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Placental Proteomics Reveal Insights into Fetal Alcohol Spectrum Disorders

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Abstract

Background—Fetal alcohol spectrum disorders (FASD) describe many of the well-known neurodevelopmental deficits afflicting children exposed to alcohol *in utero*. The effects of alcohol on the maternal-fetal interface, especially the placenta, have been less explored. We herein hypothesized that chronic binge alcohol exposure during pregnancy significantly alters the placental protein profile in a rat FASD model.

Methods—Pregnant rats were orogastrically treated daily with alcohol (4.5 g/kg, gestational day (GD) 5–10; 6.0 g/kg, GD 11–19) or 50% maltose dextrin (isocalorically matched pair-fed controls). On GD 20, placentae were collected, flash frozen, and stored until tissues were homogenized. Protein lysates were denatured, reduced, captured on a 10 kDa spin filter and digested. Peptides were eluted, reconstituted, and analyzed by a Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer.

Results—Mass spectrometry analysis identified 2,285 placental proteins based on normalized spectral counts and 2000 proteins by intensity based absolute quantification. 45 placental proteins were significantly ($P<0.05$) altered by gestational alcohol exposure by both quantification approaches. These included proteins directly related to alcohol metabolism; specific isoforms of alcohol dehydrogenase and aldehyde dehydrogenase were upregulated in the alcohol group. Ingenuity analysis identified ethanol degradation as the most significantly altered canonical pathway in placenta, and fetal/organ development as most altered function, with increased risk for metabolic, neurological, and cardiovascular diseases. Physiologic roles of the significantly altered

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proteins were related to early pregnancy adaptations, implantation, gestational diseases, fetal organ development, neurodevelopment, and immune functions.

Conclusions—We conclude that the placenta is a valuable organ not only to understand FASD etiology but it may also serve as a diagnostic tool to identify novel biomarkers for detecting the outcome of fetal alcohol exposure. Placental mass spectrometry analysis can offer sophisticated insights into identifying alcohol metabolism-related enzymes and regulators of fetal development.

Keywords

Alcohol; FASD; Teratology

Introduction

Gestational alcohol exposure may result in irreversible fetal damage manifested as a range of physical, physiological, behavioral, and intellectual deficits which are classified under the umbrella term Fetal Alcohol Spectrum Disorders (FASD; Sokol et al., 2003). Prenatal alcohol exposure affects nearly all fetal organ systems including cardiac, vascular, endocrine, neurobehavioral, and uteroplacental systems (Burd et al., 2007a, Ren et al., 2002, Ramadoss and Magness, 2012, Parnell et al., 2007, Warren et al., 2011, Riley and McGee, 2005, Lewis et al., 2015, Gautam et al., 2015, Sawant et al., 2014, Gareri et al., 2009, Gundogan et al., 2008). Fetal Alcohol Syndrome (FAS), the most severe form of FASD, is identifiable in infancy by craniofacial abnormalities and neurobehavioral deficits (Murawski et al., 2015, Jones and Smith, 1973). In contrast, less severe forms of FASD are typically not diagnosed until a child exhibits learning and behavioral problems in elementary school, thereby delaying therapeutic intervention (May et al., 2014). In the United States, 1 in 10 pregnant women consume alcohol and 1 in 33 pregnant women report binge drinking in the past 30 days (Tan et al., 2015), and it is estimated that up to 1 in 20 school children have FASD (May et al., 2009). FASD diagnosis relies on self-reported alcohol consumption during pregnancy, which is often an unreliable and inaccurate method due to social stigma and the fear of litigation (Murawski et al., 2015, Del Boca and Darkes, 2003). Therefore, specific, sensitive and objective methods for detection of *in utero* alcohol exposure are urgently needed.

