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Differential expression of proteins in fetal brains of alcohol-treated prenatally C57BL/6 mice: a proteomic investigation

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Abstract

Alcohol is known to impede the growth of the central nervous system and to induce neurodegeneration through cellular apoptosis. We have previously shown that moderate prenatal alcohol exposure results in brain defects at different stages of development. In this study, we further characterize the proteomic architecture underlying ethanol teratogenesis during early fetal brain development using chromatography in conjunction with a tandem mass spectrometry (LC-MS/MS) system. Pregnant C57BL/6 mice were exposed from embryonic day 7 (E7) to E13 with either a 25% ethanol derived calorie (ALC) or pair-fed (PF) liquid diets. At E13, fetal brains were collected from 5 dams for each group. Individual brains were homogenized and the extracted proteins were then tryptically digested and analyzed by LC-MS/MS. Label-free quantitative proteomic analyses were performed on proteomes extracted from fetal brains of both ALC and PF groups. These analyses demonstrated that prenatal alcohol exposure induced significant down-regulation ($p < 0.001$) of the expression of mitochondrial enzymes including ADP/ATP translocase 1, ATP synthase subunit α and ubiquinol-cytochrome-c reductases. In addition, mitochondrial carrier homolog 1, which plays a role in apoptosis, was significantly down-regulated ($p < 0.001$) in the ALC group. Moreover, among the cytosolic proteins that were significantly down-regulated ($p < 0.001$) are Bcl-2, 14-3-3 protein and calmodulin. Significant down-regulation ($p < 0.001$) of proteins that are critical for fetal brain development was observed such as prohibitin and neuronal migration protein doublecortin. These findings provide information about possible mechanisms underlying the effects of prenatal alcohol exposure during early embryonic stage.

Keywords

ethanol; teratogenesis; cytochrome c; mitochondria; apoptosis

Introduction

Fetal alcohol exposure (FAE) and fetal alcohol syndrome (FAS) are characterized by brain growth restriction, microencephaly, facial dysmorphology, and neurological disorders [1–10]. Prenatal alcohol exposure has been shown to impede cerebral and cerebellar growth in

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rat models [1–3, 11–14]. In addition, prenatal alcohol exposure from embryonic day 7 (E7) to E13 and E15 reduces body weight, head size, and brain weight in mouse models [for review see ref. [15]]. During the period from E7 to 15, the developing brain exhibits the highest susceptibility to alcohol exposure.

Retardation of brain growth [1–3, 11–14] and induction of morphological defects caused by prenatal alcohol exposure [15] may occur through an apoptotic mechanism [16]. Mitochondrial activation of apoptotic pathways is a major player in ethanol-mediated neuronal death [16] and the mitochondrion is a target organelle of ethanol-induced organ-system toxicity [17]. Prenatal alcohol exposure induces mitochondrial dysfunction such as diminished mitochondrial glutathione concentration, decreased activities of respiratory chain complex IV and ATP synthase, and increased mitochondrial permeability transition [18–21]. Moreover, evidence suggests that alcohol exposure may accelerate apoptosis in the developing brain either through direct activation of intrinsic mitochondrial apoptotic signaling pathways or through indirect extrinsic pathways such as receptors systems [22, 23].

One extrinsic signaling pathway involves the activation of caspase cascades [24]. However, research has suggested that this extrinsic signaling pathway is not involved in the apoptotic effect of alcohol exposure at postnatal ages [25]. The intrinsic signaling pathway may involve the translocation of proteins from the outer mitochondrial membrane to the cytosol. This process increases the permeability of the membrane and releases cytochrome c [26], which leads to activation of caspase cascades. At the mitochondrial outer membrane, apoptotic proteins including the Bcl-2 family may regulate survival/apoptosis during fetal brain development. The Bcl-2 family of proteins has been implicated in ethanol-induced apoptosis in prenatal and neonatal developing brains [25, 27–31].

Although the mechanism of alcohol-induced growth restriction is unknown, evidence suggests that alterations of nuclear proteins, mitochondrial proteins, and intrinsic/extrinsic factors may be central factor in the growth restriction process. While most studies focus on the effects of prenatal alcohol exposure on anatomical changes and enzyme activities in the cytosol and mitochondria, here we utilize quantitative proteomics to investigate the effects of prenatal alcohol exposure in the expression of proteins in the cytosol, nucleus and mitochondria at E13. Proteomics investigate the global changes in the protein expression that may occur during embryonic stages as a consequence of prenatal alcohol exposure. It is unknown whether alcohol-induced injuries affect nuclear encoded proteins, mitochondrial proteins, cytosolic proteins, or all systems. Thus, the proteomic approach is applied to provide information about the role of mitochondrial, nuclear, and cytosolic proteins in alcohol-induced fetal brain injuries.

Materials and Methods

1. Animals

C57BL/6 mice tested in this study were supplied by Harlan Inc. (Indianapolis, IN). They were housed in the Indiana University Laboratory Animal Research Center (LARC) in a vivarium with a controlled climate (temperature 22°C, 30% humidity) and 12:12 reverse light-dark cycle. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Indiana University Bloomington and are in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee of the National Institutes of Health, and the Guide for the Care and Use of Laboratory Animals.

2. Breeding and treatment procedure

Female mice were placed into male home cages for 2 hours; immediately afterward, females were checked for a sperm plug by vaginal smear. When positive, this time point was designed as E0. Weight-matched pregnant females were assigned on E7 to the following groups: (1) Ethanol liquid diet group (ALC, n=5) 25% (4.49%, v/v) ethanol derived calories (EDC) with chocolate-flavored Sustacal (Mead Johnson, Evansville, IN) supplemented with vitamins and minerals, and (2) Pair-fed control group (PF, pair-fed to ethanol-fed group, n=5) fed with a maltose-dextrin solution made isocaloric to the dose of ethanol used. ALC groups were adjusted to a pair-fed liquid diet on E6. This drinking behavior of alcohol administration has been well established in our laboratory (for review see [15]). Ten litters were used in this study (five litters from each group, ALC or PF group). On E13, animals were sacrificed, and their fetal brains were removed and frozen for proteomic analyses. For both PF and ALC groups, additional fetal brains were randomly collected from 3 of the 5 aforementioned litters to test for variability within the same dam.

