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What Choline Metabolism Can Tell Us About the Underlying Mechanisms of Fetal Alcohol Spectrum Disorders

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Abstract The consequences of fetal exposure to alcohol are very diverse and the likely molecular mechanisms involved must be able to explain how so many developmental processes could go awry. If pregnant rat dams are fed alcohol, their pups develop abnormalities characteristic of fetal alcohol spectrum disorders (FASD), but if these rat dams were also treated with choline, the effects from ethanol were attenuated in their pups. Choline is an essential nutrient in humans, and is an important methyl group donor. Alcohol exposure disturbs the metabolism of choline and other methyl donors. Availability of choline during gestation directly influences epigenetic marks on DNA and histones, and alters gene expression needed for normal neural and endothelial progenitor cell proliferation. Maternal diets low in choline alter development of the mouse hippocampus, and decrement memory for life. Women eating low-choline diets have an increased risk of having an infant with a neural tube or orofacial cleft birth defect. Thus, the varied effects of choline could affect the expression of FASD, and studies on choline might shed some light on the underlying molecular mechanisms responsible for FASD.

Keywords Choline · Epigenetics · Methyl donor · Alcohol · Single nucleotide polymorphism · Nutrition

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Introduction

Exposure of germ cells, fetus, and young infant to alcohol can cause abnormalities in behavior and organ structures that range from barely detectable, to birth defects, to fetal loss [1]. Fetal alcohol spectrum disorders (FASD) include growth retardation, distinctive facial anomalies, cardiac defects, and altered brain function; less commonly, FASD are associated with skeletal, ocular, vestibular, hepatic, skin, and immune defects [2]. Thus, the consequences of fetal exposure to alcohol are very diverse and the likely molecular mechanisms involved must be able to explain how so many developmental pathways could go awry. Though there are likely many varied mechanisms of damage, Haycock [3] recently hypothesized that epigenetic factors are among the important mechanisms underlying FASD based on the important role of epigenetic mechanisms in central nervous system development.

Though our genetic code is spelled out at the time of conception, humans retain some level of flexibility about which of our genes is active or suppressed. This is accomplished by an epigenetic code which is transmitted by DNA methylation, covalent modifications of histones and chromatin, and by RNA interference [4]. The pattern of DNA methylation is first established during gastrulation [5] but can be changed during fetal development and early life. Haycock argues that epigenetic perturbations could cause the varied sequelae observed after exposure to alcohol [3]. This concept is strengthened by evidence that chronic exposure to alcohol perturbs one-carbon metabolism, thereby altering the key substrates needed to methylate DNA and histones [6].

A parallel line of investigation seems to lead to the same conclusion—that FASD may be caused by epigenetic perturbations at the time that progenitor cells are dividing

and differentiating to form organs. Children with FASD have a perturbation in one-carbon metabolism that is reflected in a decreased choline/creatine ratio in brain (left striatum) [7]. In 2009, Thomas and colleagues [8] reported that when pregnant rat dams were exposed to ethanol during gestational days 5–20, their offspring had reduced birth weight and brain weight, delays in eye opening and incisor emergence, and alterations in the development of the righting reflex, geotactic reflex, cliff avoidance, reflex suspension, and hindlimb coordination. However, if rat dams were also treated with choline, the effects from ethanol were attenuated (on birth and brain weight, incisor emergence, and most behavioral measures) in their pups. In fact, behavioral performance of ethanol-exposed pups treated with choline did not differ from that of controls. This effect from choline occurred without changing alcohol exposure, as blood alcohol levels were not changed by choline [8]. If children with FASD have some perturbation in choline metabolism, and choline supplementation attenuates the effects of alcohol exposure, then perhaps examining choline-related mechanisms will be of use in understanding the molecular etiology of FASD (Fig. 1).

Choline and Fetal Development

Choline is an essential nutrient for humans [9], and it is needed for biological membrane formation, for methylation, and for acetylcholine biosynthesis [10]. Choline is found in a variety of foods ([11]; and see http://www.ars.usda.gov/services/docs.htm?docid=6232; last accessed 11 Nov 2010), but the richest food sources of choline (eggs and liver) are avoided by many women. In fact, in the USA, most women do not eat diets delivering the recommended intake of choline [12]. Newborns have higher tissue choline concentrations than do their mothers at term (e.g., 10–15-fold higher in rats) [13, 14] because the placenta effectively takes choline from mother and delivers it to the fetus [15–17]. Later on, mother keeps supplying the baby with choline, as the mammary gland also delivers choline from mother to baby in breast milk [18, 19]. Finally, pregnant women may have a higher capacity to form phosphatidylcholine (a source of choline) from precursors in liver because the gene *PEMT*, that encodes the enzyme phosphatidylethanolamine-*N*-methyltransferase that catalyzes this process, is induced by estrogen [20]. Thus, there are many physiological mechanisms that permit women to

