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Distinct and Additive Effects of Alcohol and Thiamine Deficiency in the Developing Brain: Relevance to Fetal Alcohol Spectrum Disorder

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Abstract

Background: Neurodevelopmental abnormalities in fetal alcohol spectrum disorder (FASD) are linked to brain insulin resistance and oxidative stress. However, the role of thiamine deficiency as a distinct or additive factor in the pathogenesis of the neurodevelopmental and metabolic derangements in FASD has not been determined. **Methods:** Control and ethanol-exposed human PNET2 cerebellar neuronal cells and rat cerebellar slice cultures were treated with vehicle or pyrithiamine (Pyr) to assess independent and additive effects of thiamine deficiency on ethanol-mediated neurotoxicity, mitochondrial dysfunction, insulin resistance, inhibition of neuronal and glial genes, and oxidative stress. **Results:** Pyr treatments (0 - 200 μM) caused dose-dependent cell loss (Crystal Violet assay) and reduced mitochondrial function (MTT assay) in PNET2 neuronal cultures. Ethanol alone (100 mM) significantly reduced PNET2 neuronal viability, MTT activity, and ATP production. Over the broad dose range of Pyr treatment, ethanol significantly reduced ATP content and cell number and increased mitochondrial mass (MitoTracker Green). *Ex vivo* cerebellar slice culture studies revealed ethanol-induced developmental architectural disruption that was substantially worsened by Pyr. The adverse effects of ethanol were linked to increased lipid peroxidation and inhibition of asparatyl-asparaginyl-β-hydroxylase (ASPH) expression. The independent and additive effects of Pyr were associated with increased cytotoxicity, lipid peroxidation, Caspase 3 activation, and Tau accumulation. **Conclusions:** During development, alcohol exposure and thiamine deficiency exert distinct but overlapping molecular pathologies that ultimate-

ly impair the structure and function of cerebellar neurons. While both insults drive cell loss and mitochondrial dysfunction with increased lipid peroxidation, ethanol's additional inhibitory effects on ASPH reflect impairments in insulin and IGF signaling. In contrast, Pyr's main adverse effects were likely due to neurotoxicity and the activation of apoptosis cascades. The findings suggest that FASD severity may be reduced by thiamine supplementation, but without additional support for insulin/IGF signaling networks, FASD would not be prevented.

Keywords

Fetal Alcohol Spectrum Disorder, Thiamine Deficiency, Cerebellum, Slice Culture, Pyrithiamine

1. Introduction

Alcohol abuse during pregnancy causes fetal alcohol spectrum disorder (FASD), which leads to long-term neurodevelopmental deficits [1]-[3]. Ethanol can mediate its adverse effects on the brain by inhibiting insulin and insulin-like growth factor (IGF) signaling at multiple levels, beginning with ligand-receptor binding [4]. These ethanol effects are evidenced by reduced: 1) insulin and IGF-1 receptor tyrosine kinase (RTK) activation; 2) transmission of signals through insulin receptor substrate (IRS) molecules [5]-[7]; 3) activation of phosphotidyl-inositol-3-kinase (PI3K) and Akt; 4) disinhibition of glycogen synthase kinase 3β (GSK-3β) [4] [6]-[9]; and 5) inactivation of phosphatases that negatively regulate RTK-stimulated pathways, e.g. protein tyrosine phosphatase 1b (PTP-1B) and phosphatase and tensin homolog (PTEN) [7] [9] [10]. The consequences include impairments of networks that regulate growth, survival, metabolism, neuronal migration, and plasticity during development [11]-[16]. Therefore, ethanol's inhibitory effects on insulin/IGF signaling mediate multiple structural and functional central nervous system (CNS) abnormalities characteristic of FASD, including motor impairments, cerebellar hypoplasia, and neuronal migration disorders [3] [4] [17] [18]. In addition to its inhibitory effects on insulin/IGF signaling, ethanol causes neurotoxic injury and promotes oxidative and endoplasmic reticulum (ER) stress, DNA damage, mitochondrial dysfunction, inflammation, and apoptosis [4] [19]-[21]. The combined effects of insulin/IGF resistance and neurotoxic injury promote cell loss and compromise a broad range of functions needed for normal brain development.

1.1. Alcohol-Related Neurotoxicity

A critical aspect of studies of alcohol-mediated neurotoxicity is to consider the consequences in relation to development, age of exposure, and mode of exposure, *i.e.* chronic versus repeated binging. Previous work provided strong evidence that CNS vulnerability to the neurotoxic effects of alcohol is highest dur-

ing development, including in the adolescent period, with worse outcomes associated with earlier exposures [22]. Correspondingly, experimental data showed that synaptic activity and plasticity needed for memory were significantly more compromised in adolescents compared with adults, resulting in greater impairments in spatial learning and memory [23]. Mechanistically, the effects of acute alcohol exposure include prominent targeting of hippocampal neurons and their connectivity both within and beyond the hippocampus, including subcortical structures, leading to deficits in cognitive-behavioral functions [24] [25]. However, besides the hippocampus, the elaborate sub-categorical nature of memory is dependent upon connections with the neostriatum and cerebellum [25].

1.2. Thiamine Deficiency Effects in the CNS

Although experimental models clearly demonstrate that ethanol exposure alone is sufficient to produce FASD with dose-dependent phenotypes [8] [26]-[28], an unresolved question is whether thiamine (Vitamin B1) deficiency contributes to neurodevelopmental abnormalities. A better understanding of this problem is needed because up to 80% of alcoholics have some degree of Thiamine deficiency [29]. Thiamine is essential to the human diet [30]. In adults, serious thiamine deficiencies can cause heart failure, neurodegeneration, dementia, psychosis, peripheral neuropathy, or death [31] [32]. During development, maternal thiamine deficiency has been linked to stillbirth [33], fetal intrauterine growth restriction [34] [35], cerebellar hypotrophy with neuronal loss [36], and hippocampal damage in regions injured by acute alcohol exposures [36]. In addition, Thiamine deficiency exacerbates the chronic deleterious effects that ethanol has on spatial memory, in part due to their similar inhibitory effects on acetylcholine release [37], and due to overlapping damage to white matter tracts [38]. Thiamine is used for transketolase, pyruvate dehydrogenase (PHD), and α-ketoglutarate dehydrogenase (α-KGDH) enzymatic activities which mediate carbohydrate metabolism and ATP production [29]. In addition, Thiamine regulates enzymes for neurotransmitter, nucleic acid, fatty acid, steroid, and complex carbohydrate biosynthesis. Although several molecular and cellular effects of thiamine deficiency have been linked to chronic alcohol abuse, the extent to which specific structural and insulin signaling-related abnormalities in alcohol-related brain injury are caused by thiamine deficiency, ethanol neurotoxicity, or both, has not been determined [29].