The fortified liquid diet contained 237 ml of chocolate-flavored Sustacal, 1.44 g Vitamin Diet Fortification Mixture and 1.2 g Salt Mixture XIV (Bio-Serv, Frenchtown, NJ). For the ethanol diet, 15.3 ml (4.49% v/v; 25% EDC) of 95% ethanol was added to the fortified Sustacal formula with water that was added to make 320 ml of diet with 1 cal/ml (ethanol). For the isocaloric control diet, 20.2 Maltose Dextrin was added to the fortified Sustacal formula and water was added to bring it to 1 cal/ml [32, 33]. One day before treatment, the ALC dams and the PF dams were adapted to the liquid diet. The dams were weighed, the volume of liquid diet consumed during the previous 24 h was recorded from 30-ml graduated screw-cap tubes, and freshly prepared diet was provided. The PF subjects had unlimited access to the EDC liquid diet each day to match the amount of consumed calories of ALC subjects.

3. Maternal Blood Alcohol Levels

Maternal blood alcohol levels were tested in a separate group of C57Bl/6 dams with the 25% (4.49%, v/v) ethanol derived calorie (EDC) diet as described previously [34, 35]. Briefly, pregnant mice were given the same feeding protocol as the other experimental dams (EDC diet provided on E7 at 900 h in a reverse dark cycle), and two 50- μ l tail blood samples were obtained (at 1100 and 1300 h) on E8 and E11. The blood samples were collected in heparinized capillary tubes and centrifuged, and 5 μ l plasma samples were analyzed for alcohol concentrations using the Analox Alcohol Analyzer, calibrated with a 100 mg/dl ethanol standard. Blood alcohol concentrations (BACs) were evaluated at 2 and 4 hours on E8 and E11. The BACs were consistently higher at 2 hours as opposed to 4 hours exposure. The average peaks obtained in the 25% EDC group at 2 hrs were ~40 mg/dl on E8 and ~55 mg/dl on E11.

4. TUNEL immunostaining reaction for cell death detection

Cell death was determined using TUNEL reactions (TdT-mediated dUTP Nick End Labeling) as described recently by us [35]. Apoptosis and cell mediated cytotoxicity are characterized by a fragmentation of the genomic DNA. The many breakpoints can be visualized with the TUNEL reaction using immunochemical TUNEL kit (Roche Pharmaceuticals, Inc, IN). To keep consistent conditions of immunostaining for both the experimental and the control groups, we embedded the fetal brains from PF and ALC groups in gelatin. All fetal brains were aligned at the same level in 10% gelatin (grade A), and serial 50- μ m coronal sections were then cut using a vibratome apparatus (Leica Microsystems Inc., Bannockburn, IL). Fetal brain sections were treated with Proteinase K (10–20 μ g/ml) (Sigma-Aldrich, St. Louis, MO) for 5 min at 37°C, rinsed with phosphate-buffered saline (PBS) 3 times for 5 min and then incubated with 3% H₂O₂ in methanol for 10 min at room

temperature. The sections were again rinsed with PBS 3 times for 5 min and then incubated in a permeabilisation solution (0.1% TX-100 in 0.1% sodium citrate) for 2 min at 4°C. After the sections were rinsed twice in PBS for 5 min, they were incubated with a TUNEL reaction mixture (50 µl from bottle 1 and 450 µl from bottle 2, TUNEL kit) for 1 h at 37°C. The control was prepared by incubation in solution from bottle 2 only. The sections were rinsed 3 times for 5 min with PBS and incubated in converter-POD for 30 min at 37°C. After the sections are rinsed with Tris-buffered saline (TBS), they were incubated in 0.05% 3'-3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO) and 0.003% H₂O₂ in TBS to reveal the peroxidase activity.

5. Proteomics

5.1. Tissue preparation/protein extraction—Frozen fetal brain tissues were homogenized in solution containing 5 M urea, 2 M thiourea, 2% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS), 2% (w/v) sulfobetaine 3-10, 40 mM Tris, 0.2% (w/v) Bio-Lyte 3/10, and 2 mM tributyl phosphine (Sigma-Aldrich, St. Louis, MO). All subsequent steps were performed at 4°C using tissue tearor™ homogenizator (BioSpec Products, Inc., Bartlesville, OK) at 30,000 rpm. The homogenate was incubated for 1 hr at 4°C and then centrifuged at 35,000 rpm for 1 hr at 4°C. Protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA) using bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO) as a standard. The amount of protein determined in each brain sample was calibrated to 15 µg. The proteins extracted from both pellet and supernatant were subjected to trypsin digestion prior to LC-MC/MS analysis.

Trypsin digestion: A 10 µg aliquot of total proteome was suspended in 8 µL of 10 mM ammonium bicarbonate ((Sigma-Aldrich, St. Louis, MO). Reduction was carried out for 1 hr at 37°C after adding 1 µL of 10 mM DTT in 50 mM ammonium bicarbonate (Sigma-Aldrich, St. Louis, MO). One µL of a 1% acid-labile surfactant (RapiGest, Waters, Milford, MA) solution was added to the solution and the mixture was heated at 95°C for 5 min. After cooling to room temperature, 2 µL of 0.1 mg/ml proteomic grade trypsin (Sigma-Aldrich, St. Louis, MO) was added, and the sample was digested overnight at 37°C. The digestion reaction was terminated by the addition of 1 µl of formic acid. Subsequently, the digested protein was subjected to LC-MS/MS analysis.