Alcohol consumption can be quantified via several diagnostic methods; however, currently no clinical test exists for gestational alcohol exposure or FASD. Studies conducted in men, nonpregnant/pregnant women, and animal models have described numerous markers for alcohol consumption which range in sensitivity, specificity, and window of detection. Candidate biomarkers for alcohol consumption include alcohol metabolites such as acetaldehyde adducts like hemoglobin-associated acetaldehyde (HAA), phosphatidylethanol (PEth), ethyl glucuronide (EtG), ethyl sulfate (EtS), and fatty acid ethyl esters (FAEEs), as well as markers of alcohol-mediated pathology like gamma-glutamyl transferase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and mean corpuscular volume (MCV; Joya et al., 2012, Cook, 2003, Peterson, 2004). Specifically in the FASD field, biomarkers are identified utilizing biological samples from the mother like serum/whole blood or directly from the offspring including neonatal hair, meconium, and dried umbilical cord blood spots (Sanvisens et al., 2016, Bakhireva et al., 2013). The placenta is a

fetal tissue which is present from implantation to parturition and is highly under-investigated despite its potential as a non-invasive diagnostic medium available at delivery.

The placenta allows for exchange of oxygen, nutrients, waste products, hormones, and antibodies between mother and the developing fetus throughout pregnancy (Sood et al., 2006). The placenta forms at the time of implantation (Bowman and Kennedy, 2014), is delivered after birth, and serves as a medium to obtain valuable information about the health of a pregnancy (Sood et al., 2006, Shukla et al., 2011). Pathological changes in the placenta may be induced by alcohol and are heavily associated with fetal morbidity (Altshuler, 1984), preeclampsia (Walsh, 1985, Pineles et al., 2007), and increased risk for chronic adult diseases such as type 2 diabetes and cardiovascular disease (Jansson and Powell, 2007, Belkacemi, 2011). A growing body of work suggests gestational alcohol-induced maternal-fetal interface adaptations play a critical role in FASD pathogenesis, yet this area of focus remains largely unexplored. Currently, only a limited understanding of the negative effects of gestational alcohol consumption on the placenta exists. In animal models, alcohol alters placental gene expression, indicating the potential of this organ as an effective tool in detecting prenatal alcohol exposure (Shukla et al., 2011, Rosenberg et al., 2010, Balaraman et al., 2014). Thus, we hypothesize that gestational alcohol exposure will significantly alter the placental protein profile in a FASD rat model. Further, we believe that the alcohol-induced placental protein profile will provide pivotal insights into the underlying FASD mechanisms, offer insights into identification of non-invasive FASD biomarkers, and identify targets for developing novel therapeutic intervention.

Materials and Methods

Animals

All experimental procedures were in accordance with National Institutes of Health guidelines (NIH Publication No. 85–23, revised 1996) with approval by the Animal Care and Use Committee at Texas A&M University. Pregnant Sprague Dawley rats were housed in a temperature-controlled room (23°C) with a 12:12-hour light–dark cycle. Rats were assigned to a pair-fed control group (n= 6) or an alcohol treatment group (n = 6). The alcohol group received a dosing regimen of 22.5% (wt/v) ethanol (4.5 g/kg) between GD 5–10. The pair-fed controls were isocalorically matched to alcohol rats by dosing with maltose dextrin to account for calories from alcohol, and the amount of diet consumed by the pair-fed animals was matched with the alcohol-fed animals. Animals received a once daily orogastric gavage in a binge paradigm. Animals were sacrificed on GD 20, one day after the last alcohol exposure. There was no significant difference in the maternal weight between the pair-fed control and alcohol-fed dams on GD 20 (Pair-fed control, 309 ± 8 g; Alcohol, 308 ± 15 g). Fetal weight was significantly decreased in the alcohol group (2.12 ± 0.11 g), compared with that in the pair-fed control (2.53 ± 0.06 g). Placental tissue was isolated, serially washed in phosphate buffered saline (PBS) to remove any residual blood, centrifuged, flash frozen, and stored at −80°C.

Sample Preparation

As previously described (Berger et al., 2015), samples were thawed on ice, washed five times in ice cold PBS, trimmed and shredded using a scalpel, and immediately transferred into ice cold PBS. After vortexing samples for 10 sec, tissues were collected by centrifugation at 4,000 rpm for 10 sec. PBS was removed and this procedure was repeated five times. Blood-free tissue was then collected with 500 µl radioimmunoprecipitation assay (RIPA) buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 158 mM NaCl, 10 mM Tris-HCl, pH 7.6, 1 mM PMSF, and protease inhibitor cocktail) and directly transferred to a glass homogenizer for homogenization. For improved cell lysis, the homogenized samples were additionally incubated on ice for 30 min.

