Highlights

- Micronutrients supplementation was studied in Ukrainian women during pregnancy
- Cardiac orienting responses assessed the impact of micronutrient supplementation
- Choline supplementation resulted in improved encoding of environmental events.
- Changes in dimethylglycine in pregnancy predicted cardiac orienting outcomes.

Table 1

Sample characteristics by prenatal alcohol use history, prenatal multivitamin/mineral supplement treatment (MVM), and choline supplement treatment

Alcohol Exposure Status	Alcohol Use				Contrast				Group Effects
	(n=35)	(n=42)	(n=23)	(n=19)	(n=46)	(n=45)	(n=27)	(n=18)	
Measure	**No MVM Treatment**	**MVM Treatment**	**MVM Only**	**MVM & Choline**	**No MVM Treatment**	**MVM Treatment**	**MVM Only**	**MVM & Choline**	**Statistics and *p* value**
Maternal Age (Yrs)	26.2 (5.2)	25.4 (6.1)	25.3 (5.9)	25.6 (6.4)	27.4 (5.3)	26.0 (4.7)	26.1 (4.1)	25.8 (5.6)	
Paternal Age (Yrs)	30.5 (5.1)	30.1 (6.0)	29.8 (5.5)	30.4 (6.6)	28.6 (5.9)	27.5 (5.5)	27.3 (4.6)	27.9 (6.8)	Alc: $F(1,164) = 6.2$, $p < .014$; Alc > No Alc Chol: $F(1,84) = 4.3$, $p < .041$; Chol > No Chol
Child's Gender (% Male)	37.1	40.5	47.8	31.6	60.9	61.4	59.3	64.7	Alc: $\chi = 8.1$, $p < .005$; Alc < No Alc
Marital Status (% with partner)	91.4	85.7	91.3	78.9	93.5	97.7	96.3	100	
Parity-Number of Children	.40 (.70)	.60 (.94)	.57 (.84)	.63 (1.1)	1.1 (1.6)	.84 (1.0)	.96 (1.1)	.65 (.79)	Alc: $F(1,164) = 7.0$, $p < .009$; Alc < No Alc
Maternal Education (years)	13.1 (2.2)	12.9 (2.6)	13.0 (2.3)	12.7 (2.9)	14.6 (1.8)	13.9 (2.3)	14.0 (2.5)	13.8 (2.0)	Alc: $F(1,164) = 13.4$, $p < .000$; Alc < No Alc Chol: $F(1,84) = 4.0$, $p < .049$; Chol < No Chol
Social Class[1]	34.9 (10.3)	33.1 (9.6)	32.9 (8.5)	33.4 (11.0)	38.5 (10.3)	36.6 (10.6)	35.9 (12.0)	37..8 (8.1)	Alc: $F(1,164) = 5.3$, $p < .022$ Alc < No Alc
Gestational Age at Enrollment (Weeks)	20.9 (6.3)	18.9 (6.8)	18.7 (6.7)	19.1 (7.1)	19.2 (6.2)	17.4 (6.3)	18.5 (7.3)	15.7 (4.0)	
Adjusted Age at Assessment (days)	192.4 (31.0)	204.5 (41.7)	209.3 (42.1)	198.6 (41.6)	200.3 (38.8)	203.3 (34.2)	209.0 (35.5)	194.8 (31.2)	
Birthweight (grams)	3088.6 (534)	3343.9 (516)	3402.3 (474)	3276.3 (566)	3300.4 (460)	3545.2 (404)	3611.5 (431)	3440 (344)	Alc: F(1,16) = 6.4, $p < .012$; Alc < No Alc Chol: $F(1,84) = 4.0$, $p < .048$; Chol < No Chol MVM: $F(1,164) = 10.1$, $p < .002$;

Alcohol Exposure Status	Alcohol Use				Contrast				Group Effects
	(n=35)	(n=42)	(n=23)	(n=19)	(n=46)	(n=45)	(n=27)	(n=18)	
Measure	No MVM Treatment	MVM Treatment	MVM Only	MVM & Choline	No MVM Treatment	MVM Treatment	MVM Only	MVM & Choline	Statistics and *p* value
									MVM > No MVM
Birth Length (cm)	50.4 (3.2)	51.8 (3.0)	52.1 (2.9)	51.4 (3.2)	51.4 (2.4)	52.3 (2.0)	52.7 (2.1)	51.8 (1.9)	MVM: $F(1,164) = 7.0$, $p < .009$; MVM > No MVM
Birth Head Circumference (cm)	33.6 (1.7)	34.1 (1.6)	34.4 (1.2)	33.8 (1.9)	33.9 (1.7)	34.8 (1.3)	35.0 (1.4)	34.4 (1.0)	MVM: $F(1,164) = 7.8$, $p < .006$; MVM > No MVM
Preconception AA/day-Mean (Oz)	.52 (.31)	.72 (.62)	.69 (.60)	.76 (.66)	.004 (.02)	0 0	0 0	0 0	Alc: $F(1,164) = 130.2$, $p < .000$; Alc > No Alc
Preconception AA/drinking day (Oz)	1.77 (1.2)	1.63 (.90)	1.65 (.91)	1.60 (.91)	.034 (.16)	0 0	0 0	0 0	Alc: $F(1,164) = 233.2$, $p < .000$; Alc > No Alc
Trimester 1 AA/day-Mean (Oz)	.018 (.045)	.030 (.054)	.025 (.042)	.035 (.067)	0 0	.00 (.001)	0 0	.00 (.001)	Alc: $F(1,164) = 20.7$, $p < .000$; Alc > No Alc
Trimester 1 AA/ drinking day (Oz)	.153 (.36)	.206 (.34)	.180 (.311)	.238 (.370)	0 0	.002 (.012)	0 0	.004 (.02)	Alc: $F(1,164) = 24.2$, $p < .000$; Alc > No Alc
Cigarettes/Day at Enrollment	1.2 (2.5)	2.2 (4.5)	3.1 (5.7)	1.0 (2.0)	.60 (3.0)	0 0	0 0	0 0	Alc: $F(1,134) = 8.3$, $p < .005$; Alc > No Alc

[1] Hollingshead Scale (Hollingshead, 2011) includes measures of educational attainment and occupation.

