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Alcohol-Induced Alterations in Maternal Uterine Endothelial Proteome: A Quantitative iTRAQ Mass Spectrometric Approach

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Abstract

Objective—To quantitate alcohol-induced alterations in the maternal uterine endothelial proteome utilizing iTRAQ-based mass spectrometry.

Study Design—Uterine artery endothelial cells from third trimester pregnant ewes were FAC sorted, validated and treated without or with binge-like alcohol. Lysates were trypsin digested, iTRAQ-labeled, and analyzed using nano LC MS/MS.

Results—Alcohol significantly upregulated 14 and downregulated 17 proteins ($P<0.05$) including those related to cell structure, transcription/translation regulation, histones, Ca^{+2}/NO, and redox balance. Gene ontology and ArrayTrack analyses revealed alterations to protein processing, binding, and nutrient metabolism pathways. Further, alcohol altered proteins previously correlated with Fetal Alcohol Spectrum Disorders (FASD) and those that regulate epigenetic, transcriptional, and translational processes.

Conclusions—Alcohol differentially alters the proteome in the maternal uterine compartment at the level of the endothelium. iTRAQ mass spectrometry provides a robust high throughput platform to comprehend the multi-mechanistic actions of alcohol and develop appropriate biomarkers and ameliorative measures for FASD.

Keywords

Alcohol; FASD; Endothelium; Pregnancy; Uterus

1. INTRODUCTION

The varying degree of deficits manifested in the developing fetus exposed to alcohol is defined as Fetal Alcohol Spectrum Disorders (FASD) [1, 2]. Though reports on FASD

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pathophysiology have primarily focused on the fetal/neonatal brain and the associated behavioral sequelae [1, 3], alcohol-induced effects on the maternal and fetal cardiovascular system including altered systemic hemodynamics [4], cardiovascular malformations [5, 6], vascular function deficits [7], as well as reproductive vascular abnormalities [8–10] have been described. However, few studies have utilized state of the art novel high throughput mass spectrometric proteomic technologies that have the potential to shed light on the multi-mechanistic perspectives underlying gestational alcohol-induced deficits including those on the maternal compartment.

Quantitative proteomic methodologies to study differentially altered protein expression profiles using mass spectrometry can be classified into three categories: non-labeled (label-free), gel-based, and label-based. Each of these methods has specific advantages and disadvantages. Label-free methods have no chemical derivatization steps and use spectral abundance or ion intensities with the inherent limitation of not utilizing signal strength for quantitation [11]. Gel-based quantification of differentially altered proteins typically uses CyDye labeling and two dimensional gel electrophoresis, followed by mass spectrometry. This method has the advantage of multiplexing using a single gel but also has disadvantages such as inadequate representation of highly acidic/basic or large proteins [12]. In contrast, labeled techniques use isotopic label-based protocols that are known to yield very little coefficient of variation in quantitative measurements [11, 13]. In the FASD field, few proteomic studies have been conducted to date [9, 14–18]. For instance, in one study, C57BL/6 mice amniotic fluid was analyzed using MALDI TOF, tandem MS and label-free quantification, demonstrating deficiency of alpha fetoprotein in response to alcohol [15]. Tryptic digestion, LC MS/MS and label-free quantification was also utilized to characterize alcohol-induced alterations in the in C57BL/6 mice fetal brains [16]. In another study, 2-D DIGE followed by MALDI TOF/TOF has been utilized to illustrate alcohol-induced alteration in proteins related to oxidative stress and vesicle transport in the maternal uterine endothelium [9]. However, no FASD study to date has utilized label-based approaches like Isobaric Tag for Relative and Absolute Quantification (iTRAQ) for differential proteomic quantitation. In this method, iTRAQ labels react with amines in the sample peptides, fractionating differentially during mass spectrometry [13], and is considered one of the most robust techniques for differential quantitative proteomic analyses and has been tested in numerous clinical settings as it offers several advantages including a low coefficient of variance of the quantitative output, faster analyses, multiplexing, no major side reaction, automatic read out, and its potential value for large scale analyses [11, 13].

Utilizing quantitative label-based proteomics for exploring FASD vascular abnormalities is important not only for identifying direct alcohol effects on maternal-fetal vascular adaptations, but also as a secondary cause for fetal brain damage. [3, 19]. During pregnancy, the reproductive uteroplacental vasculature especially plays an important role as it undergoes significant adaptations by the third trimester of gestation to accommodate the requirements of the growing fetus [20]. Further, uteroplacental vascular development and functions are altered in numerous animal models of compromised pregnancies [21] and alcohol is reported to affect the maternal-fetal interface at the level of gene, protein, as well as uteroplacental vascular function [8, 9, 22, 23]. Thus, there is a great need to pursue proteomic technologies to develop not only diagnostic tools and biomarkers, but also therapeutic treatment strategies and to illustrate the mechanism(s) underlying the teratogenic actions of alcohol exposure during critical windows of gestation. Therefore, we herein utilized for the first time label-based iTRAQ followed by LC MS/MS to quantitate alcohol-induced differentially altered proteins in the maternal uterine artery endothelium.