1.3. Thiamine Deficiency versus Alcohol-Related Injury in the CNS

Difficulties with unraveling the roles of Thiamine deficiency versus ethanol neurotoxicity stem from our inability to conduct ethical human clinical studies and the fact that under normal circumstances, thiamine-deficient states are uncommon because Vitamin B1 is readily available in the diet and its storage in the liver is prolonged. On the other hand, alcoholics suffer from variable degrees of thiamine deficiency due to impaired absorption, transport, activation, and utili-

zation [39]-[42]. To examine thiamine's role in alcohol-associated diseases, investigators typically have generated models in which the experimental animals are chronically fed with thiamine-deficient diets and co-administered Pyr to inhibit thiamine's functions [43]-[45]. Pyr is a chemical inhibitor of thiamine transketolase, which converts thiamine to thiamine pyrophosphate, its active form. Previous studies showed that Pyr rapidly depletes thiamine pyrophosphate from the brain and other organs [46], and causes a neurological disorder like Wernicke-Korsakoff syndrome [45], which is at least partially reversed by thiamine administration [47] [48]. Correspondingly, humans diagnosed with acute Wernicke's encephalopathy are effectively treated by thiamine administration together with abstinence [49]. We hypothesize that Thiamine deficiency in alcohol-abusing pregnant women may contribute to the pathogenesis of FASD and its associated long-term impairments in cerebellar structure and function.

1.4. Study Goals

In the present study, we used immature human cerebellar PNET2 cells to delineate the individual and additive deleterious effects of alcohol and thiamine deficiency on neuronal metabolic functions and indices of oxidative stress. In addition, we employed slice cultures generated in a rat FASD model to further investigate the independent and interactive effects of alcohol and Pyr on cerebellar morphology, neurotoxicity, energy metabolism, and expression of aspartyl-asparaginyl-β-hydroxylase (ASPH) [50]-[52], an insulin/IGF-responsive gene [53]-[55] that has an important role in cell motility [55] [56] including cerebellar development-related neuronal migration [26] [57]-[59].

2. Methods

2.1. Reagents and Resources

Commercial antibodies used and RRID numbers are provided in **Table S1** in **Supplementary**. Thermo Fisher Scientific (Bedford, MA, USA) was the source of superblock (TBS), bicinchoninic acid (BCA) reagents, enzyme-linked immunosorbent assay (ELISA) MaxiSorp 96-well plates, and horseradish peroxidase (HRP)-conjugated secondary antibody. Life Technologies (Carlsbad, CA, USA) was the commercial source of Amplex Red fluorophore, Vybrant Cytotoxicity assay, and 4-methylumbelliferyl phosphate (4-MUP). Pyrithiamine hydrobromide was purchased from Sigma-Aldrich (Burlington, MA). MitoTracker Red, MitoTracker Green Hoechst H33342, and ATPLite reagents were from Invitrogen (Carlsbad, CA). Molecular Devices Corp. (Sunnyvale, CA, USA) was the source of the SpectraMax M5 microplate reader. All other fine chemicals will be purchased from CalBiochem (Carlsbad, CA), Pierce (Rockford, IL), or Sigma (St. Louis, MO).

2.2. Human Neuronal Cell Cultures

Primitive neuroectodermal tumor 2 (PNET2) human cerebellar neuronal cells

were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% heat-inactivated fetal calf serum (FCS), 9 g/L glucose, 10 mM non-essential amino acids, and 4 mM glutamine. Initial studies using 96-well micro-cultures, characterized Pyr dose effects on PNET2 cell viability and mitochondrial function using the Crystal violet assay [60] and the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay [61], respectively. Further studies examined the independent and additive effects of Pyr and ethanol on culture cell number, ATP generation, and mitochondrial function. The PNET2 models were generated by first exposing sub-confluent T-75 cultures to 0mM or 50mM ethanol for 48 hours, followed by re-seeding the cells into 96-well plates at a density of 2×10^4 cells/well. The resulting micro-cultures were treated for 48 hours with pyrithiamine (Sigma-Aldrich, 0 - 240 μM) and incubated in sealed humidified chambers with water or 50 mM ethanol vaporized from a reservoir tray for ethanol treatment [62]. The result was a 4-way model: Control-vehicle; Ethanol-vehicle; Control-Pyr; Ethanol-Pyr. The cultures were analyzed for cytotoxicity, mitochondrial function, mitochondrial mass, ATP production, and cell density using the Vybrant Cytotoxicity Assay Kit of LDH release into the culture supernatant, MitoTracker Red, MitoTracker Green, ATPLite, and Hoechst H33342 fluorescence-based assays, respectively.

2.3. Organotypic Cerebellar Slice Cultures

The Lifespan Institutional Animal Care and Use Committee (IACUC), Board Reference #000615 approved using rats for this research. Long Evans rat pups were administered intraperitoneal (i.p.) injections (50 μl) of saline (vehicle) or 2.0 g/kg of pharmaceutical grade ethanol (in saline) on postnatal days (P) 3, 5, 7, and 9 [27] [63] [64]. On P10, the rats were sacrificed. Freshly harvested cerebella were chilled in ice-cold Hank's Balanced Salt Solution and individually positioned in a McIllwain tissue chopper (Mickle Laboratory Engineering Co. Ltd, UK). Slices were made at 250 μm intervals in the sagittal plane, and then carefully separated under a dissecting microscope to minimize tearing. Two or 3 slices were placed (non-overlapping) on the bottoms of BD Falcon 8 μm pore culture inserts that were seated into 24-well Nunc plates with 1 ml of pre-warmed (37˚C) DMEM/well. DMEM was supplemented with 10% fetal calf serum, 25 mM KCl, 9 g/L glucose, 10 mM non-essential amino acids, 4 mM glutamine, 120 IU/ml Penicillin, and 120 μg/ml Streptomycin.

