RESEARCH ARTICLE

Diabetes Management

# Differential rescue effects of choline chloride and soy isolate on metabolic dysfunction in immature central nervous system neurons: Relevance to fetal alcohol spectrum disorder

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## ABSTRACT

**Background:** Central Nervous System (CNS) abnormalities with insulin resistance and mediated by developmental exposures to ethanol can be avoided or remediated by consumption of dietary soy, which has insulin-sensitizing as well as antioxidant effects. However, choline supplementation has been shown to diminish Fetal Alcohol Spectrum Disorder (FASD) pathologies, and dietary soy contains abundant choline. This study was designed to determine if the therapeutic effects of soy were mediated by or independent of choline.

**Methods:** Human PNET2 cells exposed to 0 mM or 100 mM ethanol for 48 hours were seeded into 96-well or 12-well plates and treated with vehicle, choline chloride (75 μM), or 1 μM Daidzein+1 μM Genistein (D+G) for 24 h. The cells were then analyzed for viability (Hoechst 33342), mitochondrial function (MTT), and GAPDH, Tau, Acetyl Cholinesterase (AChE), Choline Acetyl Transferase (ChAT), and Aspartyl-Asparaginyl-β-Hydroxylase (ASPH) immunoreactivity.

**Results:** Choline and D+G significantly increased MTT activity (mitochondrial function) corrected for cell number relative to vehicle in control and ethanol-exposed cultures. Both choline and D+G prevented the ethanol-induced inhibition of GAPDH and ChAT and increased cellular accumulations of Tau. However, D+G significantly increased ASPH expression relative to vehicle and Choline.

**Conclusion:** Choline and D+G differentially modulated the expression of neuronal proteins, mitochondrial function, and ASPH. Importantly, the prominently increased expression of ASPH by D+G corresponds with the insulin-sensitizer actions of soy isoflavones since ASPH is an insulin-responsive molecule. The findings further suggest that dietary soy may be more effective than choline for reducing ethanol-impaired neuronal migration linked to ASPH inhibition in FASD.

## Introduction

Fetal Alcohol Spectrum Disorder (FASD) is associated with excessive chronic or binge consumption of ethanol during pregnancy [1,2] and is the most preventable etiology of human neurodevelopmental deficits [3,4]. FASD includes a collection of notable pathologies such as increased fetal demise, malformations of the Central Nervous System (CNS), skeletal and craniofacial, impaired intrauterine growth, cognitive and motor deficits, defects and Attention Deficit Hyperactivity Disorder

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- Mitochondria
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(ADHD) [5-7]. In the USA, FASD broadly afflicts 0.2-1.5 per 1000 live births but can reach 7 or 9/1000, particularly with binge drinking [8,9]. CNS structural pathologies in FASD [10-13] are characteristically distributed in the corpus callosum, prefrontal region, temporal lobe, and cerebellum [10,14], accounting for performance deficits on cerebellar motor and spatial learning and memory tasks that persist and adversely impact function through adolescence [15,16] and beyond [7,17]. Consequently, the economic and social burdens of FASD are high [18].

The mechanisms of ethanol-mediated neurodevelopmental defects have been parsed through systematic studies of experimental models. *In vitro* and *in vivo* experiments have shown that ethanol's adverse effects on CNS cells and tissues include neurotoxicity, stress oxidative injury, inflammation, and metabolic dysfunction [19-23]. Consequences include impairments in neuronal function and viability tied to compromised insulin and insulin-like growth factor type 1 (IGF-1) signaling through Akt and its downstream pathways [19, 24-31]. Associated reductions in signaling through Insulin Receptor Substrate (IRS) proteins and Akt increase glycogen synthase kinase 3β (GSK-3β) activation [25,28,32-35], which inhibits neuronal function, metabolism, and survival [24,29,31,36,37]. Consequences include reduced expression of target proteins needed for various neuronal functions [25,38-43].