Protein identifications: LC-MS/MS analyses of the tryptic digests were performed using a Dionex 3000 Ultimate nano-LC system (Dionex, Sunnyvale, CA) interfaced to OrbiTrap Mass Spectrometer (ThermoElectron, San Jose, CA). Prior to separation, a 5-µl aliquot of trypsin digestion (2 µg protein equivalent) was loaded on a PepMap300 C18 cartridge (5 µm, 300 Å, Dionex, Sunnyvale, CA) and eluted through a pulled-tip capillary column (150 mm × 75 µm i.d.) packed with 200 Å Magic C18AQ Packing Materials (Microm BioResources Inc., Auburn, CA). The tryptic digests were separated using a reversed-phase gradient from 0–35% B, 97% acetonitrile with 0.1% formic acid over 150 min, at 250 nl/min flow rate. The mass spectrometer was operated by an automated data-dependent mode that was switching between MS scan and CID-MS. In this mode, eluted LC products undergo an initial full-spectrum MS scan from m/z 400 to 2000 in the OrbiTrap at 15,000 mass resolutions, and a subsequent CID-MS (at 35% normalized collision energy) was performed in the ion trap. The activation scan steps were performed on the same precursor ion over the same m/z scan range as used for the full-scan MS spectrum. The precursor ion was isolated using the data-dependent acquisition mode with a 2 m/z isolation width to select automatically and sequentially five most intense ions (starting with the most intense ion) from the survey scan. The total cycle (6 scans) takes less than one second and is continuously repeated for the entire LC-MS run under data-dependent conditions with

dynamic exclusion. Performing MS scanning in the Orbitrap allows high mass accuracy and accurate charge state assignment of the selected precursor ions.

Between sample injections, both trapping column and separation column were subjected to a washing step. This involved the injection of a blank and washing of both columns with 85% acetonitrile aqueous mobile-phase for 45 min. Next, they were preconditioning with 10% acetonitrile aqueous mobile-phase for 10 min prior to the next sample injection. This washing step insured no sample carryover, as suggested by the failure to identify peptides from the tandem mass spectra generated during the washing step.

5.2. LC-MS/MS Quantitative Analyses

Label-free quantitative proteomics: MS/MS spectra were searched against protein sequences for rodent in the Swiss-Prot database using a licensed copy of MascotTM for peptide. The data were submitted to another search against a reversed version of the same protein database in order to estimate the false positive rate [36, 37]. The estimation was then used to set a MOWSE-based peptide filtering criteria, where the ion score threshold represented a false positive rate of less than 5%. In addition, the peptide hits found with the same query numbers in both regular and reversed databases were excluded from the results, since they are most likely false positives. Identified proteins and peptides were then processed and quantified using our in-house developed ProteinQuant Suite software (available at www.ncgg.indiana.edu) [22]. It consists of three standalone complementary computer utilities, namely Turbo RAW2MGF, ProtParser, and ProteinQuant. Turbo RAW2MGF directly processes raw data files generated by LC-MS/MS (Xcalibur, ThermoElectron, San Jose, CA) and generates a MASCOT generic file (MGF). This file format includes information required for the precise identification and quantification of peptide chromatographic peaks. ProtParser is a filtering utility designed to evaluate database search results. Filtering was performed according to the above described criteria. For quantification, ProteinQuant utilizes this parsed list of peptides and their corresponding proteins in conjunction with mzXML files generated from the raw files of the LC-MS/MS analyses. This quantification is based on the automatic detection and integration of chromatographic peaks representative of the LC-MS/MS elution profiles for identified peptides. Finally, protein abundance is calculated by adding the areas of all the peptides identified for this particular protein that fulfill the above filtering criteria. A list of proteins and their corresponding abundance is generated for each sample (supplementary Table 1) which is then imported to MarkerView (Applied Biosystems Inc., Framingham, MA) for chemometric analysis.

Data evaluation and statistical analyses: Principal component analysis (PCA) was performed using MarkerView, allowing the visualization of multivariate information. Supervised PCA methods were employed, using prior knowledge of the sample groups (PF versus ALC). MS data were weighted using the base-e logarithm of protein abundances. They were also scaled using pareto option in which each value is subtracted by the average and then divided by the square root of the standard deviation. This option is suitable for MS data as it prevents intense peaks from completely dominating the PCA process, thus allowing any peak with good signal-to-noise ratio.

The range of values obtained in this study was expressed as a standard error of mean (SEM), which accounts for the sample size. Standard deviation is the most common measure of statistical dispersion, measuring how spread-out the values are in a data set (e.g., due to limitations in measurement reproducibility). However, when working with biological samples, any observed variation might be intrinsic to the phenomenon that distinct members of a population (biochemical individuality). Consequently, the standard error (SE) or SEM

estimates the standard deviation of the sampling distribution of means based on the data from one or more random samples. SEM accounts for the number of real samples while implicating their biodiversity in the evaluation process.

Comparisons of the protein expression levels between the PF and ALC groups were done using the Wilcoxon rank sum test [38] (also known as Mann-Whitney rank sum test) and the exact method was utilized to calculate the *P* values. Due to the large number of tests, the false discovery rate [39] (FDR) was employed to correct for multiple comparisons, and statistical significance threshold was determined by controlling FDR at 1%. The exact *P* values from rank sum test for those proteins with significant changes are included in supplementary Table 2. Some of the proteins that have demonstrated statistical differences between the two groups are listed in Table 1, while the complete list of all the proteins that demonstrated significant differences is depicted in supplementary Table 1. All statistical analyses were carried out using Matlab version 7.4.0.287 (The MathWorks).

Results

1. TUNEL-staining for determination of cell death

In order to determine whether prenatal alcohol exposure induces apoptosis, we used TUNEL-staining to evaluate cell death. Among the fetal brain regions that were analyzed anatomically are the primordium frontal cortex and basal ganglia eminence. These fetal brain regions have been well studied by us in the FAE model [for review see [15]]. In the primordium frontal cortex, we found that prenatal alcohol exposure induced increases in TUNEL-positive cells in ALC group (Figure 1b) compared to PF group (Figure 1a). Apoptotic neurons are indicated by arrowheads and are clearly identified at high magnification photomicrographs in ALC (Figure 1d) versus PF groups (Figure 1c).