Protein Concentration Determination

Protein concentration of the lysates were determined by using the Bradford Assay (Bradford, 1976; Bio-Rad DC™ Protein Assay) following the manufacturer's protocol. The standard curve was established using a stock solution of 20 mg/ml bovine serum albumin (BSA) and final concentrations of 0.25 mg/ml, 0.5 mg/ml, 1 mg/ml, 1.5 mg/ml and 2.0 mg/ml. After incubation at room temperature, analysis was performed via microplate spectrophotometer (Bio-Rad Model 680) at a wavelength of 595 nm, as previously described (Berger et al., 2015).

Filter assisted sample preparation (FASP)

The filter assisted sample preparation method was carried out as previously described (Wisniewski et al., 2009, Berger et al., 2015). In short: Proteins were first denatured and reduced by adding 100 µl sample to 100 µg urea (8M) supplemented with 20 µl dithiothreitol (DTT, 100 mM in 1 M Tris/HCl pH 8.5). A nominal protein content of 100 µg was used for analysis. After alkylation of reduced cysteine side chains with 50 mM iodoacetic acid (IAA; final concentration), denatured proteins were captured on a 10 kDa molecular weight cut-off (MWCO) spin filter (MRCPRT010, Millipore) and washed twice with 50 mM ammonium bicarbonate (ABC). Protein digestion was performed with sequencing grade trypsin (V5111, Promega) at a nominal enzyme to substrate ratio of 1:50. After incubation overnight with 100 µl digestion buffer (trypsin in 50 mM ABC), resulting peptides were eluted with 300 µl 0.5 M sodium chloride (NaCl). Peptide elutes were desalted with reversed, phase-based, TARGA C-18 spin tips (SEMSS18R, Nest Group) prior to LC-MS/MS analysis. Lyophilized samples were stored at −20°C.

LC-MS/MS Analysis

A combination of liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), as previously described (Berger et al., 2015), was implemented for the analysis of placental tissue. In brief, following the ultra-performance liquid chromatography (UPLC), the first stage of MS (MS1) produced precursor ion fragmentation, and in the second stage MS (MS2) separated and detected the produced fragmented ions. Peptides were reconstituted in loading buffer (5% acetonitrile [ACN; v/v], 5% formic acid [FA; v/v]). The prepared samples were analyzed using a nanoflow UPLC system (EK425, Sciex) with an in-house packed column (75 µm × 15 cm; AQUA C18/3 µm from Michrom Bioresource)

coupled online to a Q Exactive ™ Hybrid Quadrupole-Orbitrap™ mass spectrometer (MS; Thermo Fisher Scientific), as previously described (Berger et al., 2015). Tryptic digests (~1.5 μg) were separated by a linear gradient from 93% buffer A (0.2% FA in HPLC water)/7% buffer B (0.2% FA in ACN) to 70% buffer A/30% buffer B within 120 min at a flowrate of 400 nl/min. The MS was operated in data-dependent TOP10 mode with the following settings: resolution for MS1 scan: 70,000 at 200 Th; lock mass: 445.120025 Th; resolution for MS2 scan: 17,500 at 200 Th; isolation width: 1.6 m/z; NCE 27; underfilll ratio: 1%; charge state exclusion: unassigned, 1, > 6; dynamic exclusion: 20 sec.