One such target of ethanol that is also regulated by signaling through insulin/IGF-1 pathways is Aspartyl-asparaginyl-β-Hydroxylase (ASPH) [24,36,44,45]. ASPH is an ~ 86 kD type 2 transmembrane protein that mediates cell adhesion, motility/migration, and growth [24,36,46-51]. ASPH's effects on cell motility are mediated by Notch pathway activation *via* its C-terminal catalytic domain [52,53]. Corresponding with its inhibitory effects on insulin/IGF-1 signaling, ethanol reduces ASPH expression and function [24,36,45]. For example, in FASD, ethanol-induced dose-dependent reductions in ASPH correlate with the severity of impaired cerebellar neuron migration [36].

A major goal underlying mechanistic studies of FASD is to identify preventive or harm-reduction therapeutic strategies. Given the importance of insulin/IGF-1 signaling in cerebellar development and the impact that gestational alcohol exposure has on these critical pathways, one potential therapeutic approach for preventing FASD could be to fortify the function of relevant pathways with insulin sensitizers. Previous studies showed that Peroxisome-Proliferator-Activated Receptor (PPAR) agonists, which have both insulin-sensitizing and anti-oxidant effects [54-56] can abrogate the adverse effects of ethanol exposure in the CNS [57] and liver [58-62]. PPAR agonist treatments abrogated neurobehavioral dysfunction and alterations in neuroglial expression of insulin/IGF-1 regulated brain genes and proteins in a range of experimental models, including those utilizing ethanol exposures [57,63-65]. However, despite encouraging results, the potential for translating PPAR agonist research data to human studies is restricted due to unidentified risks to the maternal-placental-fetal unit. This concern led us to consider an alternative natural insulin-sensitizer and antioxidant food, namely dietary soy. Isoflavones are among the most important constituents of soy isolate protein. Isoflavones support insulin responsiveness and help resolve states of insulin-resistance that lead to disease [66-71].

In recent reports we showed that dietary soy could prevent neurobehavioral dysfunction caused by chronic ethanol feeding in a rat model [15], and that maternal consumption of dietary soy during pregnancy prevented placental and craniofacial phenotypic pathologies in experimental FASD [72]. However, soy isolate contains substantial amounts of choline [67,68] and choline is known to benefit neurocognitive function following developmental exposures to alcohol, both in experimental models [73-75] and humans [76,77]. On the other hand, the main advantage of incorporating soy isolate over choline into the diet is that soy is a natural whole food product that provides healthful protein, which is often needed to correct nutritional deficiencies that accompany inadequate choline as well as other micronutrients intake during pregnancy in socioeconomically challenged environments. Despite these considerations, we were left with the goal of determining whether the therapeutic effects of soy were due to or the result of those of choline's inclusion in the diet as a constituent of soy isolate. This study formally compares the therapeutic effects of dietary soy with those of choline in PNET2 human CNS-derived cerebellar neuronal cells that have been used to investigate neurotoxic and metabolic effects of ethanol [26,49,78]. The short-term experimental design compared the degrees to which the soy isolate bioactive constituents, Daidzein and

Genistein, or choline chloride could support control and ethanol-exposed immature neuronal viability, function, and ASPH expression.

## Materials and Methods

### ■ Materials

Invitrogen (Carlsbad, CA, USA) was the source of Dulbecco's Modified Eagle Medium (DMEM), Amplex UltraRed, and 4-methylumbelliferyl phosphate (4-MUP). Vector Laboratories (Burlingame, CA, USA) was the source of Alkaline Phosphatase Conjugated to Streptavidin. Table 1 lists the supplements for cell culture and Table 2 lists the antibodies used and their sources. All other fine chemicals were purchased from either Sigma-Aldrich (St. Louis, MO, USA) or CalBiochem (Carlsbad, CA, USA).