2. Qualitative Proteomics

Eight fetal brains were collected at E13 from PF dams of which three pairs were collected from the same dams; similarly eight fetal brains at E13 were collected from dams that have been prenatally exposed to alcohol (ALC dams). Samples were prepared as described in the methods section. The mixture of proteins collected from all samples contained, on average, $16 \pm 0.3 \mu\text{g}$ of proteins as determined by the Bradford assay. Only $2 \mu\text{g}$ of the tryptically digested proteomes were analyzed with LC-MS/MS. The LC-MS/MS analyses of the total proteomes extracted from PF group resulted in the identification of 733 tryptic peptides originating from 246 proteins, while that of ALC group resulted in the identification of 705 tryptic peptides originating from 238 proteins. Peptide identification was based on database searching using MASCOT search engine and a filtering criteria, resulting in at least 95% identification confidence and a false positive identification rate less than 5%.

The distribution of proteins identified is shown in Figure 2. The majority of proteins consisted of 26% enzymes, 18% nuclear binding proteins, 9% ribosomal proteins, 8% regulatory proteins, 7% histones, 4% chaperones, 4% transporting proteins, 10% cytoskeletal proteins, and others. The distribution of all identified proteins is illustrated in Figure 1a. As shown in Figure 1b, the shared 26% enzymes were composed of 17% kinase, 14% dehydrogenase, 13% isomerase, 12% cytochrome oxidase c, 9% synthase, 6% oxidoreductase, 6% helicase, 4% ubiquinol-cytochrome reductase c, 4% mutase, 4% translocase, 3% transferase, and 7% other enzymes.

3. Identifications of proteins through LC-MS/MS Quantitative Analyses

The quantitative proteomics were based on a label free approach. The areas of distribution corresponding to the identified proteins were then determined using in-house developed

protein quantification software (ProteinQuant) [40]. To cluster data according to their differences, the data were imported into MarkerView, (Applied Biosystems Inc., Framingham, MA), a chemometric software that utilizes Principal Component Analysis (PCA). PCA score plots of the areas of distribution of all proteins identified in the two groups analyzed are shown in Figure 3. Statistical differences in the areas and number of proteins between PF and ALC groups resulted in a distinct data-clustering, thus yielding obvious separation in the PCA score plot. Sample ALC#2 appears to have different expression of proteins that have resulted in the clustering of this sample and PCA different than the other ALC samples. We believe that this is mainly due to the biological individuality of this animal.

In table 1, we have summarized some of the proteins that significantly contributed to the distinct clustering observed in Figure 3. Table 1 shows differences in the expression of proteins between ALC and PF groups. The changes in proteins observed in Table 1 are based on the analysis of individual fetal brain proteomes. Determination of protein expression by quantitative proteomics was performed at the level of individual fetal brains collected from ALC and PF groups at E13.

Several fetal brain proteins were affected by prenatal alcohol exposure, as shown in Table 1. Most of the proteins analyzed included nuclear, cytosolic, mitochondrial and cytoskeletal proteins. Prenatal alcohol exposure induced down-regulation of mitochondrial proteins, including NADH-ubiquinone oxidoreductase 20 kDa ($p=2.E-04$), NADH dehydrogenase 1 α subcomplex subunit 9 ($p=2.E-04$), cytochrome b-c1 complex subunit 1 ($p=3.E-04$), cytochrome b-c1 complex subunit 2 ($p=2.E-04$), mitochondrial carrier homolog 1 ($p=2.E-04$), ADP/ATP translocase 1 ($p=2.E-04$), ATP synthase α -subunit ($p=2.E-04$), and prohibitin ($p=2.E-04$). Figure 4 shows the dot-plots of the areas of some of these proteins, which were significantly different between PF and ALC groups as determined by statistical evaluation. The data suggests that prenatal alcohol exposure induced mitochondrial dysfunction via the down-regulation of these proteins. Prenatal alcohol exposure also significantly reduced the expression of cytoskeletal proteins such as microtubule-associated protein 2 (MAP2, $p=2.E-04$), β -actin ($p=2.E-04$), and tubulin α -3 chain ($p=2.E-04$).

Moreover, prenatal alcohol exposure resulted in changes in the expression of several nuclear proteins (Table 1). The nuclear proteins found to be significantly down-regulated in ALC groups were histone-binding protein RBBP4 ($p=2.E-04$), nuclear pore complex protein Nup107 ($p=2.E-04$), Elav-like protein 1 (Hu-antigen R) ($p=2.E-04$), mediator of DNA damage checkpoint protein 1 ($p=2.E-04$), 40S ribosomal protein S3 ($p=2.E-04$), and SON protein ($p=2.E-04$). However, prenatal alcohol exposure up-regulated probable ATP-dependent RNA helicase ($p=1.E-03$) and heterogeneous nuclear ribonucleoprotein H ($p=6.E-04$) (Figure 5).

Several cytosolic proteins were down-regulated in the ALC group (Table 1). Among these proteins are 14-3-3 protein zeta/delta (Protein kinase C inhibitor protein) ($p=1.E-03$), Bcl-2-associated transcription factor 1 ($p=2.E-04$), calmodulin (CaM, $p=2.E-04$), dihydropyrimidinase-related protein 3 ($p=2.E-04$), T-complex protein 1 subunit alpha B ($p=2.E-04$), elongation factor 2 ($p=2.E-04$), and neuronal migration protein doublecortin ($p=2.E-04$).

To test for variability in protein expression between individual fetal brains from the same dam, we analyzed the global protein intensity and correlated it between groups. Figures 6a,b show the correlation in protein intensities between two individual fetal brains from each dam in the PF group. Per example a significant correlation ($R^2=0.7222$) in protein intensity was found between individual fetal brains from the PF-1 dams (PF-1A versus PF-1B), Figure 6a.

