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Supplementation with the methyl donor betaine prevents congenital defects induced by prenatal alcohol exposure

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Abstract

Background—Despite decades of public education about dire consequences of prenatal alcohol exposure, drinking alcohol during pregnancy remains prevalent. As high as 40% of live-born infants exposed to alcohol during gestation and diagnosed with Fetal Alcohol Syndrome have congenital heart defects that can be life-threatening. In animal models, the methyl donor betaine, found in foods such as wheat bran, quinoa, beets and spinach, ameliorated neurobehavioral deficits associated with prenatal alcohol exposure (PAE) but effects on heart development are unknown.

Methods—Previously we modeled a binge drinking episode during the first trimester in avian embryos. Here we investigated whether betaine could prevent adverse effects of alcohol on heart development. Embryos exposed to ethanol with and without an optimal dose of betaine (5 μ M) were analyzed at late developmental stages. Cardiac morphology parameters were rapidly analyzed and quantified using optical coherence tomography. DNA methylation at early stages was detected by immunofluorescent staining for 5-methylcytosine in sections of embryos treated with ethanol or co-treated with betaine.

Results—Compared to ethanol-exposed embryos, betaine-supplemented embryos had higher late-stage survival rates and fewer gross head and body defects than seen after alcohol exposure alone. Betaine also reduced the incidence of late-stage cardiac defects such as absent vessels, abnormal atrio-ventricular (AV) valves, and hypertrophic ventricles. Furthermore, betaine co-treatment brought measurements of great vessel diameters, interventricular septum (IVS) thickness, and AV leaflet volumes in betaine-supplemented embryos close to control values. Early-

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stage 5-methylcytosine staining revealed that DNA methylation levels were reduced by ethanol exposure and normalized by co-administration with betaine.

Conclusions—This is the first study demonstrating efficacy of the methyl donor betaine in alleviating cardiac defects associated with PAE. These findings highlight the therapeutic potential of low-concentration betaine doses in mitigating PAE induced birth defects and has implications for prenatal nutrition policies, especially for women who may not be responsive to folate supplementation.

Keywords

betaine; prenatal; prevention; methylation; cardiac

Introduction

Maternal alcohol consumption during pregnancy remains a prevalent problem in the North America, even with the widespread and longstanding understanding that prenatal alcohol exposure (PAE) at any stage of pregnancy is harmful to the developing embryo and fetus. A recent survey from the CDC reported that 10% of pregnant women in the USA drink alcohol, while 3% engage in binge drinking (Tan et al., 2015). The prevalence of FASD (Fetal Alcohol Spectrum Disorders), an umbrella term encompassing all alcohol-related defects, ranges from 24–48 cases per 1000 children (May et al., 2014) but these numbers are often higher in certain communities and are suspected to be under-diagnosed. Alcohol-associated neurodevelopmental and craniofacial defects are well characterized (Williams and Smith, 2015) and reviews of the literature have also revealed a substantial number of cardiac defects in FASD patients (Burd et al., 2007; Popova et al., 2016). When Popova and colleagues (Popova et al., 2016) conducted a meta-analysis, they noted that the prevalence of cardiac defects including valvuloseptal and outflow anomalies was as high as 40–50% in patients diagnosed with Fetal Alcohol Syndrome (FAS), the most severely affected of the FASD spectrum.

In previous studies, we established a model of PAE in an avian model where binge-like concentrations of alcohol were introduced at gastrulation stages (Karunamuni et al., 2014; Karunamuni et al., 2015). This PAE model is similar to that used by others in which fertilized chicken eggs were injected with ethanol (Smith, 2008; Serrano et al., 2010) and phenocopies findings from PAE models in other animal models as well as characteristics of FAS individuals (Wilson and Cudd, 2011; Suttie et al., 2013). This stage is equivalent to the 3rd week of pregnancy when a woman may not have recognized nor confirmed that she is pregnant. Alcohol exposure severely reduced survival and increased the frequency of defects of the head and body (failure of head and body wall closure) in our avian model, and contributed to the development of a spectrum of FASD-related cardiac anomalies that would require surgical correction and long-term medical care in human patients. In theory, FASD-associated birth defects are 100% preventable as long as women abstain from alcohol consumption either while trying to conceive or during pregnancy, as recommended by the CDC and American Academy of Pediatrics (Williams and Smith, 2015; Green et al., 2016). However this may not be practical especially since drinking rates among pregnant women have remained constant over the last few decades (Denny et al., 2009; Marchetta et al., 2012;

Tan et al., 2015) despite our expanding knowledge of the inherent risks that alcohol can pose to the developing fetus. Therefore, in addition to continuing campaigns to educate the public on the hazards of PAE, it is crucial that we continue to identify safe, effective supplements and/or vitamins that can prevent alcohol-associated defects.