Data Analysis

Acquired MS raw files (RAW) were analyzed using MaxQuant (Cox and Mann, 2008, Berger et al., 2015; version 1.5.2.8). Briefly, the acquired RAW files were loaded into MaxQuant and searched against the rat UniProtKB database. For quantification, ‘intensity based absolute quantification’ (iBAQ) and ‘label-free quantification’ (LFQ) were selected. Fixed modifications were set to Carbamidomethyl (C) and variable modifications to Acetyl (N-Term), Oxidation (M), and Phospho (STY), as digesting enzyme ‘trypsin’ was specified. Otherwise, default settings (by MaxQuant) were used for the analysis: trypsin with up to two missed cleavages; mass tolerance for the first search: 0.07 Da; main search: 0.006 Da. Additionally, RAW files were converted into Mascot Generic Files (MGF) using an in-house script (ms2preproc, version 2009-01). The generated MGF files were searched against the rat database (containing all sequences in a reversed order as decoy) using Mascot (Matrix Science; version 2.3). The following parameters were used for the search: ‘enzyme’ was set to ‘trypsin’, two missed cleavages were allowed, peptide tolerance was set to 10 ppm and the MS/MS tolerance to 20 mmu, only 2^+, 3^+ and 4^+ charged ions were allowed, search was performed in Target-Decoy mode. Mascot search result files (DAT files) were loaded to Scaffold (Proteome Software Inc., version 4.3.2) for further analysis. General contaminants were not included in the subsequent data analysis. Protein abundances were determined using a normalized spectral count method as well as protein iBAQ. Identified proteins were evaluated by a blinded observer using Ingenuity Pathway Analysis to interpret the proteomic data.

Statistics

Difference in the mean protein abundance between pair-fed control and alcohol treatment groups was analyzed using a Student’s t-test. Data are presented as Mean $\pm$ SEM. Significance was established *a priori* at $P < 0.05$.

Results

Two approaches were used to quantify the placental proteins identified by mass spectrometry: a total of 2,285 proteins with greater than 95% probability and a minimum of 2 peptides per protein were identified and quantified by normalized spectral counts method with Mascot and Scaffold, and 2000 proteins by iBAQ analysis with MaxQuant. A list of identified proteins is provided in the Supplemental Information. 321 of the proteins detected by the normalized spectral method and 262 of the proteins identified by the iBAQ method were significantly ($P < 0.05$) altered by gestational alcohol exposure. A stringent approach

was utilized to validate the proteins significantly altered in response to alcohol: proteins identified by both iBAQ and normalized spectral counts methods, met validation criteria: 45 placental proteins were identified by both quantification processes as significantly different in pair-fed controls and alcohols (Figure 1).

Interestingly, proteins directly related to alcohol metabolism (Crabb et al., 2004) were upregulated in the alcohol group, including both alcohol dehydrogenase NADP(+) (ADH) and aldehyde dehydrogenase, mitochondrial (ALDH). The representative MS/MS spectra illustrated in Figure 2 depict a nearly complete y and b ion series assignment for ADH (parent mass error: 0.72 ppm; Figure 2A) and ALDH (parent mass error: −0.46 ppm; Figure 2B). The b ion series identified peptide fragments which extend from the amino (N)-terminus, whereas, the y ion series identified the peptide fragments extending from the carboxyl (C)-terminus. Peak height identified the MS signal intensity, and the distance between the peaks was used to construct the sequences of the identified peptide. The mean alcohol to pair-fed protein abundance of ADH was determined to be 3.53 by iBAQ, and 3.53 by normalized spectral count, and the mean alcohol to pair-fed protein abundance for ALDH was 1.49 by iBAQ, and 1.12 by normalized spectral count (Figure 2C).

Ingenuity Pathway Analysis identified canonical pathways, diseases, and physiological systems development and functions significantly altered in the placenta following gestational alcohol exposure (Table 1). Ethanol degradation, noradrenaline and adrenaline degradation, and synaptic long term potentiation were the top canonical pathways altered following gestational alcohol exposure. Analysis also revealed increased risk for metabolic, neurological, and cardiovascular diseases following gestational alcohol exposure. Importantly, the most altered physiologic systems were directly related to fetal organ and embryo development, hair and skin development, and auditory, vestibular, renal, and urological systems function.