Similarly a significant correlation ($R^2=0.7704$) in protein intensity was also found between individual fetal brains from the PF-2 dams (PF-2A and PF-2B), Figure 6b. In ALC groups, significant correlation ($R^2=0.8177$) in protein intensity was also observed between ALC-1A and ALC-1B individuals fetal brains from the same dam ALC-1 (Figure 6c). Similar observation was found between individual fetal brains, ALC-2A versus ALC-2B ($R^2=0.7704$, Figure 6d). These data suggest that there is no difference in protein intensity between fetal brains from the same dams irrespective of experimental group.

Discussion

We have previously shown that moderate prenatal alcohol exposure induced brain defects at different embryonic stages (for review see [15]). Here, we further characterize the proteomic architecture underlying ethanol teratogenesis at early fetal brain embryonic stage E13. We investigated differential expression of proteins in control groups and prenatally-treated alcohol groups that can be correlated to apoptotic phenomenon. Most of the information about the functionality and the distribution of each altered proteins was obtained from the ExPASy Proteomics Server (<http://www.expasy.ch/>). Additional information about the enzymes altered by prenatal alcohol exposure was obtained from Brenda, The Comprehensive Enzyme Information System (www.brenda.uni-koeln.de).

1. Effects of prenatal alcohol exposure on mitochondrial proteins

The mitochondrion is selectively targeted during ethanol-induced neurotoxicity in the fetal brain [19]. Prenatal alcohol exposure (from E6 to E15) impaired the proliferation and differentiation of E18 fetal cerebral mitochondria [21]. Moreover, prenatal alcohol exposure (from E17 to E18) caused an increase in mitochondrial permeability, which is accompanied by an elevation of cytochrome c and mitochondrial apoptotic factors in E19 fetal rat brains [19]. Acute administration of alcohol on postnatal day 4 leads to apoptotic purkinje cells at the early formations of cerebellar lobules [41]. These apoptotic neurons release cytochrome c from mitochondria, a critical event in the intrinsic pathway of apoptosis. We have recently shown that prenatal alcohol exposure increased cytosolic cytochrome c and decreased mitochondrial cytochrome c in E13 fetal brain compared to control group [35]. Thus, it is suggested that cytochrome c is activated by alcohol exposure. In relation to the present findings, we found reductions in the expression of two enzymes that are critical in the mitochondrial respiratory chain, ubiquinol-cytochrome-c reductase complex core protein I (UQCRI_MOUSE) and ubiquinol-cytochrome-c reductase complex core protein II (UQCRII_MOUSE). Reduced expression of another mitochondrial membrane respiratory chain protein, NADH-ubiquinone oxidoreductase (NDUS7_MOUSE), in ALC group may be linked to this mechanistic process. In addition, mitochondrial carrier homolog 1 (mitochondrial carrier-like protein 1) was down-regulated in ALC group compared to PF group. This is a potential mitochondrial transporter that plays a critical role in apoptosis.

We have also shown that prenatal alcohol exposure induced dramatic down-regulation of two key enzymes in ATP/ADP exchange: ADP/ATP translocase (ADT1_MOUSE) and ATP synthase subunit alpha (ATPA_MOUSE). Both enzymes are located in the mitochondrion inner membrane; the translocase catalyzes the exchange of ADP and ATP across the inner membrane, and the synthase converts ADP to ATP. Our findings validate previous works which utilize a different model of alcohol exposure [21, 42]. The latter studies demonstrated that intragastric ethanol exposure (5–6g/kg) on days 6–15 decreased the activities of respiratory chain complex I and IV and ATP synthase in the fetal cerebral mitochondria of mice. In addition, significant deficits in mitochondrial ATP synthase activity were found in postnatal day 1 brains exposed prenatally to alcohol [43]. The interaction between ADP/ATP translocase and ATP synthase is crucial for the maintenance of the mitochondrial membrane potential and, consequently, for cellular energetic metabolism. Deficits in the

concentration of these two enzymes may lead to cellular dysfunction and are likely to lead to cell death resultant from prenatal alcohol exposure.

In addition, we found a dramatic down-regulation of the mitochondrial inner membrane protein, prohibitin (PHB_MOUSE) in ALC group. This protein is highly expressed in neurons throughout gestation. Prohibitin functions as a membrane-bound chaperone that stabilizes mitochondrial proteins; it prevents misfolding in newly synthesized proteins [44]. Additionally, prohibitin interacts with respiratory chain complex I subunit, which indicates that this protein may be involved in the assembly or degradation of mitochondrial complex I [45]. Deficits in prohibitin may play a role in the alterations of mitochondrial development and function in *C. elegans* [46].

2. Effects of prenatal alcohol exposure on cytoskeletal proteins

Our quantitative proteomic analyses revealed differential expression of cytoskeletal proteins. The three most significantly down-regulated proteins in ALC group were β -actin (ACTB_MOUSE), microtubule-associated protein 2 (MAP2_MOUSE), and tubulin α -3 chain (TBA3_MOUSE) (Table 1). Actins are highly conserved cell motility proteins and β -tubulins are major constituents of microtubules. MAP2 is crucial cytoskeletal protein selectively located in the somatodendritic compartment of neurons, where it stabilizes against depolymerization [47]. MAP2 regulates both microtubule spacing and the cross linking of actin filaments. Down-regulation of MAP2 expression is in accordance with findings from an *in vitro* model of alcohol exposure that demonstrated a reduction in density of MAP2 immunostaining as a result of alcohol exposure [48]. The decrease in expression of cytoskeletal proteins might be indirectly due to a combination of apoptotic effects from prenatal alcohol exposure and other unknown mechanisms.

3. Effects of alcohol exposure in nuclear proteins

There are several nuclear proteins that have been found to be down- or up-regulated in alcohol-treated fetal brains. In the ALC group, down-regulated nuclear proteins include Elav-like protein1 (Hu-antigen R) (ELAV1_MOUSE), mediator of DNA damage checkpoint protein 1 (MDC1_MOUSE), histone-binding protein RBBP4 (RBBP4_MOUSE), and 40S ribosomal protein S3 (RS3_MOUSE). ELAV protein appears to be involved in the regulation of splicing, transport, stability, and translation of mRNA [49–51]. ELAV protein also plays a key role during fetal brain development; its involvement extends to posttranscriptional regulation during the early phase of neuronal development [52, 53]. In addition, ELAV protein is reported to be an early marker of neuronal commitment and to possess a specific temporal order of expression in the developing brain. This protein has been shown to induce the transition between stem/progenitor cells and differentiation/committed neurons. Reduced ELAV expression in an alcohol-treated fetal brain may alter this transition.