The cultures were maintained for 96 hours at 37˚C in sealed, humidified chambers equilibrated with 5% CO_2, 20% O_2, and 75% N_2. Control cultures from saline-injected pups were incubated in chambers in which water was vaporized from the reservoir tray. Cultures from ethanol-exposed pups were incubated in chambers with 50 mM ethanol supplied in the reservoir tray to permit continuous ethanol exposure [26] [65]. Culture media and the reservoir trays were changed daily. After 48 hours in vitro, control and ethanol-exposed slice cultures were co-treated with 0 or 280 μM Pyr for 48 hours. Eight replicate cultures were

generated per experimental condition. At the 96-hour experimental endpoint, the culture supernatants were assayed for LDH release using the Vybrant Cytotoxicity Assay Kit and the slice cultures were harvested. Representative tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, and microtome-sectioned (5 μm-thick) for Hematoxylin and Eosin (H&E) staining. The remaining fresh tissue samples were processed for molecular and biochemical assays.

2.4. Duplex Enzyme-Linked Immunosorbent Assays (ELISAs)

Slice culture tissue samples were homogenized in weak lysis buffer that contained 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA (pH 8.0), 50 mM NaF, 0.1% Triton X-100, and protease and phosphatase inhibitors [66]. ELISAs were performed in 96-well MaxiSorp plates. Proteins (100 ng/50 μl) were adsorbed to bottom surfaces of the wells by overnight incubation at 4˚C with gentle platform rotation, and then blocked for 3 hours with SuperBlock-TBS (Pierce Chemical Co, Rockford, IL). The samples were then incubated with primary antibody (0.1 - 0.4 μg/ml) for 1 hour at 37˚C. Immunoreactivity was detected with horseradish peroxidase (HRP)-conjugated secondary antibody and AmplexUltraRed soluble fluorophore. Fluorescence intensity was measured (*Ex* 565 *nm*/*Em* 595 *nm*) in a SpectraMax M5 microplate reader. To measure the expression of the subsequently, the samples were incubated with biotin-conjugated antibodies to large ribosomal protein as a housekeeping control molecule (RPLPO) [67], and immunoreactivity was detected with streptavidin-conjugated alkaline phosphatase (1:1000) and the 4-Methylumbelliferyl phosphate (4-MUP) fluorophore. Fluorescence (*Ex*360/*Em*450) was measured in a SpectraMax M5. Binding specificity was determined from parallel control incubations in which the primary or secondary antibody was omitted. The ratios of immunoreactivity corresponding to specific protein/RPLPO were calculated and used for inter-group comparisons [68] [69].

2.5. Molecules Assayed by Duplex ELISA

Duplex ELISAs [68] were used to examine the effects of ethanol and thiamine depletion on neuronal cytoskeletal protein (Tau and phospho-Tau), choline acetyltransferase (ChAT), acetylcholinesterase (AChE), 4-hydroxy-nonenal (HNE), activated Caspase 3, aspartyl-asparaginyl-β-hydroxylase (ASPH), and Humbug. HNE is a marker of lipid peroxidation. Activated Caspase 3 marks apoptosis. ASPH is abundantly expressed in developing cerebella, regulated by insulin/IGF-1, and previously demonstrated to be inhibited by ethanol [26] [57]. ASPH has a functional role in neuronal migration [26] [58]. Humbug is highly related to ASPH [52] but has a functional role in calcium regulation in the endoplasmic reticulum [52].

2.6. Statistics

The PNET2 experiments were performed with 8 sample wells per group. Pyr

dose effects were analyzed by linear trend analysis to test the hypothesis that the slopes significantly differed from 0. Inter-group comparisons of the mean levels of immunoreactivity or cellular/metabolic indices were made using T-tests and area-under-curve calculations. For the cerebellar slice culture experiments which included 4 groups, Control-vehicle, Ethanol-Vehicle, Control-Pyr, and Ethanol-Pyr, results were analyzed by two-way analysis of variance (ANOVA) with the Fisher multiple-comparison post-test (GraphPad Prism 10.2, La Jolla, CA).

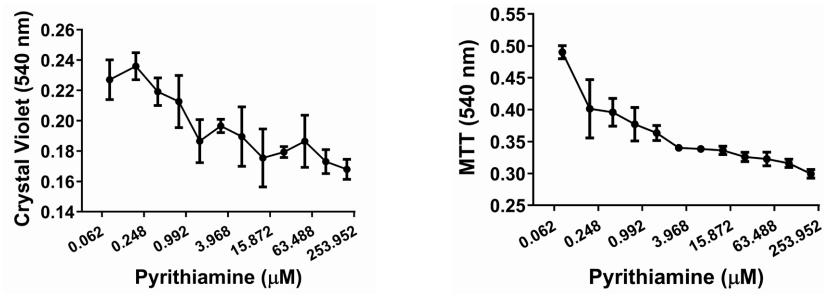
3. Results

3.1. Independent Effects of Pyr and Ethanol on PNET2 Cell Viability and Mitochondrial Function

To examine the effects of thiamine deficiency on PNET2 neuronal viability and mitochondrial function, sub-confluentmicro-cultures were treated with different concentrations of Pyr for 24 hours and then analyzed with the Crystal violet [60] and MTT [61] [70] assays. Increasing the concentration of Pyr from 0.062 to 250 μM caused progressive declines in PNET2 cell viability (**Figure 1(a)**) Linear trend analysis for Crystal violet revealed an R^2 = 0.284 with F = 18.26 for a non-zero slope ($p < 0.0001$). Similarly, increasing concentrations of Pyr resulted in progressive reductions in MTT activity (**Figure 1(b)**). Linear trend analysis for MTT yielded R^2 = 0.283 with F = 18.13 for non-zero slope ($p = 0.0001$). Treatment with 50mM ethanol caused significant reductions in viability based on the Crystal Violet assay (**Figure 2(a)**), MTT activity (**Figure 2(b)**), and ATP production (**Figure 2(c)**).