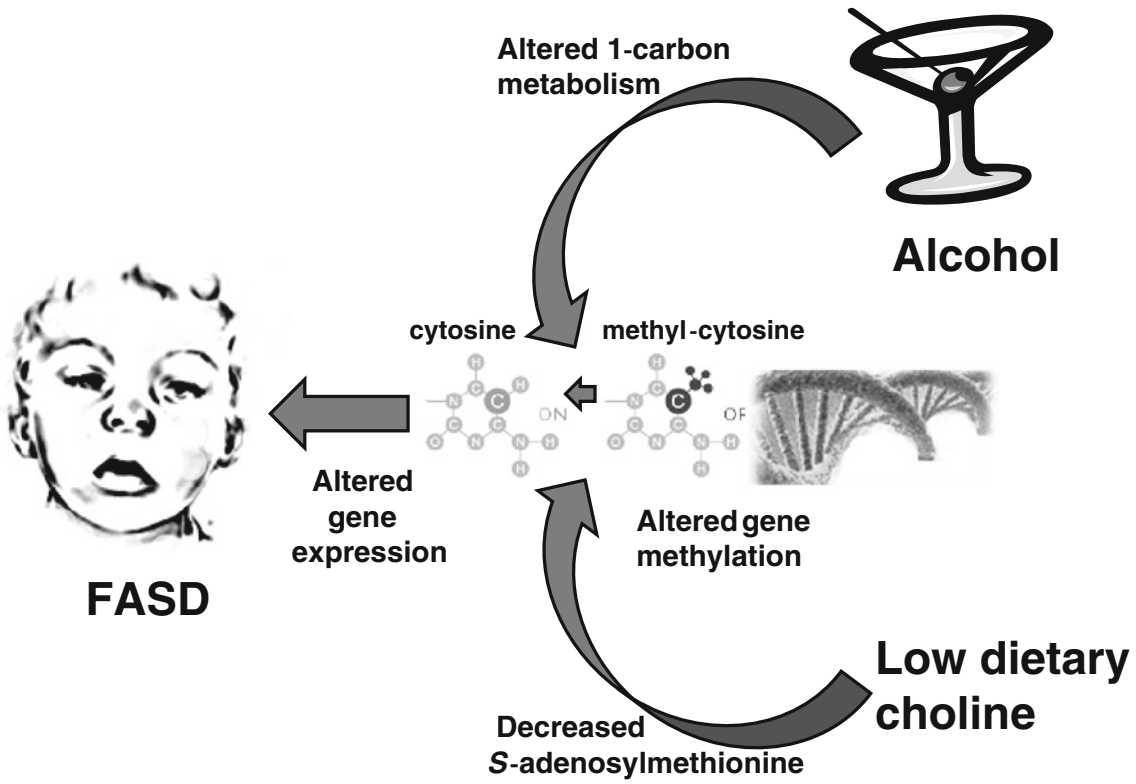

Fig. 1 Potential shared mechanisms. Diets low in choline, or genetic variations that increase the dietary demand for choline, can decrease DNA methylation (converting methyl-cytosines to cytosines in genes) and this increases gene transcription, changes progenitor cell proliferation and differentiation, resulting in birth defects and abnormal brain development. It is possible that similar mechanisms explain how alcohol exposure causes fetal alcohol spectrum disorders (*FASD*). Alcohol exposure during pregnancy perturbs one-carbon metabolism, decreasing the use of folate and increasing the use of choline as a methyl donor

deliver large quantities of choline to the growing fetus and infant and, for this to have evolved choline must play some important role in development.

Rodent dams fed diets low in choline during days 11–17 of gestation had pups with diminished neural progenitor cell proliferation in fetal hippocampus at the same time that there was increased apoptosis in these cells [21, 22]. In electrophysiologic studies, an insensitivity to long-term potentiation (LTP) was measured when these pups were adult animals [23], and in cognitive studies, they had decremented visuospatial and auditory memory as adults [24–27]. Conversely, rodent dams fed diets supplemented with choline (about four times the normal chow levels) during days 11–17 of gestation had pups with increased sensitivity to LTP when they were adult animals [23], and enhanced visuospatial and auditory memory as adults [24–27]. During gestation, maternal diets low in choline were also associated with increased neural tube closure defects and orofacial defects in rodent and human fetuses [28–31]. Apparently, choline availability is important for normal fetal development throughout gestation.