The mediator of DNA damage checkpoint protein 1 was also down-regulated in ALC group. DNA damage checkpoints are associated with biochemical pathways that end, delay, or arrest cell-cycle progression [for review [54]]. These checkpoint proteins interact with damage sensor proteins, such as the Rad9-Rad1-Hus1 complex and the Rad17–RFC complex, during DNA damage detection and signal transduction. The mediator of DNA damage may serve as a scaffold for the recruitment of DNA repair and signal transduction proteins to discrete foci of DNA damage marked by ‘Ser-139’ phosphorylation of histone H2AFX. Down-regulation of the mediator of DNA damage in the alcohol-treated group suggests the negative effects of alcohol exposure implicated in DNA damage.

In addition, the histone-binding protein RBBP4 was down-regulated in ALC group. This protein is a core histone-binding subunit that plays a critical role in conjunction with chromatin assembly proteins, chromatin remodeling factors and histone deacetylases. The core histone deacetylase complex promotes histone deacetylation and represses transcription. Alcohol exposure disrupts histone H1 and histone-binding proteins and leads to epigenetic imprinting, which is now considered to be a major problem in fetal alcohol exposure.

The heterogeneous nuclear ribonucleoprotein H (HNRH1_MOUSE) was up-regulated in ALC groups. This protein is a component of the heterogeneous nuclear ribonucleoprotein complexes that are involved in pre-mRNA alternative splicing. It is unclear about the causality of alcohol exposure in up-regulation of this protein. This might be explained by the fact that cells and neurons evade apoptosis as a regulatory mechanism against the insult of alcohol exposure. Further studies are warranted to substantiate this assumption.

4. Effects of alcohol exposure on cytosolic proteins

Quantitative proteomic analyses identified several cytosolic proteins that were down-regulated in the ALC group. Among these proteins were, 14-3-3 protein zeta/delta (14-3-3Z_MOUSE), Bcl-2-associated transcription factor 1 (BCLF1_MOUSE), calmodulin (CALM_MOUSE), elongation factor 2 (EF2), neuronal migration protein doublecortin (DCX_MOUSE), and dihydropyrimidinase-related protein 3 (DPYL3_MOUSE). Bcl-2 is located at the mitochondrial outer membrane and regulates survival/apoptosis during brain development [30]. Bcl-2 has been implicated in alcohol-induced apoptosis during the period of high vulnerability [25, 27–29]. Alcohol exposure at postnatal day 4 induced altered transcription and expression of pro- and anti-apoptotic Bcl-2 family members that lead to cell death [28, 29]. Thus, our study reports that down-regulation of Bcl-2 expression may reflect the effect of alcohol-induced apoptosis in E13 fetal brains.

The 14-3-3 proteins are homodimeric cytosolic proteins that regulate subcellular localization and activity of over 70 distinct proteins in their phosphorylated forms [55]. The 14-3-3 proteins are a family of serine/threonine binding proteins that have anti-apoptotic functions and exert their anti-apoptotic activity through the Bad protein [56]. The anti-apoptotic function of 14-3-3 proteins was abolished by activation of c-jun N-terminal kinase (JNK) which is one of the proteins of the signaling pathways involving in alcohol-induced cell death. JNK activation promotes translocation of Bax to mitochondria through phosphorylation of 14-3-3 proteins [57]. A recent study revealed that JNK regulates the interaction between 14-3-3 and Bad [36]. These findings demonstrated that 14-3-3 interaction with Bad was reduced in the cerebral cortices of alcohol-treated rats. The diminution of this interaction promotes Bad protein/Bcl-xl binding which leads to Bax release and the induction of cell death. Our present findings provide ample information about the mechanisms of cell death that are associated with down-regulation of 14-3-3 protein as a consequence of alcohol exposure.

Dihydropyrimidinase-related protein 3 (which is also known as collapsing response mediator protein) is implicated in the developmental processes of the brain. This protein plays key roles in axon guidance, neuronal growth, and cell migration during fetal brain development. We have previously demonstrated that FAE altered brain growth and retarded the migration of 5-HT neurons [58]. Down-regulation of collapsing response mediator proteins in ALC group provides great insight to the deficit shown in our previous findings (for review see [58]). In addition, down-regulation of doublecortin expression, a neuronal migration protein, may explain the effects of alcohol-induced retardation of neuronal migration during embryonic stages. This protein is required for initial step of neuronal migration during embryonic stages.

Conclusions

This study utilized quantitative proteomic analyses to investigate differential protein expression in fetal brains of ALC group compared to PF control group. Most of the proteins analyzed were nuclear, cytosolic, mitochondrial, and cytoskeletal proteins. The down-regulation of several important mitochondrial enzymes: ADP/ATP translocase, ATP synthase, NADH dehydrogenase, and ubiquinol-cytochrome-c reductase, explains the mitochondrial dysfunction induced by alcohol exposure. Interestingly, concurrent down-regulation of prohibitin indicates that prenatal alcohol exposure affects mitochondrial DNA synthesis in the fetal brain. Down-regulation of some of the cytoskeletal proteins such as MAP 2 and β -actin, provides information about the effects of prenatal alcohol exposure on cell migration and neurite extension. Prenatal alcohol exposure induced down-regulation of many nuclear proteins, such as ELAV protein, which plays a key role in fetal brain development. It is suggested that this protein might be involved in posttranscriptional regulation in the early phases of neuronal development, which is crucial for triggering neuronal diversity. Moreover, there were several cytosolic proteins affected by alcohol exposure. Most of these proteins are involved in the growth and development of the fetal brain. Understanding the regulation of proteins in FAE or FAS models will ultimately provide new mechanistic explanations for alcohol-related brain abnormalities. These findings may provide important information for the identification of mechanisms of neuroprotection, and allow for the development of intervention strategies that could protect or attenuate the deleterious effects of alcohol exposure during pregnancy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Prenatal alcohol exposure increases cell death in primordium frontal cortex (FCx) and basal ganglia eminence (BGE) (b) as compared to PF group (a) at E13 as indicated by TUNEL staining. High power photomicrographs show sections of FCx that are pointed by arrows in PF (c) and ALC (d) groups. Arrowheads indicate cell undergoing apoptosis. Scale bars: 100 μm.