We classified the significantly altered proteins based on their physiological functions (Figure 3). A number of proteins related to early pregnancy adaptations and implantation were downregulated, such as 15 kDa selenoprotein and calumenin. Proteins involved in preeclampsia, gestational diseases, and fetal organ development, including AMP deaminase 3 and glycine amidinotransferase, were also downregulated. Proteins involved in neurodevelopment were also altered by alcohol: high mobility group protein B was downregulated, and serine/threonine-protein phosphatase PP1-beta was upregulated. Importantly, proteins essential for alcohol metabolism and nutrition, such as alcohol dehydrogenase and aldehyde dehydrogenase, were upregulated in the alcohol group. Proteins involved in immune functions, such as UDP-glucose:glycoprotein glycosyltransferase and collectin-12, were downregulated in the placenta following gestational alcohol exposure.

Discussion

To the best of our knowledge, this is the first study to investigate the placental protein profile in the FASD field. We herein demonstrate that chronic binge alcohol exposure has specific and substantial effects on the placenta; of the more than 2000 placental proteins identified,

45 proteins were significantly altered in the alcohol treatment group compared to the pair-fed control group when analyzed by both normalized spectral count and the iBAQ approaches. Ethanol degradation was the most significantly altered canonical pathway in the placenta. Placental proteins altered by alcohol indicated increased risk for metabolic, neurological, and cardiovascular diseases. Our data also demonstrate that the placental proteins may act as a window into fetal/organ development in the FASD rat model.

Alcohol freely disperses from maternal circulation across the placenta into the fetal compartment, producing fetal BACs and amniotic fluid concentrations equivalent to maternal BACs, yet alcohol remains for a longer duration within the amniotic fluid (Nava-Ocampo et al., 2004). The placenta has multiple functions during pregnancy (Burd et al., 2007b) and previous studies have shown that alcohol (1) induces placental vasoconstriction, which persists for the duration of exposure (Taylor et al., 1994) and adversely impacts nutrient and oxygen delivery to the fetus (Burd et al., 2007b); (2) alters placental metabolism by decreasing glucose utilization (Rice et al., 1986, Burd et al., 2007b); (3) disrupts endocrine function by inhibiting growth factor production (Karl and Fisher, 1994); and (4) activates an immune response by upregulating cytokine expression (Svinarich et al., 1998). Together these alcohol-induced changes impact multiple maternal placenta-associated syndromes; women who drank during pregnancy were more likely to develop placental abruption, have an infant that is small for gestational age, and have a preterm infant when compared with mothers who abstain (Salihu et al., 2011). The mechanism(s) underlying alcohol-induced placental pathogenesis requires further study.

We analyzed the physiological functions of placental proteins altered by gestational alcohol exposure and found they had critical roles in alcohol metabolism, pregnancy, placental function, and fetal development. Ethanol degradation was the most significantly altered canonical pathway in the placenta. ADH and ALDH have integral roles in alcohol metabolism (Tawa et al., 2016), and were determined to be upregulated by mass spectrometry analysis. Proteins involved in alcohol-related disease were also upregulated such as sideroflexin-1 (Rosenberg et al., 2010), which is linked to alcoholic hepatotoxicity and iron transport (Kim et al., 2015). The fetal liver is very limited in its ability to metabolize ethanol and additional studies are necessary to further decipher the expression, distribution, and activity of ALDH and ADH in the placenta (Sanchis and Guerri, 1986, Cederbaum, 2012, Pikkarainen and Raiha, 1967, Heller and Burd, 2014). This study demonstrates that ALDH and ADH are expressed in the placenta following chronic alcohol exposure and these enzymes may have the ability to metabolize alcohol. Future studies are warranted to assess the placental metabolic clearance of alcohol. Mass spectrometry analysis determined GATM to be significantly upregulated following gestational alcohol exposure. GATM is involved in the biosynthesis of guanidinoacetate, a precursor of creatine (Item et al., 2001). Diseases associated with GATM mutations have resulted in deficiencies in creatine synthesis which may result in mental and behavioral disorders (Fons and Campistol, 2016). Consequently, GATM may have a critical role in neurodevelopment and its disruption may affect the neuropathogenesis of FASD.