Folate or folic acid is already present in prenatal supplements and as a food supplement in North America for protection against congenital neural tube defects, including those associated with alcohol exposure (Serrano et al., 2010; Shi et al., 2014; Bailey et al., 2015). However, current recommended supplement levels may not be sufficient to prevent alcohol-induced congenital heart defects (CHDs) (Huhta and Linask, 2015). Increased folate intake could be problematic in some patient populations since it has been linked to higher cancer risks (Cole et al., 2007; Ebbing et al., 2009). Another consideration is that certain women may not be responsive to folate supplementation, especially if they carry gene variants, such as those found in the gene for methylenetetrahydrofolate reductase (MTHFR), an enzyme involved in folic acid metabolism. The variant *C677T* reduces enzyme activity as much as 30% and its frequency can be as high as 20% in certain populations (Botto and Yang, 2000). Thus it would be beneficial to identify alternative low-dose supplements in preventing birth defects including CHDs.

Potential candidates include choline and its metabolite betaine, which like folate, function as a methyl donor in the methionine-homocysteine cycle (Figure 1) and are found naturally in foods such as liver, broccoli, spinach, beets, shrimp and quinoa (Craig, 2004; Young et al., 2014). Choline plays a role in several processes, including neurotransmission (via acetylcholine), cell membrane integrity (as phosphatidylcholine), and methyl group donation via its metabolite betaine (Young et al., 2014). A recent national survey (Zeisel and da Costa, 2009) found that less than 10% of the US population, including pregnant women, are getting their adequate intake (AI) of choline (450 mg/day) despite the fact that the metabolic demand for choline is increased during pregnancy especially in relation to fetal brain development (Zeisel, 2005). There is no recommended daily dose for betaine but as a methyl donor it serves vital purposes. Among the many roles for betaine, it donates methyl groups for the metabolism of homocysteine to methionine, an important step in the progression of the one-carbon cycle which methylates DNA and proteins and thus regulates the expression of genes that are crucial for embryogenesis (Miller, 2003).

It has already been established that prenatal alcohol exposure (PAE) can alter the DNA methylation program and one-carbon metabolism in mouse models (Liu et al., 2009; Zhou et al., 2011; Ngai et al., 2015), which could contribute to abnormal fetal development. Using methyl donors such as choline and betaine in alcohol exposure models has already shown great promise in alleviating associated anomalies. Bekdash and colleagues (Bekdash et al., 2013) showed that gestational choline supplementation in rats prevented neuronal damage induced by alcohol, while pre- or peri-natal choline ameliorated behavioral and learning deficits in a PAE rat model (Thomas et al., 2004; Ryan et al., 2008; Thomas et al., 2010). Prenatal betaine supplementation also protected against alcohol-related neurodegeneration in rat pups (Kusat Ol et al., 2016), and co-culture with betaine reduced alcohol-induced apoptosis in cultures of neural crest cells (Wang and Bieberich, 2010), which are thought to be severely impacted by fetal alcohol exposure (Smith et al., 2014). However, detailed

analysis of the effects of such supplementation on cardiac morphogenesis is sadly lacking, which we will address in this study. Previously in our model of PAE, survival rates and defect rates of the head and body were assessed at late stages to compare cohorts of embryos (Karunamuni et al., 2015). In this study, analysis of these parameters was conducted for varying concentrations of betaine and choline with alcohol exposure (Table 2, Supplementary Table 1), revealing that betaine was able to improve survival and reduce defect rates at a lower concentration (5 μ M) than choline (100 μ M). Thus our subsequent analyses of cardiac development and DNA methylation focused on betaine as a rescue agent. In our earlier study (Karunamuni et al., 2015), we used optical coherence tomography (OCT) imaging technology that allowed us to rapidly capture data and streamline analysis to demonstrate and quantify the different cardiac defects associated with ethanol exposure. This is now the first OCT study to investigate the efficacy of a low-dose supplement (betaine) in preventing PAE-associated heart defects. These findings could have the potential to significantly impact public health policy related to prenatal nutrition recommendations.

Materials and Methods

Ethics Statement

IACUC approval was not required for this study which involves the use of avian embryos that were collected at Embryonic Day 8. Quail embryos typically hatch at Embryonic Day 17. According to IACUC guidelines at Case Western Reserve University, the Policy on the use of Avian Embryos states that “If embryos will be sacrificed prior to 3 days before hatching, the research will not be subject to IACUC review.”