**TABLE 1. Choline chloride and Soy Isolate Constituents Used for Culture Supplementation.**

| Compound | Source | Catalog # | M.W. | Solvent | Concentration |
|---|---|---|---|---|---|
| Choline Chloride | Fisher Scientific | AC1102950 | 139.62 | DMSO | 75 µM |
| Daidzein (>98%)* | Sigma-Aldrich | D7802 | 254.2 | DMSO | 1 µM |
| Genistein (>95%)** | Sigma-Aldrich | G0897 | 432.4 | DMSO | 1 µM |

**Note:** *Daidzein was a >98% pure synthetic molecule. **Genistein was from Glycine max (soybean) and determined to be >95% pure by HPLC. Vehicle control cultures were treated with DMSO

**TABLE 2. Antibodies used for duplex ELISA studies.**

| Antibody | Source | Company | Type | Concentration/ Dilution | RRID# |
|---|---|---|---|---|---|
| Glucose-6-Phosphate Dehydrogenase (GAPDH) | Mouse | Santa Cruz, Dallas, TX | Monoclonal | 0.2 µg/ml | AB_10847862 |
| Tau | Rabbit | Agilent/Dako, Santa Clara, CA | Polyclonal | 6.2 µg/ml | AB_10013724 |
| Choline Acetyltransferase (ChAT) | Rabbit | Abcam, Waltham, MA | Polyclonal | 2.125 | AB_2244866 |
| Acetylcholinesterase (AChE) | Mouse | Abcam, Waltham, MA | Monoclonal | 0.25 µg/ml | AB_303316 |
| Aspartyl-asparaginyl β-hydroxylase (ASPH); A85E6 and A85G6 | Mouse | 21st Century Biochemicals, Marlborough, MA | Monoclonal | 1.4305556 | (36) |
| Large acidic ribosomal protein (RPLPO) | Mouse | Santa Cruz, Dallas TX | Monoclonal | 0.1 µg/ml | [98-100] |

### ■ Cell culture

Human PNET2 primitive neuroectodermal tumor cerebellar neuronal cells were cultured in DMEM containing 10% heat-inactivated 10% Fetal Bovine Serum (FBS), 4.5 g/L glucose, and 4 mM L-glutamine in a standard 5% $CO_2$ cell culture incubator (37°C). For the ethanol exposure model, sub-confluent cultures, freshly seeded in 75 $cm^2$ flasks, were treated with 0 mM or 100 mM ethanol for 48 hours, and then after re-seeding into 96-well ($1.4 \times 10^4$ viable cells/well) or 12-well plates ($1.35 \times 10^5$ viable cells/well), the cultures were treated for 24 h with vehicle (DMSO), 75 µM Choline Chloride (CC), or 1 µM each of Daidzein and Genistein (D+G). Cell viability was assessed by Trypan blue exclusion. Cultures were analyzed for viability, mitochondrial function, and protein expression.

### ■ Protein extraction

The 12-well cultures were harvested in 5.0 volumes of buffer that contained 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM NaF, 5 mM EDTA, pH 8.0, 0.1% Triton X-100, phosphatase inhibitor (10 mM $Na_3VO_4$), and protease inhibitors (1 mM PMSF, 0.1 mM TPCK, 2 µg/ml aprotinin, 2 µg/ml pepstatin A, 1 µg/ml leupeptin). The supernatants obtained by centrifuging the culture homogenates at 14000 rpm for 15 min at 4°C were used to measure immunoreactivity by Enzyme-Linked Immuno Sorbent Assay (ELISA). The Bicinchoninic Acid (BCA) assay was used to measure protein concentration.

Duplex ELISAs were used to quantify immunoreactivity corresponding to Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), Tau, Choline Acetyl Transferase (ChAT), Acetylcholinesterase (AChE), and Aspartyl-asparaginyl-β-Hydroxylase (ASPH) such that the results were corrected/normalized to large acidic Ribosomal Protein (RPLPO) as previously described [79,80]. In brief, duplicate 50 µl aliquots containing 50 and ng of protein were allowed to adsorb to the bottoms of 96-well MaxiSorp plates by overnight incubation at 4°C. Superblock (TBS) Blocking Buffer was used to mask non-specific sites. Primary antibodies diluted to 0.2–5.0 µg/ml, were incubated with the proteins overnight at 4°C. HRP-conjugated secondary antibody and the Amplex UltraRed soluble fluorophore were used to detect and quantify immunoreactivity. Fluorescence intensities were measured in a SpectraMax (Ex 530 nm/Em 590 nm). Then, RPLPO immunoreactivity, a loading control, was measured by incubating the same samples with biotinylated anti-RPLPO, the binding of which was detected with streptavidin-conjugated alkaline phosphatase and 4-MUP (Ex 360 nm/Em 450 nm). The ratios of target protein to RPLPO fluorescence were used for inter-group comparisons. N=6 replicate cultures per group.