3.2. Pyr versus Ethanol + Pyr Effects on Mitochondrial Mass and Mitochondrial Function-PNET2 Cells

To assess the additive effects of Pyr plus ethanol, PNET2 cells were treated with a dose range of Pyr, or Pyr plus 50 mM ethanol. MitoTracker Red (**Figure 3(a)**) and MitoTracker Green (**Figure 3(b)**) fluorescence assessed mitochondrial



(a) (b)

Figure 1. Pyr dose-effects on neuronal viability and mitochondrial function. PNET2 96-well micro-cultures were treated with progressively higher concentrations (μM) of Pyr for 48 h. (a) The Crystal violet assay assessed viability (viable cells/culture), and (b) the MTT assay was used as an index of mitochondrial function. Data points reflect mean ± S.D. of results from 8 replicate cultures. Linear trend analysis yielded an R^2 = 0.284 ($p < 0.0001$) for CV and R^2 = 0.283 ($p = 0.0001$) for MTT.

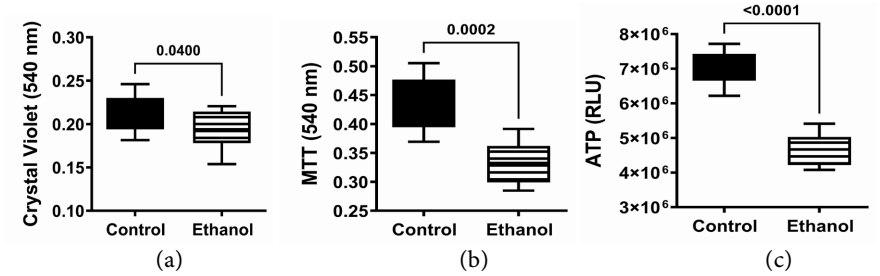


Figure 2. Ethanol inhibits immature neuronal cell viability and mitochondrial function. PNET2 cells treated with 50 mM ethanol for 48 h in 96-well plates were assayed for (a) viability using the Crystal violet assay, (b) MTT activity, and (c) ATP content. Inter-group comparisons were made by T-test analysis of results from 8 replicate cultures per assay.

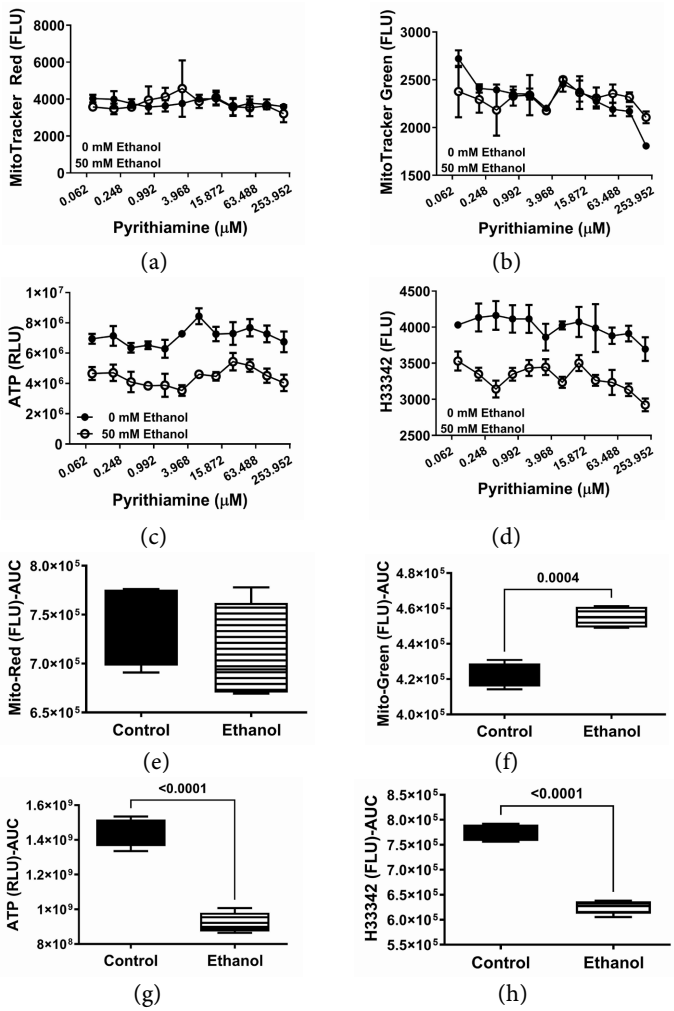


Figure 3. Pyr versus Pyr + ethanol effects on mitochondrial function and culture viability. PNET2 cells were treated with a dose range of Pyr or Pyr + 50 mM ethanol for 48 h. The cells were assayed for (a) MitoTracker Red, (b) MitoTracker Green, (c) ATP, and (d) Hoechst H33342 fluorescence. Graphs in Panels (a) - (d) depict the mean ± S.E.M. of results obtained from 8 replicate cultures per treatment/group. Panels (e) - (h) show results of area-under-curve calculations (mean ± S.D.) used to assess the overall effects of Pyr + ethanol versus Pyr only. T-tests were used for inter-group statistical comparisons.

function and mitochondrial mass, respectively. ATP content (Figure 3(c)) was quantified using the ATP-Lyte assay, and cell number (DNA content) was measured by Hoechst H33342 fluorescence (Figure 3(d)). Inter-group comparisons were made by T-test analysis of the calculated area-under-the-curve results from 8 replicate cultures per Pyr dose (Figures 3(e)-(h)). These studies demonstrated similar levels of MitoTracker Red fluorescence in the Pyr and Pyr + Ethanol-treated cultures across the dose range of Pyr (Figure 3(a), Figure 3(e)), but significantly higher levels of MitoTracker Green in Pyr + Ethanol versus Pyr-exposed cultures (Figure 3(b), Figure 3(f)). The consequences of combined Pyr + Ethanol were striking with respect to ATP luminescence (oxidative energy metabolism) (Figure 3(c)) and H33342 fluorescence (culture cell number) (Figure 3(d)). Co-treatment with 50 mM ethanol sharply reduced ATP content and H33342 fluorescence across the full dose range of Pyr. The aggregate effects shown by area-under-curve calculations confirmed the significant reductions in ATP (Figure 3(g)) and H33342 (Figure 3(h)) resulting from Pyr + Ethanol versus Pyr-only treatments.