Ethanol Exposure and Betaine Supplementation

Fertilized quail eggs (*Coturnix coturnix communis*; Boyd’s Bird Company, Inc., Pullman, WA) were incubated in a humidified incubator (G.Q.F. Manufacturing Co., Savannah, GA) at 38°C until HH Stage 4–5 (gastrulation), at which stage the embryo has been found to be susceptible to the induction of congenital heart defects (Serrano et al., 2010; Karunamuni et al., 2014; Karunamuni et al., 2015). Solutions were injected into the egg using an insulin needle (28 gauge) and holes were sealed with tape before eggs were re-incubated. Ethanol-exposed eggs were injected with 40 μ l of 50% ethanol (v/v with saline) and control eggs were either injected with 40 μ l of saline or left intact. Ethanol dosage was based on previously published protocols (Fang et al., 1987; Bruyere and Kapil, 1990; Bruyere et al., 1994; Serrano et al., 2010; Karunamuni et al., 2014; Karunamuni et al., 2015) as being equivalent to one binge-like drinking episode in humans (4–5 standard drinks on one occasion) and has reliably produced FAS-associated CHDs (Serrano et al., 2010; Karunamuni et al., 2014; Karunamuni et al., 2015). The ethanol dose [40ml of 50% ethanol (v/v)] injected into an 8.8 ml (wet volume) quail egg is equivalent to 0.179 % (g/dL) or 40 mM. Intoxicated individuals have a blood alcohol content (BAC) of 0.08%–0.268% (g/dL) (Urso et al., 1981; NIAAA, 2004). Thus, the dose we use is within range of BACs detected after 1 bout of heavy alcohol consumption. The dose experienced by our avian embryos is relevant to the dose experienced by a human fetus because alcohol passes easily through the placenta and the BAC of the mother would be similar to that experienced by the embryo/fetus. Investigators using the pregnant sheep model concluded that “the placenta does not

significantly retard maternal/fetal transfer of ethanol.” after comparing the alcohol levels in the blood of the mother sheep and the fetal sheep (Lafond et al., 1985).

Betaine supplemented eggs were injected at gastrulation with 40 μ l of 50% ethanol along with varying concentrations of betaine (Table 2) (Sigma Aldrich; St. Louis, MO) to establish a dose response curve after which an optimal dose of betaine (5 μ M in the 40 μ l) was chosen based on improved survival and head/body defect rates at late stages (HH Stage 34). This is equivalent to a dose of 0.023 μ M. A single injection of 40 μ l of this mixture of 50% ethanol and 5 μ M betaine was used per egg in the subsequent rescue experiments.

Statistical Analyses

The statistical analysis of the Table data are explained in the result section.

Optical Clearing

Cohorts of control, ethanol-exposed, and betaine-supplemented embryos were allowed to develop until HH stage 34, when the heart would normally acquire a four-chambered morphology and developed cardiac valve leaflets. Hearts from the different exposure groups were dissected, incubated in 0.5M potassium chloride (to relax the heart muscle), washed in phosphate buffered saline, and fixed in 10% formalin overnight. Samples were then optically cleared using the Clear-T protocol, involving a series of formamide solutions (20% formamide for 30 minutes, 40% for 30 minutes, 80% for 2 hours, and 95% for 30 minutes and then overnight) (Kuwajima et al., 2013; Karunamuni et al., 2015), before being imaged with the OCT system.

OCT Imaging of Hearts

Embryos were imaged using a custom-built spectral-domain OCT system with a quasi-telecentric scanner (Hu and Rollins, 2005; Hu and Rollins, 2007; Ma et al., 2014; Peterson et al., 2014). The OCT light source is a superluminescent diode centered at 1310 nm with a full-width at half-maximum bandwidth of 75 nm. The OCT system uses a linear-in-wavenumber spectrometer and a line-scan camera with a line rate of 47 kHz (Hu and Rollins, 2007). The axial resolution as well as the lateral resolution is approximately 10 μ m in tissue (Hu and Rollins, 2007). Thus OCT imaging offers high spatial resolution and extremely high temporal resolution, while providing appropriate penetration depth (1–3 mm in cardiac tissues) (Drexler, 2015). For this study, embryo hearts were placed in 95% formamide in a small plastic dish for imaging. OCT volumes (1000 lines/frame, 400 frames/volume, 4mm $\times$ 4mm field of view) were recorded for each sample. For each heart, 3 volumes were taken: two from the front focusing on the great vessels and the atrio-ventricular (AV) valves respectively, and one from the back also focusing on the AV valves.

Post-processing and Analysis of OCT Images

Customized MATLAB programs (MathWorks; Natick, MA) were used to generate OCT images from the raw data, which were then analyzed with AMIRA software (FEI Visualization Sciences Group; Burlington, MA). The frontal plane was used for the identification of septal defects while the transverse plane was used for the identification of outflow defects and the measurement of vessel diameters and septum thicknesses, as

previously (Karunamuni et al., 2015). Our co-author Dr. Strainic, a pediatric cardiologist with expertise in echocardiography, also analyzed the OCT images to confirm identification of heart defects. Vessel diameters were measured proximally after the immediate branching into the individual arteries. The cardiac valves were manually segmented in AMIRA in order to measure their volumes. Statistical analyses of vessel diameters and valve volumes were performed with ANOVA using MATLAB.