### ■ Statistical analysis

Results were analyzed by two-way analysis of variance (ANOVA). Post hoc Tukey multiple comparisons tests compared individual differences (GraphPad Prism 9.4, San Diego, CA). Significant ($P<0.05$) and trend-wise ($0.05<P<0.10$) differences are tabulated. Significant differences are marked in the graphs.

## Results

### ■ MTT activity

MTT activity was lowest in vehicle-treated cultures with no effects of ethanol. Choline and D+G significantly increased MTT activity relative to vehicle, although ethanol muted that response in choline-treated cultures (Figure 1A). Significant effects of ethanol, treatment, and ethanol x treatment interactions were detected by two-way ANOVA (all $P<0.0001$) (Table 3).

### ■ Hoechst H33342

The levels of H33342 fluorescence were similar in the vehicle- (control and ethanol-exposed), and choline-treated control cultures, whereas the lowest levels were measured in D+G-treated cultures (Figure 1B). In addition, H33342 was significantly reduced by ethanol in choline- and D+G-treated cultures. The mean level of H33342 in ethanol-exposed, D+G-treated cultures was significantly lower than in all other groups. Two-way ANOVA revealed significant effects of ethanol ($P<0.0001$), treatment ($P<0.0001$), and ethanol × treatment interactions ($P=0.0087$) (Table 3).

### ■ MTT/H33342

Mitochondrial activity corrected for cell number is reflected by this ratio. The mean levels of MTT/H33342 increased from vehicle to Choline to D+G, without ethanol effects in the vehicle-treated cultures. In contrast, significant effects of ethanol with choline (reduced) or D+G (increased) occurred relative to the corresponding controls (Figure 1C). The mean levels of MTT/H33342 were significantly higher in Choline-

versus vehicle-treated, and in D+G-treated versus all other groups. Two-way ANOVA demonstrated significant effects of treatment (P<0.0001) and ethanol × treatment interactions (P<0.0001), but not ethanol alone (Table 3).

## ■ Neuronal function markers

Duplex ELISAs were used to compare the effects of choline and D+G on GAPDH, Tau, ChAT, AChE, and ASPH immunoreactivity in control and ethanol-exposed PNET2 cells. The data were analyzed by two-way ANOVA tests (Table 3) and depicted graphically in Figures 2 and 3.

## ■ GAPDH

Ethanol significantly reduced GAPDH expression in vehicle-treated but not Choline- or D+G-treated cultures (Figure 2A). The mean levels of GAPDH/RPLPO were significantly lower in the Ethanol-Vehicle relative to all other groups except Ethanol-D+G. Correspondingly, the two-way ANOVA test demonstrated a significant effect of ethanol, but not treatment with choline or D+G, or an ethanol x treatment interaction (Table 3).

## ■ Tau

Ethanol significantly increased cellular Tau expression in vehicle-treated, but not Choline- or D+G-treated cultures (Figure 2B). The mean level of Tau/RPLPO was significantly elevated relative to Control-Vehicle and Control-D+G. Two-way ANOVA detected a significant effect of ethanol, but not treatment with choline or D+G, or an ethanol x treatment interaction (Table 3).

## ■ ChAT

Ethanol significantly decreased neuronal ChAT expression in vehicle-treated cultures. However, the levels of ChAT were similarly reduced in Control-Choline, and Control-D+G relative to Control-Vehicle (Figure 2C). In contrast, in the Ethanol-Choline and Ethanol-D+G cultures, ChAT was elevated and not significantly different from Control-Vehicle. Two-way ANOVA detected significant ethanol x treatment interaction effects but no significant effects of ethanol or choline/D+G treatment (Table 3).

## ■ AChE

The mean levels of AChE were similar across all culture exposures and treatments. However, modest tight reductions in AChE expression in the Ethanol-D+G cultures rendered the differences statistically significant relative to the Control- and Ethanol-Choline (Figure 2D). Two-way ANOVA detected a significant effect of Choline/D+G treatment, but not ethanol or ethanol x treatment interaction (Table 3).