Mechanisms for the Actions of Choline

One of the likely mechanisms for these effects of choline on fetal development is epigenetically mediated. Choline is a major source of methyl groups [10], and, as discussed earlier, methylation of DNA and histones are important components of the epigenetic code [4]. Thus, DNA methylation is altered by availability of dietary choline [32]. DNA can be methylated on cytosines, and when this nucleotide is methylated in a specific location with the promoter of a gene, methyl-binding proteins are attracted, forming a complex that prevents access by transcription factors needed to activate expression of the gene [4]. Thus, usually methylation inhibits gene expression and hypomethylation increases gene expression. In the rodent experiments on dietary choline and memory that were described earlier, dams fed diets low in choline during days 11–17 of gestation had pups with diminished DNA and histone methylation in genes of the hippocampus [33, 34]. Specifically, a gene (*cdkn3*) that normally inhibits cell cycling, was hypomethylated and overexpressed in the prime germinal zone of the dentate gyrus of the fetal hippocampus [34, 35]. The product of this gene, kinase-associated phosphatase (Kap) protein levels were increased and this subsequently activated the retinoblastoma protein (Rb) pathway that inhibits cyclin-dependent kinase [34, 35].

In mice, maternal diets low in choline during pregnancy also altered histone methylation in fetal neural progenitor cells in the areas of the hippocampus where neurogenesis was occurring (supraventricular and ventricular zones; [33]). Transcriptional repressor neuron-restrictive silencing factor (REST) binding to gene promoter regions mediates the inhibition of expression of numerous neuronal genes and is a very important regulator of brain development [36]. REST recruits a group of co-repressors (histone deacetylase, methyl CpG binding protein 2 [37], and G9a histone methyltransferase [38]) to the repressor element 1 (RE1) in the promoter of various genes, resulting in inhibition of neuronal gene expression. The binding of REST at the RE1 site is facilitated by a specific histone methylation pattern [39]. When neural progenitor cells were exposed to a low-choline environment in vitro, there was diminished REST binding to RE1 [33]. Thus, availability of choline modulated histone methylation and thereby gene expression in fetal neural progenitor cells.

Choline effects are not limited to neural progenitor cells. We examined whether maternal dietary choline modulates angiogenesis in fetal brain [40]. In fetuses from mouse dams fed a low-choline diet during pregnancy, proliferation of endothelial cells in hippocampus was decreased because endothelial progenitor cells differentiated prematurely and stopped dividing [40]. These changes were associated with >25% decrease in the number of blood vessels in fetal hippocampus in the low-choline group ($p<0.01$ vs. control). In developing brain, it is important to maintain a balance between angiogenesis and neurogenesis, and this is accomplished by careful modulation by local cues (growth factors, extracellular matrix) [41–45] that include vascular endothelial growth factors (VEGF) and their receptors and by angiopoietin (ANGPT)/endothelial receptor tyrosine kinase signaling (Tie-2) [46–48]. Cytosines in the promoter regions of vascular endothelial factor C (*VEGFC*) and angiopoietin 2 (*ANGPT2*) were hypomethylated in neural progenitor cells exposed to choline compared to controls, providing an explanation for why these genes were over expressed [40]. Expression of genes for these angiogenic signals was increased in fetal hippocampus in the low-choline group (*VEGFC*, 2.0-fold, $p<0.01$ vs. control and *ANGPT2*, 2.1-fold, ($p<0.01$ vs. control)) [40]. Thus, maternal dietary choline intake and epigenetic changes also alter angiogenesis in the developing fetal hippocampus.

Shared Mechanisms for Effects of Choline and Alcohol?

Several types of tissues (nerves and blood vessels; perhaps others) are capable of changing epigenetic marks when presented with a low-choline environment. Choline availability during gestation in rodents modifies DNA methylation, DNA methyltransferase expression, histone H3 methylation, and histone methyltransferase expression in fetal tissues [33, 34, 49, 50]. However, these effects may occur only during critical windows of time during gestation

when progenitor cells are exposed to proliferation-promoting signals and before they differentiate. Hippocampal neural progenitor cell proliferation occurs from day 11–17 of mouse gestation, and that period is when choline exerts its effects. Restoration of choline later in development may not correct the epigenetic marks. Though not yet proven, it is likely that other types of progenitor cells, developing at different times during gestation, are also sensitive to choline. This could explain choline effects on neural tube and cleft palate defects. Exposure to alcohol also changes epigenetic marks. Maternal exposure to ethanol during pregnancy resulted in genome-wide hypomethylation in fetuses [51], and to differential modulation of H19 DNA methylation (controls an important growth factor gene) in the paternal and maternal alleles in the placenta [52]. Paternal alcohol exposure altered methylation of imprinted genes in the male gamete and decreased cytosine methyltransferase mRNA levels in paternal sperm [53, 54]. Thus, alcohol (and perhaps choline) might exert different effects on the epigenetic regulation of gene expression in the gametes, fetus, and placenta.