5-Methylcytosine Immunofluorescence

HH stage 12 quail embryos (3 controls, 3 ethanol-exposed embryos and 3 betaine/ethanol-exposed embryos) were collected and fixed in 4% PFA, washed in PBS and incubated in sucrose overnight. This stage corresponds to a time when cardiac neural crest cells (CNCCs) are migrating laterally from the dorsal neural tube towards the circumpharyngeal ridge. Embryos were then cryo-sectioned transversely (10µm thickness). Sections were chosen for each embryo at similar transverse levels, caudal to the end of the otic vesicle (regions of CNCC migration), and washed 3 times in phosphate buffered saline (PBS) for 5 minutes each. Sections were incubated at room temperature with blocker (0.1% Triton-X, 5% normal goat serum, diluted in PBS) for 1 hour to permeabilize tissue and then overnight at 4°C with the primary antibody (anti-5-methylcytosine antibody from Genway Biotech; San Diego, CA, diluted 1:1000 in blocker). Sections were then washed in PBS 3 times for 5 minutes each and incubated with the secondary antibody (Alexa Fluor 488 Goat Anti-mouse from Molecular Probes; Eugene, OR, diluted 1:1000 in blocker) for 2 hours at room temperature. Sections were washed again in PBS and coverslipped using DAPI-containing mounting medium (Vector Labs; Burlingame, CA), after which they were imaged using an inverted Nikon Diaphot 200 microscope and QCapture Pro software (Surrey, BC; Canada). ImageJ (National Institutes of Health; Bethesda, MD) was used to count 5-methylcytosine-positive cells and also measure the intensity of the DAPI-positive nuclei. Relative numbers of 5-methylcytosine-positive cells were compared amongst the 3 cohorts using ANOVA.

Results

At later developmental stages (HH Stage 34, four-chambered heart), control embryos, either uninjected or saline-treated, had high survival rates (89%) and low defect rates (0%) of the head and body (Table 1) consistent with our previous published findings. In contrast, ethanol-injected embryos had low survival rates (46%) and high defect rates (50%), often presenting with anomalies in eye development and cranial/chest wall closure as we previously found (Karunamuni et al., 2015).

The overall difference in the numbers of survivors among the three groups uninjected, saline or ethanol-injected, was statistically significant using both the Fisher's Exact test ($p=2.075 \times 10^{-13}$) and the Chi-square test ($p=2.56 \times 10^{-12}$) as was the difference in the numbers of survivors with defects from these three groups. To examine where the difference came from, we performed an *equivalence test* (Chow et al., 2007) for the difference ϵ between the two control groups: 'Uninjected' and 'Saline' treatment groups, and *Fisher's Exact and Proportional tests* for all possible pairs of the three groups. The equivalence test supported the bioequivalence of the 'Uninjected' vs. 'Saline' groups; specifically it rejected the null

hypothesis $|e| \geq \delta$ in favor of the alternative hypothesis $|e| < \delta$ for a small $\delta=0.005$ at the significance level of $\alpha=0.05$. By both the Fisher's exact and proportion tests (i.e. 2 types of tests) with a Bonferroni correction for the multiplicity, we found that the numbers of survivors, and the numbers of survivors with defects (i.e. 2 types of measures), were strongly and significantly different between the group treated with 'Ethanol' and any of the two controls groups: 'Uninjected', and 'Saline' (i.e. 2 different contrasts) with $p<0.00017$ for all 8 comparisons ($8=2$ contrasts $\times$ 2 tests $\times$ 2 measures), but not between the two controls ($p=1$ by both tests). In summary, injecting ethanol strongly and statistically affected the embryos in comparison to injecting saline or providing no injection (Table 1).

A dose response curve (Table 2) was obtained for the methyl donor betaine which was introduced with the ethanol injection at gastrulation stages (HH stage 4–5). The lowest optimal betaine concentration in ethanol was revealed to be 5 μ M (calculated for the 40 μ L injection), which induced improvements in both survival and defect rates for the embryos (73% and 27% respectively). To examine the toxicity of this concentration, the 5 μ M betaine dose was injected in only saline ($n = 26$ embryos), resulting in survival and defect rates that fell within control ranges (81% and 0% respectively). The methyl donor choline, from which betaine is metabolized, was also used to supplement the ethanol-exposed embryos with similar positive effects but at higher non-toxic concentrations (100 μ M) (Supplementary Table 1). Further experiments were therefore conducted using betaine at a concentration of 5 μ M because low-dose supplements could present less risk for the expectant mother.