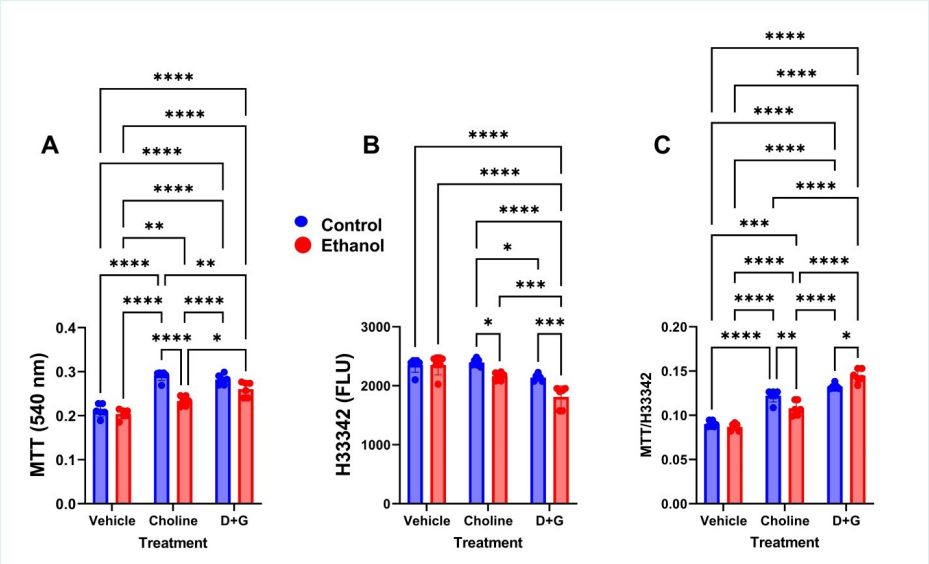


**FIGURE 1: Effects of choline chloride or daidzein+genistein (D+G) treatment on (A) MTT activity (mitochondrial); (B) H33342 fluorescence (cell number); and (C) MTT/H33342 in PNET2 human CNS neuronal cell cultures exposed to 0 mM or 100 mM ethanol for 48 hours and then treated with Vehicle (DMSO), 75 µM Choline Chloride, or 1 µM Daidzein+1 µM Genistein (D+G) for 24h. Note: Graphs show the mean ± S.D. with scatter-plotted results (N=6 cultures/group). Data were analyzed by two-way ANOVA with post-hoc repeated measures Tukey tests (See Table 3). *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. (● )-Ethanol and ( ● )-Control.**

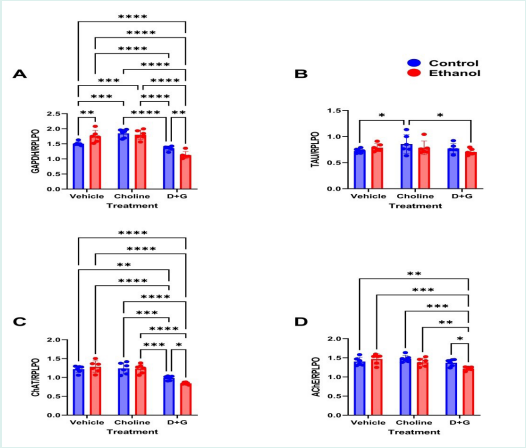


**FIGURE 2: Choline chloride and daidzein+genistein (D+G) treatment effects on (A) GAPDH, (B) Tau, (C) ChAT, and (D) AChE immunoreactivity in control and ethanol-exposed PNET2 CNS-derived neuronal cells Immunoreactivity was measured by duplex ELISA (N=6 replicate cultures/group). Scatter plots within each bar Reflect individual values. Inter-group comparisons were made by two-way ANOVA and repeated measures Tukey tests (See Table 3). *P<0.05; **P<0.01. Note: (🔴)-Ethanol and (🔵)-Control.**