3.3. Ethanol and Pyr Cerebellar Neurotoxicity Studies

Histological sections of control samples exhibited the normal immature 4-layer cerebellar cortical architecture with clearly delineated external and internal granule cell, Purkinje cell, and molecular layers (Figure 4(a) and Figure 5(a)). Pyr (Figure 4(b) and Figure 5(b)) or ethanol (Figure 4(c) and Figure 5(c)) treatment reduced cell densities in the internal granule and Purkinje cell layers, but ethanol was more disruptive to the organization of the cortical laminar architecture and caused more extensive dropout of Purkinje cells. The combined ethanol + Pyr treatments resulted in severe distortion of the cortical architecture together with extensive cell loss (Figure 4(d) and Figure 5(d)).

LDH release into the culture supernatants was measured at the 96-hour time point, just prior to harvesting the cerebellar slices. The two-way ANOVA test demonstrated significant effects of Pyr and ethanol, but not an ethanol × Pyr interaction (Table 1). Post-test analysis revealed significantly higher levels of LDH release in Pyr-treated relative to control, and ethanol + Pyr relative to control or ethanol (Figure 6(a)). Lipid peroxidation and apoptosis were assessed by duplex ELISA measurements of 4-hydroxy-2-nonenal (HNE) and activated Caspase 3 respectively. Results were normalized to RPLPO. Two-way ANOVA revealed significant ethanol and Pyr effects on HNE, and significant Pyr and ethanol × Pyr effects on activated Caspase 3 (Table 1). The post-tests demonstrated higher levels of HNE in ethanol-, Pyr-, and ethanol + Pyr-treated relative to control samples (Figure 6(b)). In addition, HNE levels were higher following ethanol ± Pyr, relative to Pyr alone, yet additive effects of the dual exposures were not detected (Figure 6(b)). Therefore, ethanol with or without Pyr significantly increased lipid peroxidation in cerebellar slice cultures relative to control and Pyr. In contrast, activated Caspase 3 was significantly increased by Pyr and Pyr +

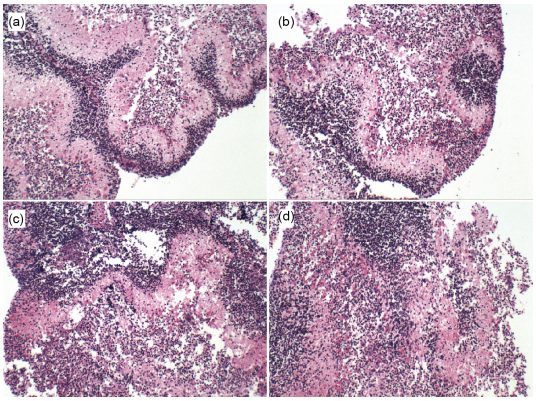


Figure 4. Histological features of cerebellar organotypic slice cultures (Low magnification images). Cultures generated from control and ethanol-exposed P10 rats were treated for 48 h with (a) vehicle (saline), (b) Pyr, (c) ethanol, or (d) Pyr + ethanol. The cerebellar slices were fixed in formalin and embedded in paraffin. Histological sections were stained with Hematoxylin and Eosin (H&E). Panels depict representative areas of cerebellar cortex. External granule cell layer = egc; molecular layer = m; Purkinje cell layer (arrows); internal granule cell layer = igc. Note the relatively neat laminar organization of control cerebellar folia, disrupted, fragmentation of the cortex associated with Pyr-treatment, marked fragmentation, architectural disruption, and thicker egc due to impaired neuronal migration associated with ethanol-exposure, and extensive loss of architectural organization and prominent parenchymal fragmentation associated with Pyr + ethanol treatment. Original magnifications, 100×.

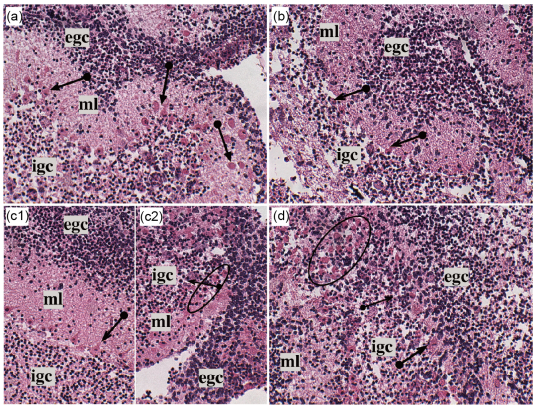


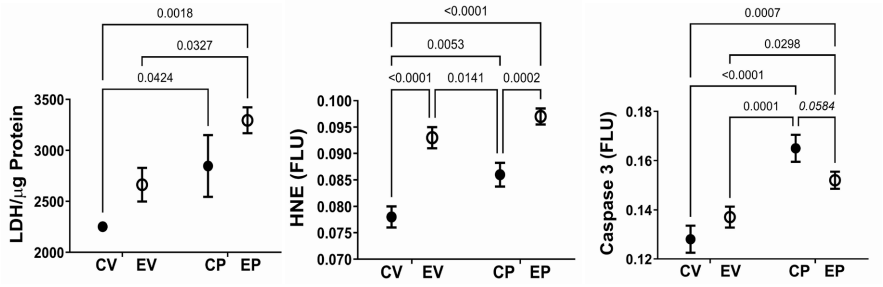
Figure 5. Histological features of cerebellar organotypic slice cultures (High magnification images). Cultures generated from control and ethanol-exposed P10 rats were treated for 48 h with (a) vehicle (saline), (b) Pyr, ((c1), (c2)) ethanol, or (d) Pyr + ethanol and then processed to generate paraffin-embedded H&E-stained sections. Panels depict representative areas of cerebellar cortex. External granule cell layer = egc; molecular layer = m; Purkinje cell layer (arrows with balls); internal granule cell layer = igc. (a) Note the relatively sharp delineations of the thin egc, uniform ml, abundant Purkinje cells, and cellular igl. (b) Pyr treatment thickened the egc, rendered the ml less uniform, reduced the Purkinje cell population, and resulted in igc cell loss. ((c1), (c2)) Ethanol exposures resulted in prominent expansion of the egc, reduced densities of Purkinje cells (arrows and circle in (c2)), and modest, irregular cell loss in the igc relative to control. (d) Combined Pyr + ethanol exposures caused substantial disruption of the basic cerebellar cortical architecture with irregular thickening and necrosis in the egc, Purkinje cell displacement/disorganization (circled) and loss, and poor preservation of the ml and igc. Original magnifications, 400×.

ethanol relative to control or ethanol only, reflecting dominant effects of Pyr on Caspase 3 activation (Figure 6(c)).