Embryos from three experimental groups ('Control', 'Ethanol', and 'Ethanol + 'Betaine') were then collected so that their hearts could be analyzed for late-stage cardiac defects using OCT. We chose to harvest a smaller cohort of ethanol-exposed embryos since they still presented with a full complement of defects and significantly different phenotypes in comparison to control embryos. 98% (42/43) of the controls developed normal hearts with no apparent defects, and the one abnormal embryo exhibited abnormal ventricular rotation that was not found in any of the other groups (Table 3). In contrast, only 27% (3/11) of the ethanol-exposed group had normal hearts. The most prevalent ethanol-induced cardiac defect in this cohort was the absence of certain great vessels at a level proximal to the heart where they would normally branch (Table 3), involving the right pulmonary artery (RPA), the right brachiocephalic artery (RBA), or both (Figure 2A–B). Other associated anomalies included abnormal atrio-ventricular (AV) valve morphologies (Figure 3A–D), hypertrophic right ventricles (thicker walls with smaller right ventricular chambers) (Figure 4A–B), ventricular septal defects, and misalignment of the aorta. With betaine supplementation however, 89% (32/36) of ethanol-exposed embryos developed normal hearts, indicating a partial rescue of cardiac defects even in the presence of ethanol. These embryos did not develop abnormal AV valves or VSDs, and also presented with reduced prevalence of missing vessel defects and hypertrophic right ventricles (Table 3). One ethanol+betaine embryo developed a tiny collateral artery at the level where we analyzed the great arteries. These findings suggest that betaine can protect substantially against ethanol-induced impairments in the right ventricle and great vessel morphogenesis, as well as AV valve and interventricular septum (IVS) formation.

Previously, we were able to use OCT to rapidly delineate and quantify subtle, complex anomalies in cardiac structures that had been affected by ethanol (Karunamuni et al., 2015), including great vessel diameter (lumen and outer), left AV valve volume, and interventricular septum (IVS) thickness. In this study, we used OCT in a similar way to economically and rapidly determine the effect of betaine on these parameters in the PAE model. In comparison to both the controls and ethanol+betaine embryos, ethanol-exposed embryos had significantly reduced lumen diameters (Figure 2) for the LPA, RPA, LBA, RBA, and AA (24.4%, 36.0%, 14.8%, 28.5%, and 20.6% decrease respectively, compared to controls, $p < 0.05$). However, only the LPA, RPA, and AA displayed significant reductions in outer diameter after ethanol exposure (14.9%, 22.0%, and 14.7% decrease respectively, compared to controls, $p < 0.05$). In contrast, there was no significant difference in the lumen or outer diameter for the great vessels in the ethanol+betaine embryos compared to control specimens (Figure 2). Furthermore, ethanol-exposed embryos had significantly reduced mural, septal, and total AV valvular volumes compared to controls and betaine-supplemented embryos (25.4% decrease, 24.5% decrease, and 25.4% decrease, respectively, compared to controls with $p < 0.05$) (Figure 3). Mural AV valve volumes were not significantly different for the control and ethanol+betaine groups, but septal valvular volumes were significantly increased in the betaine-exposed embryos (14.5% increase compared to controls, $p < 0.05$). However, there no significant increases in total valvular volume (8.9% increase compared to controls, $p > 0.05$) (Figure 3). Finally, ethanol-exposed embryos had significantly reduced IVS thicknesses compared to controls and betaine-supplemented embryos (24.7% reduction compared to controls, $p < 0.05$), but ethanol +betaine supplemented embryos displayed normalized IVS numbers (Figure 4).

For the statistical analysis of the data presented in the first three columns of Table 3, we compared the differences in 3 treatments (Control (C), Ethanol (E), Ethanol+Betaine (E+B)) in terms of the numbers of normal hearts and 7 cardiac defects, and the numbers at the 2 locations (Valve Defects vs. Vessel and Septation Defects), as well as the number of defects per embryo, in addition to an equivalence test between the numbers of normal hearts from the Control (C) and the Ethanol+Betaine (E+B) groups. Specifically, the bioequivalence between the Control group and the Ethanol+Betaine group in the numbers of normal hearts was found at $\delta=0.09$ and $\alpha=0.05$. Although this bioequivalence ($\delta=0.09$) is not as strong as that of the two control groups: Uninjected and Saline, in Table 1 ($\delta=0.005$), it showed that adding betaine helps to reduce cardiac defects in ethanol-exposed embryos, which is confirmed by detailed comparisons below.

Across the three treatments (C, E, E+B), there were 8 comparisons in terms of the numbers of normal heart and 7 cardiac defects. Fisher's exact test with a Bonferroni correction indicated that there were statistically significant differences among the three treatments in the numbers of "normal heart", defects due to "missing great vessel" and "abnormal valve morphology" (noted as ** in Column 5 in Table 3), and less but still significant differences in the numbers of defects identified as "hypertrophic right ventricle" and "ventricular septal defects" (*). There was no evidence from this data for significant differences in terms of other defects, which may require a larger study to detect.

Since there was bioequivalence between the Control and Ethanol+ Betaine groups we reasoned that the differences detected in the three-treatment comparison should come from the difference between E and E+B groups, or between E and C groups. To confirm this, comparisons between E and E+B were conducted using the Fisher's exact test across the 8 numbers (1 normal and 7 defects) (Table 3), and also for the numbers (x) of defects per embryo with $x = 0, 1$, and 2 . The significant results between E and C would be implied by those between E and E+B (Column 6 in Table 3) since the differences between E and C are clearly bigger than those between E and E+B. However, we did compare the overall difference among the three groups and both differences between E and C, and between E and E+B groups, using the proportional test for the two locations (valve or vessels). In the 8 comparisons, the numbers of 'Normal Hearts' ($p=0.00019$), or the numbers of embryos having a 'Missing Great Vessel' ($p=0.0049$) were found to be clearly significantly different between the E and E+B groups with a Bonferroni correction while the numbers with "Abnormal Valve Morphology" were significantly different but with a larger P-value (0.01018). We then separated defects by location (valve and vessel) and conducted the proportion test for all 3 groups; these were significantly different with p-values of 0.000159 and 0.0002368. We hence tested for 2 groups, between E and E+B ($p=0.0277$ and $p=0.0255$), and between E and C ($p=0.0151$ and $p=0.00049$) for both valve and vessel defects, respectively. In summary, there is strong statistical evidence that adding betaine reduced the number of a variety of cardiac defects caused by ethanol.