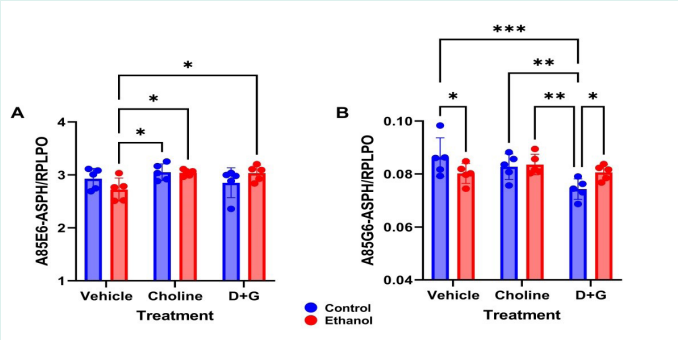


**FIGURE 3: Choline chloride and daidzein+genistein (D+G) treatment effects on aspartyl-asparaginyl-β-hydroxylase (ASPH) immunoreactivity in control and ethanol-exposed PNET2 neuronal cells. (A) ASPH-A85E6 and (B) ASPH-A85G6 immunoreactivities were measured by duplex ELISA. Inter-group differences were analyzed by two-way ANOVA with post hoc Tukey tests (See Table 3). *P<0.05; **P<0.01; ***P<0.001). Note: (🔴)-Ethanol and (🔵)-Control.**

**TABLE 3. Summary of Ethanol and Treatment (Choline or Soy Isoflavone) Effects on Markers of Neuronal Function.**

| Protein | Ethanol Factor | | Treatment Factor | | Ethanol x Treatment Interaction | |
|---|---|---|---|---|---|---|
| | F-Ratio | P-value | F-Ratio | P-Value | F-Ratio | P-Value |
| MTT | **46.54** | **<0.0001** | **84.7** | **<0.0001** | **11.94** | **0.0002** |
| H33342 | **20.53** | **<0.0001** | **32.04** | **<0.0001** | **5.588** | **0.0087** |
| MTT/H33342 | 0.667 | N.S. | **217.5** | **<0.0001** | **15.46** | **<0.0001** |
| GAPDH | 5.097 | 0.033 | 1.802 | N.S. | 1.601 | N.S. |
| Tau | 6.237 | 0.02 | 0.338 | N.S. | 2.394 | N.S. |
| ChAT | 0.089 | N.S. | 1.252 | N.S. | **4.822** | **0.017** |
| AChE | 0.604 | N.S. | **5.08** | **0.014** | 0.337 | N.S. |
| ASPH-A85E6 | 0.026 | N.S. | **3.379** | **0.05** | ***2.6*** | ***0.095*** |
| ASPH-A85G6 | 0.039 | N.S. | **5.147** | **0.014** | **4.473** | **0.022** |

**Note:** Two-way ANOVA Results for MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) activity, H33342 (Hoechst H33342 stain for nuclei of living cells) fluorescence, MTT/H33342 (relative MTT activity corrected for cell number in the cultures) and GAPDH, Tau, ChAT, AChE, ASPH-A85E6 and ASPH-A85G6 immunoreactivities (measured by Duplex ELISA) (N=6/group). Degrees of freedom: F (1, 30) for ethanol or treatment versus vehicle effects; F (2, 30) for ethanol x treatment interactive effects. Bold font marks significant P-values. Bold italics font marks P-values with statistical trends (0.05≤P≤0.10).

**ASPH expression was measured with two different monoclonal antibodies:** A85E6 and A85G6. A85E6 binds to a region within the N-terminus of the molecule, and detects full-length ASPH. A85G6 binds to the C-terminus of ASPH which contains the catalytic domain needed for cell motility [36]. Ethanol caused modest reductions in A85E6-ASPH in vehicle-treated cultures whereas Choline and D+G prevented the ethanol-associated decline, resulting in significantly higher levels of A85E6-ASPH immunoreactivity relative to Ethanol-vehicle (Figure 3A). Two-way ANOVA detected a significant effect of treatment (Choline or D+G) and a statistical trend for ethanol × treatment interaction, but no significant effect of ethanol (Table 3).