Alcohol and Choline Metabolism

Choline, folate, and methionine metabolism are highly inter-related, and these pathways intersect at the formation of methionine from homocysteine [10]. Homocysteine can be methylated using methyltetrahydrofolate or the choline metabolite betaine as the methyl group donor [10]. Manipulations that reduce the availability of methyl groups from folate increase the demand for choline and vice versa [55–57]. In rats, ethanol feeding inhibits the use of folate methyl groups to make methionine (methionine synthetase activity inhibited) and increases the use of betaine to make methionine (activity of the enzyme betaine–homocysteine methyl transferase is increased) [58]. Acute ingestion of alcohol in humans lowers brain concentrations of choline as measured by magnetic resonance spectroscopy (the choline/creatinine rations measured in such imaging likely measures a mixture of choline-containing compounds in brain) [59]. In the micropig, transcription of genes and activity for several other enzymes in one-carbon metabolism is altered by exposure to alcohol (i.e., methyltetrahydrofolate reductase, methionine adenosyltransferase 1A, glycine *N*-methyltransferase, *S*-adenosylhomocysteine hydrolase) [60]. In alcoholic liver disease, methionine metabolism is impaired, and *S*-adenosylmethionine (formed from methionine) concentrations in liver are decreased [61]. *S*-adenosylmethionine is the methyl donor needed for methylation of DNA and histones. Alcohol exposure also diminishes the availability of methyltetrahydrofolate, thereby increasing the demand for choline. Diets of alcoholics are especially deficient in folate [62]. Very low dietary folate intake (<180 μg per day) was 2.5-fold more common among women who drank 30 g alcohol regularly [63]. Heavy alcohol users malabsorb folate [64] and increase the loss of folate in the urine through a reduction in renal tubular reabsorption [65]. It is interesting that, in some animal models, dietary betaine supplementation attenuates several of the metabolic abnormalities associated with alcohol, correcting the abnormal hepatic *S*-adenosylmethionine and homocysteine concentrations and mitigating some of the damage to the liver [66, 67].

Could Mechanisms Related to Epigenetics and Choline be the Cause of FASD?

The consequences of fetal exposure to alcohol include growth retardation; distinctive facial anomalies; cardiac defects; altered brain function; and skeletal, ocular, vestibular, hepatic, skin, and immune defects [2]. Choline deficiency during gestation in animal models is associated with growth retardation, altered brain function, skeletal abnormalities, hepatic and muscle damage, as well as orofacial and neural tube birth defects [10]. Decreased choline availability to the fetus decreases hippocampal neurogenesis and increases apoptosis [68]. Exposure of the fetus to alcohol also decreases hippocampal neurogenesis and decreases cell survival [69, 70], resulting in reduced numbers of hippocampal pyramidal cells [71]. Though there are differences in the genes and tissues studied in both models, both choline deficiency and ethanol alter genes of cell cycling by altering DNA methylation of these genes [35, 72]. Prenatal alcohol exposure perturbs behavioral development leading to hyperactivity, motor incoordination, alterations in social processing, and to deficits in cognitive functioning, and prenatal supplementation with choline prevents or mitigates these effects [73]. Could there be shared molecular mechanisms that explain how so many developmental pathways could be altered?

Choline and epigenetics can also influence brain function. As discussed earlier, choline availability to pregnant rodents alters memory function in their juvenile, adult, and aged offspring [74]. In a mouse model of Down Syndrome, mice born to mothers supplemented with choline during the perinatal period had significant improvements in cognitive function and emotion regulation [75]. Epigenetic mechanisms underlie a number of syndromes in which brain function abnormalities are apparent, including Rett syndrome, an autism spectrum disorder that is characterized by cognitive impairments [76]. This syndrome results from mutations in the methyl DNA-binding protein MeCP2 [77]. Also, there are data suggesting that epigenetic factors are associated with hyperactivity in children [78] and certain psychiatric disorders [79]. As a whole, the available data suggests that the underlying mechanism responsible for the

effects of choline could also explain how many developmental pathways could go awry in FASD.

Clues that Might be of Interest for Future Studies in FASD

Humans have widely varying dietary requirements for choline, in part explained by genetic variation. As discussed earlier, several metabolic pathways influence how much choline is required from diet, and single nucleotide polymorphisms (SNPs) in specific genes influence the efficiency of these pathways. Specifically, some polymorphisms in the folate pathways limit the availability of methyltetrahydrofolate and thereby increase use of choline as a methyl donor; polymorphisms in the *PEMT* gene alter endogenous synthesis of choline; and polymorphisms in other genes of choline metabolism influence dietary requirements by changing the utilization of choline moiety [80, 81]. At this time we do not know whether maternal or fetal SNPs (or some combination of both) are most important in setting demand for choline. The fact that these SNPs are very common suggests that they could be overcome by high choline in the diet, perhaps because humans can eat choline to overcome the inefficiencies associated with the SNPs. If FASD shares some common mechanism with the effects of choline, then it is possible that people with SNP-induced metabolic inefficiencies who also eat a low-choline diet are at greater risk for FASD.

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