Figure 2.

Classification of identified proteins expressed as total percentage (a). Majority of identified proteins are enzymes (26%), nucleotide binding proteins (18%) and other isoforms. Further classification of the enzyme subgroup includes 17% kinase, 14% dehydrogenase, 13% isomerase, and 12% cytochrome c oxidase (b).

Figure 3.

PCA score plot of the areas of the identified proteins for the two groups analyzed: control group (Paired, PF) and alcohol-treated group (ALC). Insets are enlarged regions of the plot.

Figure 4.

Dot-plots of the areas of some mitochondrial proteins that have demonstrated statistical differences between the two groups, ADP/ATP translocase (a), ATP synthase α -subunit (b), prohibitin (c), and ubiquinol-cytochrome-c reductase complex core protein I (d).

Figure 5. Dot-plots of the areas of some nuclear proteins that have demonstrated statistical differences between the two groups, heterogeneous nuclear ribonucleoprotein H (a), mediator of DNA damage checkpoint protein 1 (b), and probable ATP-dependent RNA helicase (c).

Figure 6.

Scatter plots of protein intensities between two individual fetal brains from the same litter in the PF group (a, b) and ALC group (c, d). Significant correlation of protein intensities was found between two individual fetal brains in both PF and ALC group.

Table 1

Proteins among others that have been significantly down-regulated or up-regulated in their expression as a consequence of prenatal alcohol exposure in E13 fetal brains. Values are expressed as protein areas and their standard error of the mean, while the Wilcoxon rank sum test and the exact method was utilized to calculate the P values. False discovery rate was employed to correct for multiple comparisons, and statistical significance threshold was determined by controlling FDR at 1%.

Protein	Function	PF group ($\times 10^8$)	ALC group ($\times 10^8$)	p-value
MITOCHONDRIAL PROTEINS OR FACTORS				
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9 (NDUA9_MOUSE)	Accessory subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I), that is believed to be not involved in catalysis. Complex I functions in the transfer of electrons from NADH to the respiratory chain. The immediate electron acceptor for the enzyme is believed to be ubiquinone.	0.07 $\pm$ 0.01	ND	2.E-04
NADH-ubiquinone oxidoreductase 20 kDa subunit (NUKM_MOUSE)	Core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I) that is believed to belong to the minimal assembly required for catalysis. Complex I functions in the transfer of electrons from NADH to the respiratory chain. The immediate electron acceptor for the enzyme is believed to be ubiquinone	0.1 $\pm$ 0.03	ND	2.E-04
Cytochrome b-c1 complex subunit 1, Ubiquinol- cytochrome-c reductase complex core protein I (UQCR1_MOUSE)	This is a component of the ubiquinol- cytochrome c reductase complex (complex III or cytochrome b-c1 complex), which is part of the mitochondrial respiratory chain. This protein may mediate formation of the complex between cytochromes c	1.13 $\pm$ 0.2	0.33 $\pm$ 0.04	3.E-04
Cytochrome b-c1 complex subunit 2, Ubiquinol- cytochrome-c reductase complex core protein II (UQCR2_MOUSE)	This is a component of the ubiquinol- cytochrome c reductase complex (complex III or cytochrome b-c1 complex), which is part of the mitochondrial respiratory chain. The core protein 2 is required for the assembly of the complex.	0.28 $\pm$ 0.07	0.001 $\pm$ 0.0004	2.E-04
Mitochondrial carrier homolog 1, Mitochondrial carrier-like protein 1 (MTCH1_MOUSE)	Potential mitochondrial transporter. May play a role in apoptosis.	0.06 $\pm$ 0.04	ND	2.E-04
ATP synthase subunit alpha (ATPA_MOUSE)	Mitochondrial membrane ATP synthase produces ATP from ADP in the presence of a proton gradient across the membrane which is generated by electron transport complexes of the respiratory chain	1.2 $\pm$ 0.22	ND	2.E-04
Prohibitin (PHB_MOUSE)	Prohibitin inhibits DNA synthesis. It has a role in regulating proliferation	1.2 $\pm$ 0.2	0.2 $\pm$ 0.04	2.E-04
ADP/ATP translocase 1 (ADT1_MOUSE)	Catalyzes the exchange of ADP and ATP across the mitochondrial inner membrane	0.49 $\pm$ 0.09	0.26 $\pm$ 0.05	2.E-04
ADP/ATP translocase 2 (ADT2_MOUSE)	Catalyzes the exchange of ADP and ATP across the mitochondrial inner membrane.	0.7 $\pm$ 0.13	0.24 $\pm$ 0.04	5.E-03
Malate dehydrogenase, mitochondrial [Precursor] (MDHM_MOUSE)	(S)-malate + NAD ⁺ = oxaloacetate + NADH.	0.1 $\pm$ 0.05	ND	2.E-04
CYTOSKELETAL COMPONENTS				
Actin, cytoplasmic 1, Beta-actin (ACTB_MOUSE)	Function: Actins are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells.	14.7 $\pm$ 2.04	10.2 $\pm$ 1.47	2.E-04