Our data also demonstrate that the placental proteins may act as a window into fetal/organ development in the FASD rat model and they indicate risk for alcohol-induced metabolic,

neurological, and cardiovascular diseases. Gestational alcohol exposure altered proteins critical for early pregnancy adaptations and implantation: murinoglobulin-1, secreted from uterine stromal cells and involved in trophoblast invasion (Esadeg et al., 2003) was downregulated, while 15 kDa selenoprotein (Mistry et al., 2012) and calumenin (Mahnke-Zizelman et al., 1997) were upregulated. Proteins related to preeclampsia, gestational diseases, and fetal organ development were also upregulated, including AMP deaminase 3, a regulator of skeletal muscle and liver development (Mahnke-Zizelman et al., 1997), and glycine amidinotransferase, critical for embryonic and central nervous system development (McMinn et al., 2006). Furthermore, proteins involved in neurodevelopment were dysregulated following gestational alcohol exposure: high mobility group protein B (Abraham et al., 2013) was upregulated, while serine/threonine-protein phosphatase PP1-beta (Chiappetta et al., 1996) was downregulated. FASD-induced infection susceptibility may be imprinted during early development, as we identified proteins involved in the immune response were upregulated, such as UDP-glucose:glycoprotein glycosyltransferase (Zhang et al., 2011), collectin-12 (Ohtani et al., 2012), and serine/threonine-protein kinase 10 (Yamamoto et al., 2011). In summary, our data demonstrate that alcohol modifies protein profiles comprising early pregnancy and implantation, preeclampsia, gestational diseases, fetal organ development, and immune functions.

In conclusion, the placenta is unique in that it subsists exclusively from implantation until birth, and is subjected to nearly identical environmental exposures as the developing fetus. Additional studies are warranted for further validation and for assessment of placental metabolic clearance of alcohol. We anticipate that placental alterations following gestational alcohol exposure may accurately reflect pathological changes occurring within the fetus. Thus, the placenta may serve as a medium for early detection and subsequent intervention for deficits where detection may be invasive, inaccessible, or not apparent until later in life, as is the case with many alcohol-induced neurobehavioral abnormalities. The findings of this study build on earlier reports in the field and help delineate how placental protein dysregulation may provide insights into FASD phenotypes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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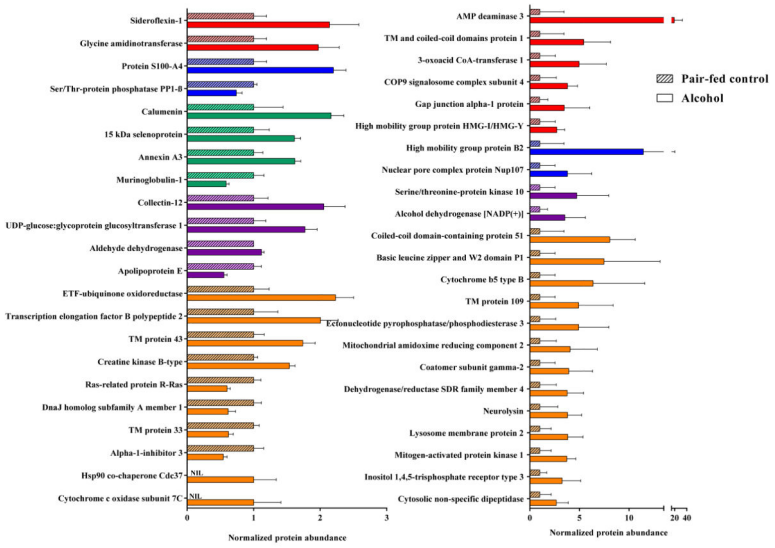

Figure 1.

Mass spectrometry analysis of alcohol-induced alteration of placental proteins. 45 proteins were identified as significantly different ($P < 0.05$) between the pair-fed control and alcohol groups when quantified by both normalized spectral counts method with Mascot/Scaffold, and by iBAQ analysis with MaxQuant. Colors depict protein function categorization: Red – Gestational diseases, fetal development; Blue – Neurodevelopment; Green – Implantation; Purple – Alcohol metabolism, nutrition, and immune function; and Orange – Cellular function. Data are represented as mean ± SEM.