Ethanol caused a significant reduction in the mean level of A85G6-ASPH in vehicle-treated cultures (Figure 3B). However, A85G6-ASPH was most prominently reduced in Control-D+G relative to Control-Vehicle, Control- and Ethanol-Choline, and Ethanol-D+G. In contrast, Choline and D+G spared PNET2 cells from ethanol-mediated reductions in A85G6-ASPH expression. Two-way ANOVA detected significant effects of Choline/D+G treatment and ethanol × treatment interaction but not ethanol-only effects on ASPH-A85G6 (Table 3).

## Discussion

FASD comprises a group of developmental abnormalities linked to gestational alcohol misuse, and although it is preventable through abstinence, a host of social, cultural, and educational forces continue to challenge public health approaches. However, research in experimental models and humans has shown the value of choline supplementation for reducing FASD [73-77] and was built on ample evidence that choline is needed for neurodevelopment, energy metabolism, and brain functions, including the generation of acetylcholine [81,82], yet its deficiency is common [83]. Choline supplementation in pregnant women and infants at high risk for FASD has yielded promising results [76,77].

Previous studies focused on underlying mechanisms of alcohol-mediated neurodevelopmental defects identified inhibition of insulin signaling as a critical mediator of FASD-related pathologies including neuronal loss, impaired neuronal migration, increased oxidative stress, deficits in mitochondrial function and energy metabolism, and cognitive-motor dysfunctions [19,24-31]. Preclinical studies showed that many adverse effects of prenatal alcohol exposure can be prevented or reduced by treatment with peroxisome Proliferator-Activated Receptor (PPAR) agonists which are small molecules with both insulin sensitizer and antioxidant actions [57,63-65]. Importantly, PPAR agonists that target both the delta and gamma receptors are highly protective for preventing or reducing permanent neurobehavioral and motor dysfunctions in FASD and other models with brain insulin resistance [57,64,65,84,85]. However, this treatment strategy is potentially problematic for humans due to unknown long-term effects on pregnant women and their offspring. Our alternative approach was to consider intervention with natural food, namely dietary soy, which like PPAR agonists, has known insulin-sensitizer and antioxidant actions [70,71,86].

Two recent preclinical studies support the use of dietary soy as a strategy for preventing long-term adverse effects of excessive alcohol exposure during development. In an adolescent model, dietary soy replacement of casein in the standard rodent diet prevented long-term neurocognitive and motor dysfunctions linked to chronic heavy ethanol exposure [15]. In a chronic gestational alcohol exposure model, dietary soy prevented FASD-associated impairments in placentation, and both craniofacial dysmorphic features and intrauterine growth restriction in the fetuses [72]. Mechanistically, dietary soy enhanced insulin and IGF-1 signaling through metabolic, growth, and antioxidant pathways required for placentation and fetal growth [15,72]. However, dietary soy contains abundant choline and conceivably, its therapeutic effects could be mediated by choline rather than soy. On the other hand, there is established evidence that soy isoflavones have positive effects on insulin-resistance diseases [67,68,70,71]. This study was designed to compare the supportive effects of choline and soy in a short-term *in vitro* human CNS neuronal model of early developmental alcohol exposure. However, to delineate the effects of soy, the study utilized purified soy isoflavones, namely daidzein and genistein. Daidzein and genistein (D+G) were administered together because exploratory studies showed greater efficacy with their combined versus individual use for supporting MTT activity in PNET2 neuronal cells.

Both choline and D+G enhanced MTT activity

and MTT/H33342 relative to vehicle treatment, irrespective of ethanol exposure. The D+G effects were distinguished from those of choline based on the: 1) significantly greater increases in MTT/H33342, a reflection of mitochondrial activity corrected for cell number; 2) significantly smaller increases in MTT and MTT/H33342 in choline-treated that D+G-treated ethanol-exposed cultures; 3) and the significantly lower mean cell densities (H33342) in D+G-treated versus choline- or vehicle-treated cultures. The lower cell densities in D+G-treated cultures, as well as in ethanol-exposed relative to corresponding choline- or D+G-treated cultures may reflect reduced cellular proliferation or increased cell loss and turnover due to enhanced metabolic activity (MTT/H33342). Together, the findings with respect to MTT and H33342 suggest that both choline and D+G support CNS immature neuronal function, but D+G is more effective and supportive of ethanol-exposed cells.