Table 1. Ethanol and pyrithiamine effects on LDH release, cytotoxicity, and protein expression in cerebellar slice cultures.

	Ethanol Effect		Pyrithiamine Effect		Ethanol × Pyrithiamine	
Protein	F-ratio	p-value	F-ratio	p-value	F-ratio	p-value
LDH	5.39	0.038	10.97	0.006	0.01	NS
HNE	44.15	<0.0001	9.40	0.003	1.04	NS
Caspase 3	0.18	NS	29.78	<0.0001	5.33	0.024
Tau	1.88	NS	10.97	0.0016	0.21	NS
pTau	2.06	NS	0.38	NS	1.05	NS
ChAT	0.00	NS	6.74	0.012	0.36	NS
AChE	0.29	NS	3.08	NS	0.57	NS
ASPH	43.01	<0.0001	5.36	0.024	*3.72*	*0.059*
Humbug	0.84	NS	0.37	NS	1.49	NS

Note: Two-way ANOVA test results (D.F. 1, 60 for all cells) corresponding to assays of glucose-6-phosphate dehydrogenase (G6PD) release, 4-hydroxy-2-nonenal (HNE), activated Caspase 3, Tau, phospho-Tau (T205), choline acetyltransferase (CHAT), acetylcholinesterase (AChE), aspartyl-(asparaginyl)-β-hydroxylase (ASPH), and Humbug immunoreactivity in control, ethanol-exposed, pyrithiamine treated, and ethanol + pyrithiamine treated cerebellar slice cultures. Significant p-values ($p \leq 0.05$) and statistical trend wise differences ($0.05 < p < 0.10$; italics) are indicated. NS = not significant. *Post hoc* test results are shown in Figure 6 and Figure 7.



(a) (b) (c)

Figure 6. Ethanol and Pyr effects on cytotoxicity in cerebellar slice cultures. Cultures from control and ethanol-exposed P10 rats were treated for 48h with vehicle (saline) or Pyr with 0mM or 50mM ethanol. (a) LDH release into the culture supernatants was measured using the Vibrant Cytotoxicity assay with results normalized to tissue protein content in the wells. (b) HNE and (c) activated Caspase 3 were measured by direct binding duplex ELISA. The groups are designated as control (C) or ethanol (E) based on *in vivo* and *ex vivo* exposures, and V (Vehicle/saline) or P, corresponding to vehicle or Pyr *ex vivo* treatments. Graphs depict mean ± S.E.M. for 4 replicate cultures in each group. Inter-group comparisons were made by two-way ANOVA (Table 1) and the Fisher post-test Results corresponding to significant ($p \leq 0.05$) and statistical trend effects ($0.05 < p < 0.10$; italics) are shown in panel (c).

3.4. Impact of Ethanol and Pyr on Cerebellar Neuron Protein Expression

Two-way ANOVA tests demonstrated significant Pyr effects on tau and ChAT. ASPH expression was significantly impacted by ethanol or Pyr, and statistical trend-wise altered by interactive effects of ethanol × Pyr (Table 1). The post hoc tests revealed significantly higher levels of Tau in Pyr-treated cultures (Figure 7(a)), higher levels of ChAT in ethanol + Pyr versus ethanol alone (Figure 7(c)), and lower expression of ASPH in ethanol ± Pyr relative to control or Pyr only (Figure 7(e)). There were no significant effects of Pyr, ethanol or, ethanol + Pyr on cerebellar expression of pTau (Figure 7(b)), AChE (Figure 7(d)), or Humbug (Figure 7(f)).

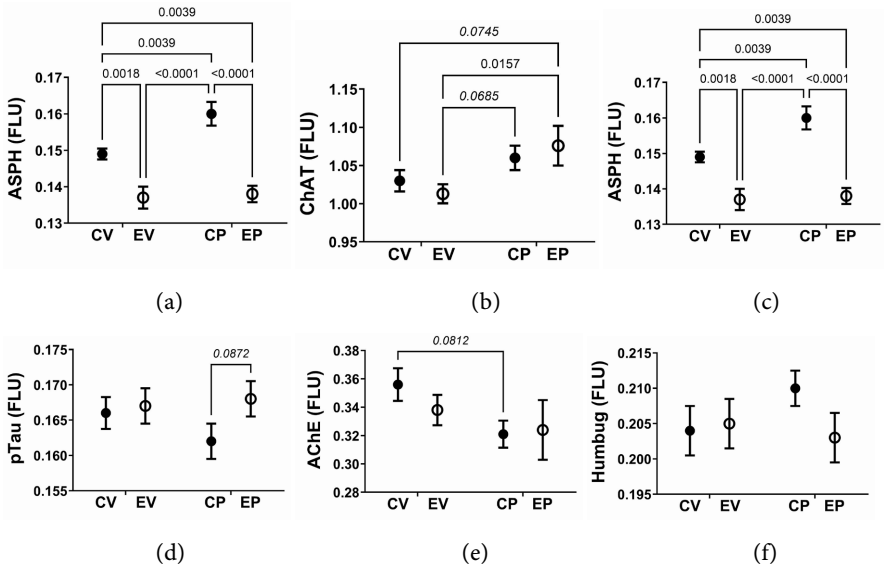


Figure 7. Ethanol and Pyr effects on cerebellar neuronal protein expression. Direct binding duplex ELISAs measured (a) Tau, (b) pTau, ChAT, (d) AChE, (e) ASPH, and (f) Humbug immunoreactivity in control (CV), ethanol (EV), Pyr (CP), and ethanol + Pyr (EP) treated cultures. Results were normalized to RPLPO measured in the same wells. Graphs depict mean ± S.E.M. for 4 replicate cultures in each group. Inter-group comparisons were made by two-way ANOVA (Table 1) and the *post hoc* Fisher post-test. Results corresponding to significant ($p \leq 0.05$) and statistical trend effects ($0.05\ p < 0.10$; italics) are shown in panels (b), (d) and (e).