After late-stage cardiac parameters were analyzed, it was apparent that the methyl donor betaine had alleviated the impact of ethanol on cardiac morphogenesis at HH stage 34. These findings raised the question of whether betaine had affected DNA methylation at earlier developmental stages as part of its role as a rescue agent for alcohol exposure. To address this issue, HH stage 12 embryos were collected for each treatment group, corresponding to a time when cardiac neural crest cells (CNCCs, with crucial roles in cardiac development) are actively migrating away from the dorsal neural tube. Transverse sections were selected from CNCC migration locations (just caudal to the otic vesicle) and stained for the DNA methylation marker 5-methylcytosine (Zhou et al., 2011). Relative numbers of 5-methylcytosine-positive cells were obtained using ImageJ software and analyzed for comparison. In comparison to controls, there was a trend towards reduced levels of 5-methylcytosine staining in the ethanol-exposed group with measurements being similar between the control and betaine-supplemented embryos (Figure 5). A statistically significant difference was detected between the ethanol-exposed and the ethanol plus betaine groups ($p < 0.05$) with the addition of betaine bringing the levels to control levels. This is consistent with early-stage DNA methylation being altered after ethanol exposure and could contribute to the progression of defects observed at later stages. The details of which cell types and which genes are affected by the methylation changes will be the subject of future studies.

Discussion

In brief, the beneficial effects of supplementation of the methyl donor betaine in our avian model of PAE were manifold (Figure 6). Within 24 hours of gastrulation (injection stage), immunostaining for the DNA methylation marker 5-methylcytosine in regions of active

cardiac neural crest cell migration confirmed that cellular methylation levels were reduced after alcohol exposure but normalized with the co-introduction of betaine. This finding suggests that the exogenous addition of betaine protected DNA methylation even with ethanol exposure. At later developmental stages (4-chambered heart), betaine/alcohol-exposed embryos showed prominent improvements in survival rates and defect rates of the head and chest wall. Furthermore, these embryos presented with more normal hearts than the group exposed to alcohol alone, with reductions in cardiac defects such as absent vessels, abnormal AV valve morphologies, and ventricular defects. Cardiac parameters such as IVS thickness, great vessel diameters and AV valve volumes in the betaine/alcohol group were also normalized, with the exception of the septal AV leaflet volume which was increased compared to controls. This mild leaflet enlargement did not appear to negatively impact embryo survival or other features of development, and could be the result of compensatory mechanisms that were activated in response to the ethanol insult.

Despite these novel and promising results, it is important to consider the limitations of the study. Our study design was limited to one timepoint of betaine administration at the same time as the ethanol exposure. In an ideal situation the mother would already be on a regimen including betaine supplements before having a binge drinking episode in the early stages of pregnancy. However, our investigations revealed that two separate egg injections significantly impaired embryo health and survival, thus necessitating reliance on a single injection of the mixture of alcohol and betaine at gastrulation. Thus, we did not test whether pre-conception intake of betaine might be even more effective in preventing congenital defects. In our model, supplementing with methyl donors such as choline or betaine improved alcohol-impacted survival rates and defect rates approaching but not achieving the numbers for control specimens, which suggests that another mechanism crucial to embryogenesis may also have been deleteriously affected by alcohol. The details of all the mechanisms by which betaine had its positive effects on preventing alcohol-induced defects was not investigated here. These considerations do not however change the fact that betaine, a naturally occurring alternative to folate, had positive effects on reducing PAE-related defects and should be investigated further in other animal models and eventually the clinical setting.