GAPDH is an insulin-responsive enzyme that has a key role in energy metabolism [87]. The inhibitory effects of ethanol on GAPDH expression observed in vehicle-treated cultures correspond with previous reports [29,44,88,89]. The major effects of choline and D+G were to normalize GAPDH expression in ethanol-exposed cultures. These findings reinforce the observations made with respect to the MTT/H33342 responses and support the notion that both choline and D+G can enhance and normalize CNS neuronal metabolic function following ethanol exposure.

Tau is a major neuronal cytoskeletal protein that can accumulate with neurodegeneration or toxic responses leading to loss of axonal transport synaptic connections [90,91]. Intra-neuronal tau build-up can promote oxidative stress with attendant neuronal dysfunction [92]. Choline and D+G prevented intra-neuronal Tau build-up, and in that respect were similarly neuroprotective.

ChAT is enzymatically responsible for the generation of acetylcholine. Previous studies linked reductions in ChAT immunoreactivity to impairments in insulin signaling in the brain and cultured neuronal cells [25,39,57,93]. Corresponding with previous reports, ethanol significantly reduced ChAT expression in vehicle-treated cultures. Choline and D+G treatments prevented ethanol-mediated significant reductions in ChAT relative to all control groups suggesting that either treatment would support cognitive-motor functions in ethanol-exposed immature CNS neuronal cells.

AChE has an important role in modulating the degradation of acetylcholine. Previous studies showed reductions in temporal lobe AChE following chronic ethanol exposure [34,38,94]. However, following the short-term *in vitro* ethanol exposures, AChE immunoreactivity was not significantly altered relative to corresponding control cultures. The only significant effect detected was a modest reduction in AChE immunoreactivity in ethanol-exposed, D+G-treated relative to choline-treated cultures. Otherwise, the effects of ethanol, choline, and D+G exposures/treatments were nil.

ASPH immunoreactivity was assessed using two different monoclonal antibodies, A85G6-ASPH and A85E6-ASPH [36] that respectively bind to the C-terminal and N-terminal regions of the molecule. The rationale is that the C-terminus, which contains the Notch-activating catalytic domain, can be cleaved and function apart from the full-length protein [47,95]. Previous studies demonstrated ethanol inhibition of ASPH expression in FASD and other models of chronic ethanol exposure [36,96,97]. The ethanol-associated modest reductions in A85E6-ASPH and small but significant reductions in A85G6-ASPH, were not as robust as observed in previous reports [36], perhaps due to the short-term nature of the exposures utilized herein. Nonetheless, the studies showed that: 1) choline and D+G normalized A85E6-ASPH relative to the vehicle-treated control cells, and 2) the ethanol-vehicle suppression of A85G6-ASPH was abrogated by choline and D+G relative to their corresponding controls. In essence, ASPH's functions in immature CNS neuronal cells would likely be supported by either choline or D+G treatment *vis-à-vis* ethanol exposure[98-100].

## Conclusion

In a short-term *in vitro* ethanol exposure model utilizing CNS human immature neuronal cells (PNET2), both choline and D+G were effective in preventing several adverse effects of ethanol on metabolic function and neuronal protein expression. The use of D+G was designed to compare the therapeutic effects of soy isoflavones to those of choline which is abundantly present in dietary soy. The protective effects of choline and D+G were similar but not identical. In particular, for ethanol-exposed neuronal cells, the enhancements of MTT and MTT/H33342

were greater with D+G than choline, whereas GAPDH and ChAT expression were more prominently up-regulated by choline than D+G. Altogether, the results suggest that both approaches are neuroprotective *vis-à-vis* alcohol exposure, indicating that soy isoflavones in the absence of additional choline, can support neuronal functions. However, *in vivo*, dietary soy may be more effective given the combined administration of isoflavones (bioactive insulin sensitizers) with choline (a natural component of soy), and the added benefit of providing nutritional support beyond choline micronutrient supplementation, particularly in states of malnutrition.

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## Conflicts of Interest

The authors have no conflicts of interest.

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