4. Discussion

FASD is the most common preventable and costly cause of mental retardation, cognitive-motor disabilities, and attention deficit hyperactivity disorders that afflicts up to 5% of school age children in the United States [71] [72]. Excessive maternal chronic or binge alcohol consumption during pregnancy exerts teratogenic effects that adversely impact fetal brain development, fetal growth leading to low birth weight, fetal survival causing increased rates of preterm delivery and fetal demise [1] [3] [17] [28] [73] and placentation [74] [75], which is needed to support fetal growth and survival [76] [77]. Within the CNS, the cerebellum is

prominently targeted by developmental exposures to alcohol/ethanol [78]-[82]. Our previous studies linked cerebellar hypoplasia and neuronal migration disorders in experimental FASD to neuronal insulin resistance and oxidative stress [4] [26]. Additional consequences of ethanol-induced brain insulin resistance include reduced neuronal survival, growth, mitochondrial function, and neurotransmitter gene expression [4] [83]-[85]. Ethanol-mediated oxidative stress is caused by the inhibition of insulin/IGF signaling through metabolic pathways, and the toxic effects of acetaldehyde [86].

Despite strong evidence for ethanol's neurotoxic and dysmetabolic effects on the CNS, epidemiological and experimental studies indicate that the pathogenic mechanisms of FASD are complex. FASD's occurrences and severities can be modulated by cofactors such as maternal smoking [87]-[91] and nutritional deficiencies [33] [92]-[94]. In adults, thiamine deficiency is known to exacerbate the clinical manifestations of alcohol-related brain degeneration and it represents a major pathogenic mediator of Wernicke-Korsakoff encephalopathy [29] [31] [42] [95]-[97]. Importantly, with borderline vitamin and micronutrient intake, it is still possible to develop thiamine deficiency in the setting of heavy alcohol misuse since alcohol inhibits intestinal absorption of thiamine [42] [98]. The goal of this study was to use experimental models to investigate the independent and potentially additive effects of thiamine deficiency in relation to neurodevelopment as it relates to FASD. Research concerning the potential co-factor role of thiamine deficiency in FASD has been scant [34] and thus far has not addressed the potential teratogenic and neurotoxic effects in the CNS.

The PNET2 studies demonstrating dose-dependent Pyr-induced reductions in Crystal violet vital staining and MTT activity indicate that Pyr exerts neurotoxic effects, and that thiamine deficiency is sufficient to impair viability and mitochondrial function in immature human CNS neuronal cells. Corresponding with previous reports [12] [21], ethanol exposure alone significantly reduced viability and mitochondrial function in PNET2 cells. The next goal was to compare the neurotoxic/metabolic effects of Pyr with those of Pyr plus ethanol to assess their additive adverse effects on mitochondrial mass (MitoTracker Green), mitochondrial membrane potential (MitoTracker Red), energy metabolism (ATP generation), and cell loss (Hoechst's H33342). The similar levels of MitoTracker Red fluorescence over a broad Pyr dose range, with or without co-treatment with ethanol indicate that Pyr and ethanol effects were not additive. In contrast, ethanol additively increased MitoTracker Green over the Pyr dose range, reflecting expanded mass of mitochondrial oxidative activity. Most likely this response reflected enhanced oxidative stress, which is known to increase mitochondrial mass in the setting of apoptosis [99] [100]. However, the two main additive inhibitory effects of ethanol and Pyr pertained to ATP generation and cell culture density, which were both significantly lower in the ethanol + Pyr relative to Pyr treated cultures. Altogether, the results depicted in **Figures 1-3** suggest the mechanisms of mitochondrial dysfunction and cell loss are not identical for ethanol

and Pyr, and that while thiamine deficiency likely worsens neuronal damage caused by ethanol, the consequences of heavy alcohol consumption vis-à-vis thiamine deficiency would be reduced but not be fully abrogated by thiamine repletion.

The *ex vivo* cerebellar slice culture experiments were more informative because the effects of ethanol, Pyr, and combined exposures were assessed in relation to all cell types and likely correspond to *in vivo* responses in FASD. The histopathological studies revealed greater effects of cell loss and necrosis contributing to the architectural disruption in Pyr treated cerebella, versus thickening of the external granule cell layer and simplification of foliation in the ethanol-treated cultures. Previous *in vivo*, *ex vivo* and in vitro experiments demonstrated that ethanol has substantial inhibitory effects on cerebellar neuron growth, migration, and viability [26] [80] [91] [101]-[103], accounting for the pathology noted herein. The combined exposures clearly caused substantially greater damage to the immature cerebellum than either Pyr or ethanol alone, suggesting that the long-term effects on motor functions would be compromised to greater extents than with either thiamine deficiency or developmental alcohol exposure. Corresponding with the findings with respect to PNET2 cells, these results also suggest that if thiamine deficiency were present, then its supplementation would likely reduce but not prevent the adverse effects of prenatal alcohol exposure on cerebellar dysgenesis and dysfunction linked to FASD.

Further studies delineated the mechanisms of ethanol versus Pyr versus Ethanol + Pyr mediated cerebellar neurotoxicity. Ethanol and Pyr differed in how they exerted their neurotoxic effects that led to cell loss such that Pyr significantly compromised membrane integrity, increased lipid peroxidation, and activated Caspase 3, whereas ethanol selectively increased lipid peroxidation. Therefore, the differential effects of thiamine deficiency included reduced membrane integrity and increased apoptosis, while lipid peroxidation resulted from ethanol exposure or thiamine deficiency. Of note is that lipid peroxidation compromises cell membrane integrity and serves as an important source of oxidative damage, cell loss, and ultimately impaired neuronal function. This phenomenon could account for the additive effect of ethanol + Pyr with respect to LDH release.

The significant elevation of activated Caspase 3 linked to Pyr, with or without ethanol co-exposure, indicates that thiamine deficiency promotes CNS cell loss due to apoptosis. This conclusion corresponds with the known inhibitory effects that thiamine deficiency has on oxidative phosphorylation pathways utilized for energy metabolism [42] [46] [104], together the findings herein that Pyr caused substantial reductions in ATP and cell number in PNET2 cultures. Mechanistically, previous studies showed that in the CNS, thiamine deficiency inhibits alpha-ketoglutarate dehydrogenase which is needed for brain glucose and energy metabolism [104], promotes endoplasmic reticulum stress with activation of the unfolded protein response, which leads to cell death [105], and upregulates the

expression of pro-apoptosis-inducing genes leading to Caspase 3-mediated apoptosis [106].