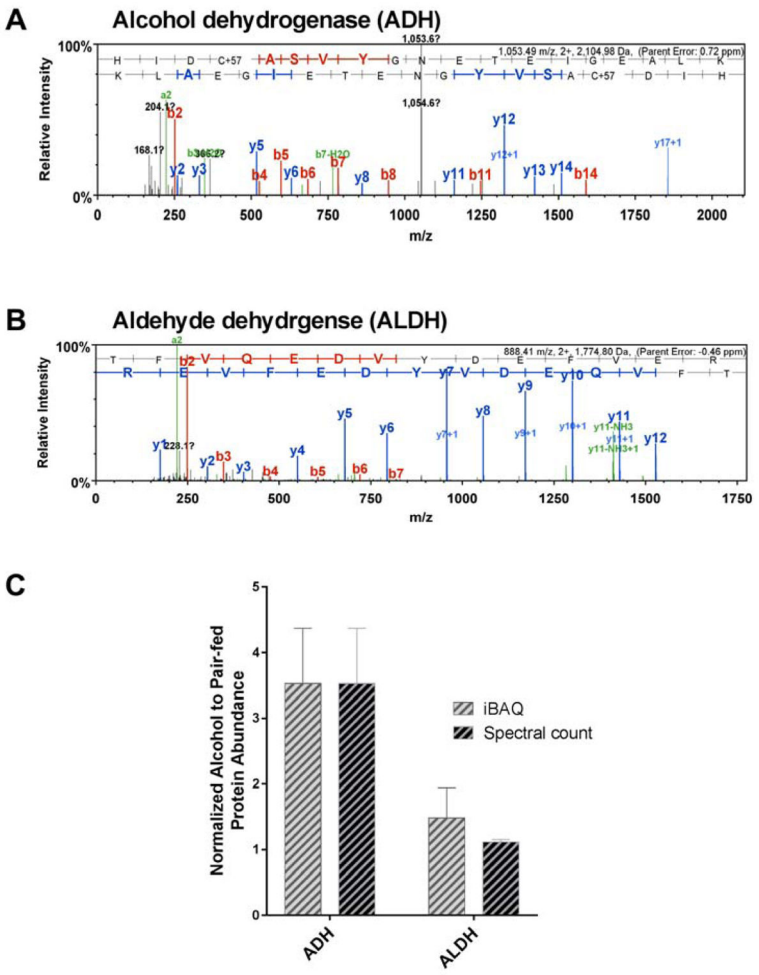

Figure 2.

Proteins directly related to alcohol metabolism were upregulated in the alcohol group, which included both alcohol dehydrogenase NADP(+) (ADH) and aldehyde dehydrogenase, mitochondrial (ALDH). One representative MS/MS spectra of (A) ADH (parent mass error: 0.72 ppm) and (B) ALDH (parent mass error: −0.46 ppm) from alcohol-exposed placenta illustrate the ions detected following protein fragmentation. Each vertical peak identifies the relative abundance of the ion and the corresponding peptide sequence, based on the mass to charge ratio (m/z). The distance between the peaks is used to construct the sequences of the identified peptide following protein fragmentation. The b ion series (red) identifies peptide fragments extending from the amino (N)-terminus, and the y ion series (blue) identifies peptide fragments extending from the carboxyl (C)-terminus. (C) The mean alcohol to pair-fed protein abundance of ADH was determined to be 3.53 by iBAQ, and 3.53 by normalized spectral count, and the mean alcohol to pair-fed protein abundance for ALDH was 1.49 by iBAQ, and 1.12 by normalized spectral count.