Processes other than DNA methylation may also have been impaired by ethanol exposure. One possibility is histone modification. *In vitro* studies by Zhong and colleagues (Zhong et al., 2010) revealed that high levels of ethanol exposure in cardiac progenitor cells altered both histone H3 acetylation and subsequent expression of genes related to heart development. Other researchers found that ethanol exposure in fetal neuronal stem cells altered levels of H3K4me3 and H3K27me3, which can affect transcription and expression of genes underlying the progression of FASD-associated defects (Veazey et al., 2013). Another promising line of investigation could target mechanisms of oxidative stress. Typically, reactive oxygen species (ROS) are byproducts of normal cellular metabolism in the mitochondria and cytoplasm. However, the presence of environmental stressors or toxins such as ethanol can result in elevated ROS levels, which can overwhelm endogenous antioxidant defenses and lead to cell damage and death through the oxidation of DNA, proteins, and lipids (Brocardo et al., 2011). Betaine has also been shown to repair damage to sulfur containing amino acids such as SAM (Zhang et al., 2016) that have direct antioxidant

activities as ROS scavengers. So while betaine does not serve as an antioxidant itself, it indirectly promotes antioxidant activities. Furthermore, cardiac neural crest cells, which are crucial to normal heart development and function, have been found to exhibit low levels of antioxidant enzymes and are thus particularly vulnerable to increased oxidative stress (Davis et al., 1990; Morgan et al., 2008; Smith et al., 2014). It has also been well documented that the introduction of antioxidants in models of PAE can alleviate certain alcohol-related defects due to their ability to quench ROS and other free radicals (Reimers et al., 2006; Brocardo et al., 2011; Chen et al., 2013; Joya et al., 2015). Examples of such compounds with antioxidant capacity include vitamins C and E, sulforaphane, catalase, superoxide dismutase, epigallocatechin gallate, and glutathione. It might therefore be advantageous to consider combining methyl donors and antioxidants in a multi-supplement that could provide enhanced protection against PAE-associated birth defects. This method of using avian embryos and OCT allows rapid and economical screening for compounds and combinations of compounds with potential to prevent PAE induced defects.

Betaine has many roles in addition to serving as a methyl donor for DNA and protein methylation that may explain its effect in preventing congenital heart defects (Day and Kempson, 2016). For example, the downstream product SAM can be synthesized to phosphatidylcholine by the enzyme phosphatidylethanolamine N-methyltransferase, leading to membrane integrity and enhanced signaling. As a methyl donor for the folate cycle, betaine could also impact purine and pyrimidine synthesis that is important for DNA and RNA synthesis crucial during embryogenesis.

In conclusion, this is the first study to demonstrate that supplementation with betaine, found naturally in several foods, can alleviate many of the negative effects of PAE, such as the prevention of congenital heart defects which often have a devastating impact on survival and quality of life. These findings may potentially inform prenatal nutrition recommendations for women who have ingested or are likely to ingest alcohol in the early stages of pregnancy, women who have a deficiency in folic acid cycle enzyme activity, or those who are more vulnerable to altered folic acid levels. The use of methyl donors such as betaine could also have therapeutic value in the ongoing development of a multi-supplement for further protection against FASD birth defects. This approach does not diminish the need for the continued public education of the community on the risks of alcohol exposure during any part of gestation (Williams and Smith, 2015; Green et al., 2016).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Diagram of the methionine-homocysteine cycle. Line arrows represent metabolism. Block arrows represent the donation of methyl groups. 5-MTHF = 5-methyltetrahydrofolate, SAM = S-adenosylmethionine, SAH = S-adenosylhomocysteine.

Figure 2.

Vessel defects induced by ethanol in HH Stage 34 quail embryos. (A) Transverse OCT slice in a control embryo depicting the five great vessels that typically branch off close to the base of the heart: the left pulmonary artery (LPA), the right pulmonary artery (RPA), the left brachiocephalic artery (LBA), the right brachiocephalic artery (RBA), and the aortic arch (AA). Scale bar = 0.5 mm. (B) Example of vessel defect where there are only three vessels at the proximal branching points: the LPA, the LBA, and the AA. (C) Comparison of lumen and outer diameters of the great vessels for control (n = 43), ethanol-exposed (n = 11), and ethanol+betaine supplemented (Eth+Bet, n = 36) embryos, black bars represent standard error of the mean (SEM). Statistical analysis was performed using standard ANOVA (single factor) using the function of MATLAB.

* for the ethanol group indicates statistical significant differences compared to controls or ethanol+betaine group, in both cases $p < 0.05$.

Figure 3.

Left atrio-ventricular (AV) valve defects induced by ethanol in HH Stage 34 quail embryos. (A) AMIRA 3-D reconstruction of septal (blue) and mural (purple) leaflets of left AV valve in control embryo. Valve leaflets are typically at the same level, making contact with each other in a V-shaped pattern. (B) Rotated angle of valve reconstruction shown in panel A. (C) AMIRA 3-D reconstruction of abnormal valve in ethanol-exposed embryo, where the mural leaflet is resting on top of the septal leaflet, indicating abnormal apposition. A rotated angle of this valve is shown in (D) for comparison with panel (B). (E) Comparison of mural, septal, and total valvular volumes for control (n = 43), ethanol-exposed (n = 11), and ethanol +betaine supplemented (Eth+Bet, n = 36) embryos, black bars represent SEM. * for ethanol group indicates statistical significance compared to controls or ethanol+betaine group, p < 0.05. Statistical analysis was performed using standard ANOVA (single factor) using the function of MATLAB. * for ethanol+betaine group indicates statistical significance compared to controls, p < 0.05.

Figure 4.