Protein	Function	PF group ($\times 10^8$)	ALC group ($\times 10^8$)	p-value
Microtubule-associated protein 2 (MAP2_MOUSE)	The exact function of MAP2 is unknown but MAPs may stabilize the microtubules against depolymerization. They also seem to have a stiffening effect on microtubules.	0.03 $\pm$ 0.01	ND	2.E-04
tubulin alpha-3 chain (TBA3_MOUSE)	Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non- exchangeable site on the alpha-chain.	0.18 $\pm$ 0.05	ND	2.E-04
NUCLEAR PROTEINS OR COMPONENTS				
Elav-like generic protein 1 (Hu-antigen R) (ELAV1_MOUSE)	Binds avidly to the AU-rich element in FOS and IL3/interleukin-3 mRNAs. In the case of the FOS AU-rich element, HUR binds to a core element of 27 nucleotides that contain AUUUA, AUUUUA, and AUUUUUA motifs.	0.26 $\pm$ 0.08	0.006 $\pm$ 0.001	2.E-04
Heterogeneous nuclear ribonucleoprotein H (HNRH2_MOUSE)	This protein is a component of the heterogeneous nuclear ribonucleoprotein (hnRNP) complexes which provide the substrate for the processing events that pre-mRNAs undergo before becoming functional, translatable mRNAs in the cytoplasm. Binds poly(RG).	0.2 $\pm$ 0.02	0.62 $\pm$ 0.08	6.E-04
Probable ATP-dependent RNA helicase DDX48 (DDX48_MOUSE)	Binds to spliced mRNAs and is involved in nonsense-mediated decay of mRNAs containing premature stop codons.	0.03 $\pm$ 0.01	0.16 $\pm$ 0.02	1.E-03
Nuclear pore complex protein Nup107 (NU107_MOUSE)	Essential component of nuclear pore complex. Required for the assembly of peripheral proteins into the nuclear pore complex	0.03 $\pm$ 0.009	ND	2.E-04
Histone-binding protein RBBP4, Chromatin assembly factor I p48 subunit (RBBP4_MOUSE)	Core histone-binding subunit that may target chromatin assembly factors, chromatin remodeling factors and histone deacetylases to their histone substrates in a manner that is regulated by nucleosomal DNA. Component of several complexes which regulate chromatin metabolism. These include the chromatin assembly factor I (CAF-1) complex, which is required for chromatin assembly following DNA replication and DNA repair; the core histone deacetylase complex, which promotes histone deacetylation and consequent transcriptional repression.	0.18 $\pm$ 0.05	ND	2.E-04
SON protein (SON_MOUSE)	Transcriptional repressor. Binds to the consensus DNA sequence: 5'-GA[GT]AN[CG][AG]CC-3'. Might protect cells from apoptosis. Might be involved in pre-mRNA splicing	0.30 $\pm$ 0.09	0.07 $\pm$ 0.02	2.E-04
40S ribosomal protein S3 (RS3_MOUSE)	Belongs to the ribosomal protein S3P family	0.27 $\pm$ 0.05	0.04 $\pm$ 0.009	2.E-04
Mediator of DNA damage checkpoint protein 1 (MDC1_MOUSE)	Required for checkpoint mediated cell cycle arrest in response to DNA damage within both the S phase and G2/M phases of the cell cycle. May serve as a scaffold for the recruitment of DNA repair and signal transduction proteins to discrete foci of DNA damage marked by 'Ser-139' phosphorylation of histone H2AFX. Also required for downstream events subsequent to the recruitment of these proteins.	0.41 $\pm$ 0.1	0.08 $\pm$ 0.01	2.E-04
CYTOSOLIC PROTEINS				

Protein	Function	PF group ($\times 10^8$)	ALC group ($\times 10^8$)	p-value
14-3-3 protein zeta/delta (Protein kinase C inhibitor protein) (1433Z_MOUSE)	Adapter protein implicated in the regulation of a large spectrum of both general and specialized signaling pathway. Binds to a large number of partners, usually by recognition of a phosphoserine or phosphothreonine motif. Binding generally results in the modulation of the activity of the binding partner.	0.38 $\pm$ 0.08	0.02 $\pm$ 0.01	1.E-03
Bcl-2-associated transcription factor 1 (BCLF1_MOUSE)	Death-promoting transcriptional repressor	0.05 $\pm$ 0.03	ND	2.E-04
Calmodulin (CaM) (CALM_MOUSE)	Calmodulin mediates the control of a large number of enzymes and other proteins by Ca(2+). Among the enzymes to be stimulated by the calmodulin- Ca(2+) complex are a number of protein kinases and phosphatases. Together with CEP110 and centrin, is involved in a genetic pathway that regulates the centrosome cycle and progression through cytokinesis.	0.33 $\pm$ 0.05	ND	2.E-04
Elongation factor 2 (EF2_MOUSE)	This protein promotes the GTP-dependent translocation of the nascent protein chain from the A-site to the P-site of the ribosome.	1.68 $\pm$ 0.24	1.06 $\pm$ 0.1	2E-04
Neuronal migration protein doublecortin (DCX_MOUSE)	Seems to be required for initial steps of neuronal dispersion and cortex lamination during cerebral cortex development. May act by competing with the putative neuronal protein kinase DCAMKL1 in binding to a target protein. May in that way participate in a signaling pathway that is crucial for neuronal interaction before and during migration, possibly as part of a calcium ion-dependent signal transduction pathway. May be part with LIS-1 of a overlapping, but distinct, signaling pathways that promote neuronal migration. Expressed already at embryonic day 11.	0.09 $\pm$ 0.03	ND	2.E-04
T-complex protein 1 subunit alpha B (TCPA2_MOUSE)	Molecular chaperone; assists the folding of proteins upon ATP hydrolysis. Known to play a role, in vitro, in the folding of actin and tubulin.	0.11 $\pm$ 0.02	ND	2.E-04
Dihydropyrimidinase- related protein 3 (DPYL3_MOUSE)	Necessary for signaling by class 3 semaphorins and subsequent remodeling of the cytoskeleton. Plays a role in axon guidance, neuronal growth cone collapse and cell migration.	0.29 $\pm$ 0.14	0.03 $\pm$ 0.007	2.E-04