Table 2

Mean and standard deviations of the indices of the cardiac orienting response by group status

Alcohol Exposure Status	Alcohol Use – PAE				Contrast				Group Effects
	(n=35)	(n=42)	(n=23)	(n=19)	(n=46)	(n=45)	(n=27)	(n=18)	
Measure	**No MVM Treatment**	**MVM Treatment**	**MVM Only**	**MVM & Choline**	**No MVM Treatment**	**MVM Treatment**	**MVM Only**	**MVM & Choline**	**Statistics and *p* value**
Auditory Baseline HR (BPM)	143.6 (13.3)	142.9 (10.4)	142.9 (9.2)	142.8 (12.0)	139.2 (13.6)	140.4 (13.0)	141.8 (13.5)	138.4 (12.3)	
AUD-HAB OR %	100	100	100	100	95	97.4	95.8	100	
AUD HAB-Average HR Change	9.23 (9.4)	10.30 (7.6)	8.61 (6.7)	12.27 (8.2)	12.00 (10.1)	7.58 (7.1)	9.14 (7.31)	5.38 (6.37)	
AUD HAB-Latency	3.5 (1.57)	3.27 (1.16)	3.40 (1.14)	3.13 (1.20)	3.33 (1.68)	3.88 (1.70)	3.67 (1.79)	4.18 (1.57)	
AUD-DISHAB OR %	100	81.8	76.5	87.5	100	86.8	87.0	86.7	
AUD DISHAB-Average HR Change	.70 (9.8)	−.08 (10.4)	−3.06 (8.35)	3.27 (11.7)	−.79 (9.0)	−.46 (8.9)	.57 (9.15)	−1.94 (8.5)	
AUD DISHAB-Latency	5.88 (2.3)	5.63 (2.6)	6.09 (2.7)	5.10 (2.51)	5.73 (2.3)	5.39 (2.5)	5.36 (2.54)	5.43 (2.50)	
Visual Baseline HR (BPM)	144.2 (14.0)	146.1 (13.4)	147.1 (12.3)	145.0 (14.9)	144.2 (11.5)	140.8 (15.3)	138.1 (13.5)	145.0 (11.6)	
VIS-HAB OR %	94.7	87.5	81	94.7	90.7	82.2	77.8	88.9	
VIS HAB-Average HR Change (bpm)	.80 (6.7)	1.14 (5.8)	1.07 (5.68)	1.23 (6.16)	2.11 (5.5)	−.17 (10.8)	−2.91 (12.7)	3.93 (5.0)	Chol (Wald Chi-Square [1, 149] = 10.9, $p < .001$); Chol > No Chol
VIS HAB-Latency (sec)	5.06 (2.08)	5.45 (2.06)	5.73 (2.18)	5.11 (1.90)	5.02 (1.97)	5.39 (2.49)	5.96 (2.27)	4.52 (2.15)	Chol (Wald Chi-Square [1, 150] = 9.0, $p < .003$); Chol < No Chol
VIS-DISHAB OR %	80.0	66.7	45.5	68.8	80.6	69.2	54.5	93.3	
VIS DISHAB-Average HR Change	−1.10 (8.6)	−1.38 (9.9)	−4.64 (8.9)	1.11 (10.17)	2.67 (13.8)	−1.84 (13.9)	−5.71 (15.7)	3.83 (8.40)	Chol (Wald Chi-Square [1, 139] = 6.1, $p < .013$); Chol > No Chol
VIS DISHAB-Latency	5.72 (2.49)	6.02 (2.60)	6.00 (2.64)	6.04 (2.64)	5.06 (2.71)	5.94 (2.79)	6.71 (2.67)	4.82 (2.65)	Chol (Wald Chi-Square (1, 137) = 4.9,

Alcohol Exposure Status	Alcohol Use – PAE				Contrast				Group Effects
	(n=35)	(n=42)	(n=23)	(n=19)	(n=46)	(n=45)	(n=27)	(n=18)	
Measure	No MVM Treatment	MVM Treatment	MVM Only	MVM & Choline	No MVM Treatment	MVM Treatment	MVM Only	MVM & Choline	Statistics and *p* value
									$p < .027$; Chol < No Chol

Table 3

Correlations between cardiac OR outcomes and choline and its metabolites in PAE and contrast groups combined

	Visual Habituation/Dishabituation Paradigm			
	Habituation Average HR[1] Δ	Dishabituation Average HR[1]	Habituation Latency	Dishabituation Latency
ΔCholine[2]	.19*	ns	ns	ns
ΔBetaine[2]	.18T	ns	ns	ns
ΔDMG[2]	.23*	.19T	−.20*	−.18T

[1] Indicates change in HR from baseline to the average between 3–8 sec post-stimulus onset.

[2] Indicates the change in nutrient from enrollment into the study until the subsequent prenatal follow-up

* $p < .05$,

T $p < .10$