Ventricular defects induced by ethanol in HH Stage 34 quail embryos. (A) Sagittal OCT slice of control embryo heart with normal sized right ventricular chamber, scale bar = 0.5 mm. (B) Example of ethanol-exposed embryo heart with hypertrophic right ventricle (under-developed right ventricular chamber). Yellow dotted lines delineate right ventricular chamber. (C) Comparison of interventricular septum (IVS) thicknesses for control, ethanol-exposed, and ethanol+betaine supplemented (Eth+Bet) embryos, black bars represent SEM. Statistical analysis was performed using standard ANOVA (single factor) using the function of MATLAB.

* indicates statistical significance compared to controls or ethanol+betaine group, $p < 0.05$.

RV = right ventricle.

Figure 5.

Analysis of 5-methylcytosine levels in untreated and experimental embryos. (A) 5-methylcytosine immunofluorescence in transverse section of HH Stage 12 embryo caudal to the otic vesicle, scale bar = 100 mm. (B) DAPI staining in the same section. (C) Relative levels of 5-methylcytosine were assessed as the number of 5-methylcytosine-positive cells in the section normalized to the total intensity of DAPI staining in the same section, as quantified by ImageJ software. 5-Methylcytosine (5-MeC) levels were reduced with ethanol exposure, but with co-administration of betaine, 5-MeC levels were closer to control

(untreated) levels. Statistical analysis was performed using standard ANOVA (single factor) using the function of MATLAB. * indicates significance compared to ethanol + betaine group, with $p < 0.05$. NT = neural tube.

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Figure 6. Summary of the effects of betaine supplementation on ethanol-induced congenital defects at early and late developmental stages. IVS = interventricular septum.

Table 1

Survival and defect rates for control and ethanol-exposed embryos.

Treatment	Number of Survivors	% of survivors with head/body defects
Uninjected (n = 47)	42 (89%)	0 (0%)
Saline (n = 35)	31 (89%)	0 (0%)
Ethanol (n = 48)	22 (46%) *	11 (50%) *

* values different from Uninjected and Saline cohorts at $p < 0.00017$.

Table 2
Survival and defect rates for ethanol-exposed embryos with betaine supplementation

Eth = Ethanol. 5 μ M of betaine was the lowest dose that provided optimal rescue of ethanol-impacted survival and defect rates.

Treatment	Survivors	% of survivors with head/body defects
1 μ M betaine/Eth (n=30)	19 (63%)	8 (42%)
5 μM betaine/Eth (n = 62)	45 (73%)	12 (27%)
10 μ M betaine/Eth (n = 59)	39 (66%)	10 (26%)
50 μ M betaine/Eth (n=77)	55 (71%)	13 (24%)
100 μ M betaine/Eth (n=60)	38 (63%)	11 (29%)
250 μ M betaine/Eth (n=15)	6 (40%)	2 (33%)
500 μ M betaine/Eth (n= 15)	5 (33%)	1 (20%)
1 mM betaine/Eth (n= 15)	5 (33%)	0 (0%)
1.5 mM betaine/Eth (n= 29)	19 (66%)	5 (26%)
2 mM betaine/Eth (n= 29)	19 (66%)	4 (21%)
2.5 mM betaine/Eth (n= 15)	9 (60%)	3 (33%)
3 mM betaine/Eth (n= 29)	18 (62%)	4 (22%)

Table 3
Comparison of cardiac defects at ED 8 (HH Stage 34) for Control, Ethanol-exposed, and Ethanol+Betaine supplemented embryos
 A mixture of 5 μ M of betaine in 50% ethanol/PBS was administered in in one 40 μ l injection/egg at gastrulation.

	Control (C)	Ethanol (E)	Ethanol+Betaine (E+B)	Fisher's P-value comp. C, E, E+B	Fisher's P-value comp. E vs. E+B
Embryo Number	43	11	36		
Normal heart	42 (98%)	3 (27%)	32 (89%) **	4.396×10^{-7} **	0.0001927 **
Missing great vessel	0 (0%)	5 (45%)	2 (6%) **	4.389×10^{-5} **	0.004898 **
Abnormal valve morphology	0 (0%)	3 (27%)	0 (0%) **	0.001404 **	0.01018 *
Hypertrophic right ventricle	0 (0%)	2 (18%)	1 (3%) *	0.01826 *	0.1323
Ventricular septal defects	0 (0%)	2 (18%)	0 (0%) *	0.01373 *	0.05088 *
Abnormal rotation of ventricle	1 (2%)	0 (0%)	0 (0%)	1	1
Misaligned aorta	0 (0%)	1 (9%)	0 (0%)	0.1222	0.234
Collateral artery	0 (0%)	0 (0%)	1 (3%)	0.5222	1

** Ethanol compared to Ethanol+Betaine p-value 0.005

* Ethanol compared to Ethanol+Betaine p-value 0.05

Fisher's Exact Test with B=Bonferroni corrected q-critical value $0.05/8=0.00625$ (pairwise comparison)