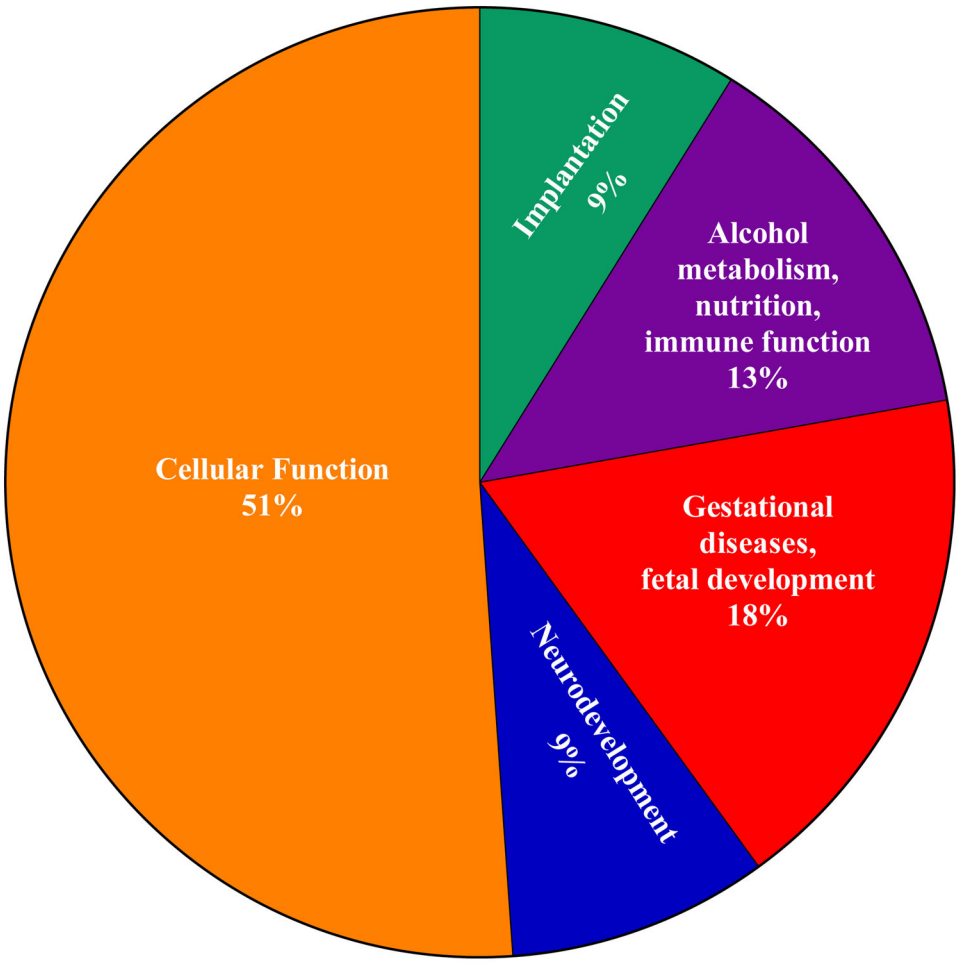

Figure 3.

Categorization of proteins significantly altered by gestational alcohol exposure based on their physiologic function during pregnancy. Specifically, 51% of the proteins were associated with general cellular functions, 18% with gestational diseases and fetal organ development, 13% with alcohol metabolism, nutrition and immune function, 9% with implantation, binding, and gestational adaptations, and 9% with fetal neurodevelopment.

Table 1

Ingenuity analysis identified ethanol degradation as the most significantly altered canonical pathway in the placenta with significantly increased risk for metabolic, neurological, and cardiovascular diseases. The most altered physiologic systems were directly related to fetal organ and embryo development.

INGENUITY Pathway Analysis	
Top Canonical Pathways	**P Value**
Ethanol Degradation II	4.01×10^{-5}
Noradrenaline and Adrenaline Degradation	5.09×10^{-5}
Synaptic Long Term Potentiation	6.33×10^{-5}
Top Diseases	
Metabolic Disease	$1.43\times10^{-2} – 2.14\times10^{-5}$
Cardiovascular Disease	$1.76\times10^{-2} – 2.84\times10^{-5}$
Neurological Disease	$1.79\times10^{-2} – 1.42\times10^{-5}$
Physiological System Development and Function	
Renal and Urological System Development and Function	$1.25\times10^{-2} – 1.41\times10^{-4}$
Auditory and Vestibular System Development and Function	$1.79\times10^{-2} – 3.27\times10^{-4}$
Embryonic Development	$1.79\times10^{-2} – 1.13\times10^{-5}$
Organismal Development	$1.79\times10^{-2} – 4.72\times10^{-5}$
Hair and Skin Development and Function	$3.60\times10^{-3} – 1.13\times10^{-5}$