A novel aspect of this study was that we characterized ethanol versus Pyr treatment effects on the expression of neuronal proteins that are critical to cerebellar development and function. Tau is an important neuronal cytoskeletal protein needed to maintain the structure and connectivity of neurons. Processing of Tau for transport from the perikaryal to cell processes is mediated by physiological phosphorylation via various kinases. In neurodegenerative disease, tau accumulation and aggregation increase oxidative stress and contribute to neurotoxic responses. Hyper-phosphorylation of Tau signals aberrant kinase activation which occurs with oxidative stress (often transiently) and can signal protein ubiquitination and aggregation with attendant cellular damage. The studies demonstrated significantly elevated levels of Tau in Pyr-treated cerebella, irrespective of ethanol co-exposure. This effect likely corresponds with the increased Caspase 3 activation leading to cellular retraction and disconnection prior to apoptosis.

ASPH, an ~86 kD Type 2 transmembrane protein [50] [52], is an important insulin and IGF-regulated molecule that is expressed on cell surface and endoplasmic reticulum membranes [51] [52] [107] and has a pivotal role in regulating cell motility in development and neoplasia [51] [53] [55] [107]-[113]. ASPH expression is inhibited by ethanol [26] [58] [74] [114] and consequently down-regulated in chronic alcohol disease-related states including FASD, corresponding with impairments in neuronal migration [26] [58] [74] [75] [114]. Several ASPH-related molecules have been characterized, including Humbug, a truncated form that is nearly identical to the N-terminal region of ASPH but lacks the C-terminal catalytic domain [52]. Humbug has roles in regulating calcium flux and cell adhesion [53] [115]. ASPH's cell migration functions are linked to its C-terminal catalytic domain which hydroxylates β-carbons of Asp and Asn residues in EGF-like domains [51] [107] of molecules such as Notch and Jagged [53] [55]. The resulting activation of Notch pathways increases cell motility and adhesion of immature and neoplastic cells [51] [53] [56] [116]. In addition, ASPH signals through hypoxia-inducible factor 1 alpha (HIF-1α), driving cell motility under conditions of oxidative stress [56] [57] [117].

Corresponding with previous studies, we observed significant ethanol inhibition of ASPH, with similar degrees of inhibition in the presence or absence of Pyr. In contrast, Pyr significantly increased ASPH. Inhibition of ASPH corresponds with the highly disordered cerebellar cortical architecture together with reductions in neuronal density, *i.e.* conspicuous cell loss observed in the slice cultures. On the other hand, the over-activation of ASPH in Pyr-only treated cerebellar cultures is of uncertain significance, although it could reflect toxic responses with increased oxidative stress mediated by HIF-1α up-regulation [56]. It is of interest that Humbug expression was not significantly modulated by the

exposures, suggesting that ethanol's inhibitory effects were selective.

5. Conclusion

In summary, this study showed that ethanol and thiamine deficiency states adversely impact the viability and function of CNS neuronal cells, and significantly distort developing cerebellar architecture. Both ethanol and Pyr impaired cerebellar neuronal survival, but ethanol had selective inhibitory effects on ASPH. Attendant impairments in neuronal migration account for the associated cerebellar dysgenesis. Ethanol's and Pyr's distinct mechanisms of compromising energy metabolism account for their additive inhibitory effects on ATP generation, cell number, cytotoxicity, and lipid peroxidation. In contrast, Pyr differentially activated Caspase 3, promoted Tau accumulation, and increased ASPH, which could represent excitotoxic responses linked to higher levels of oxidative stress. The findings suggest that neurodevelopmental impairments associated with FASD, including inhibition of neuronal migration (reduced ASPH expression) and lipid peroxidation, would not be prevented by thiamine repletion alone, and instead would require maternal abstinence from harmful ethanol exposures. On the other hand, the findings that alcohol's neurotoxic effects on energy metabolism, cytotoxicity, apoptosis pathway activation, and neuronal cytoskeletal dysfunction were exacerbated by thiamine deficiency suggest that these mediators of FASD pathogenesis could be minimized by thiamine supplementation during pregnancy.

Authors' Contributions

SMdlM conceived the research, planned the experiments, reviewed all data and figures, and prepared the original and final versions of the manuscript. MT conducted the experiments under the guidance and supervision of SMdlM, was fully involved in data analysis, and reviewed the manuscript. ES participated in the experiments including their planning and execution and helped with sample analysis and review of the manuscript.

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Datasets Availability Statement

The data supporting the findings of this study are available on request from the corresponding author.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Supplementary

Table S1. Antibodies used for duplex ELISA studies.

Antibody	Source	Type	Final Concentration or Dilution	Commercial Source	Catalog# or RRID#
4-hydroxynonenal (HNE)	Goat	Polyclonal	1.0 µg/ml	Abcam, Waltham, MA	REF A0024
Activated Caspase 3	Rabbit	Polyclonal	2 ug/ml	A Abcam, Waltham, MA	AB2302
Tau (Tubulin associated unit)	Rabbit	Polyclonal	1.55 µg/ml	Agilent/Dako, Santa Clara, CA	AB_10013724
pTau (T205) (Phosphorylated Tubulin associated unit)	Rabbit	Polyclonal	0.8 µg/ml	Abcam, Waltham, MA	AB_304676
ChAT (Choline Acetyltransferase)	Rabbit	Polyclonal	1:3000	Abcam, Waltham, MA	AB6168-100
AChE (Acetylcholinesterase)	Mouse	Monoclonal	0.25 µg/ml	Abcam, Waltham, MA	AB2803
ASPH (aspartyl-asparaginyl-β-hydroxylase)	Mouse	Monoclonal A85G6	0.2 µg /ml	RI Hospital-Providence, RI	[26]
Humbug-Truncated ASPH	Mouse	Monoclonal A85E6	0.2 µg /ml	RI Hospital-Providence, RI	[26]
Large acidic ribosomal protein (RPLPO)	Mouse	Monoclonal	0.1 µg/ml	Santa Cruz, Dallas TX	sc-293260b [67]