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2. MATERIALS AND METHODS

2.1. Alcohol Binging

The Animal Care and Use Committee of the University of Wisconsin-Madison approved procedures for obtaining uterine arteries from pregnant ewes (Day 120–130; term = 147) for endothelial cells isolation using collagenase digestion procedures [24]. This period corresponds to the third trimester-equivalent of human fetal brain development when the first order velocity of fetal brain weight (first derivative of weight with reference to time) [19, 25, 26], and the maternal uterine blood flow [27–29] peak, and when specific deficits like developmental cerebellar Purkinje cell and hippocampal pyramidal neuronal losses are observed [30–32]. Further, we utilized the uterine endothelial cells as pregnancy-induced increases in uterine blood flow and associated vascular programming are endothelium-dependent [28]. For instance, the programming of the nitric oxide system occurs only at the level of the uterine endothelium and the downstream vascular smooth muscle signal transduction pathways are unaltered [33]. Four pairs of cell lines derived from four different pregnant ewes were utilized. The procedure for alcohol binging has been described elsewhere [9]. In brief, cells were purified using Fluorescence Activated Cell Sorting (FACS), devoid of vascular smooth muscle cell contamination and maintained in culture to passage 4. Cells were cultured to ~70% confluence in the absence (0 mg/dl; Control) or presence of alcohol (300 mg/dl), a dose similar to the peak blood alcohol concentrations (BACs) in previous FASD studies in the ovine model system [4, 31]. Before commencing the study, the media alcohol concentration at the end of each bout of exposure was validated using an enzymatic assay kit (Quantichrom® ethanol assay kit; BioAssay Systems, Hayward, CA). Cells were exposed to a binge-like alcohol exposure paradigm in sealed compensating system equilibrated with aqueous alcohol for three hours on three consecutive days for two weeks [31, 34], a pattern common among drinking women of child bearing age [35–38]. Cell viability was validated prior to commencement of the study. At the end of the experiment, the endothelial cells were scraped and collected in a lysis buffer containing $Na_4P_2O_7$ (4 mM), HEPES (50 mM), NaCl (100 mM), EDTA (10 mM), NaF (10 mM), Na_3VO_4 (2 mM), pH (10.5), with freshly added PMSF (2 mM), Triton X100 (1% V/V), aprotinin (5 μg/ml), leupeptin (5 μg/ml), and microcystin (4 μl in 10 ml).

2.2. Sample Solubilization

Lysates were precipitated with trichloroacetic acid (TCA) and diluted with 1:1 v/v MilliQ DI water containing a protease inhibitor cocktail (Roche, Basle, Switzerland). Samples were centrifuged at 4500 rpm at 4°C for 15 minutes and the supernatant was collected. 500μL of each sample was then mixed thoroughly with 500μL of 20% TCA (w/v), 90% acetone and allowed to precipitate overnight at −20°C. The samples were then centrifuged at 15000 rpm for 30 minutes at 4°C. The supernatant was decanted and the pellets washed with 200μL ice cold acetone (90% v/v) and 200μL ice cold acetone (80% v/v). Pellets were air dried for approximately 15 minutes. 50 μL of 0.5M triethylammonium bicarbonate dissolution buffer containing 0.2% SDS was added to each pellet. Pellets were subsequently disrupted with manual pipette action agitation followed by shaking in incubator at maximum speed at 65°C for 30 minutes. The samples were then centrifuged at maximum speed and the manual disruption and shaking steps were repeated. The samples were again centrifuged at maximum speed for 15 minutes and the supernatants removed and protein quantitation performed using Qubit fluorometry (Invitrogen, Carlsbad, CA).

2.3. Trypsin Digestion

Trypsin digestion on 25μg of each sample was performed as per manufacturer's instructions for downstream iTRAQ labeling. Samples were reduced in 50 mM of reducing agent to a final concentration of 4.3–4.4 mM and incubated at 60°C for 1 hour. Samples were alkylated

in manufacturer's cysteine blocking reagent to final concentration of 8–10 mM and incubated for 10 minutes at room temperature. Modified sequence grade trypsin (Promega Corporation, Madison, WI) was added to each sample at a 1:12.5 ratio (2 μg trypsin: 25 μg target) and digested overnight at 37°C.

2.4. iTRAQ Labeling

Each isobaric tag was solubilized in 50μL isopropanol. Tags (113, 114, 115, 116, 117, 118, 119, 121) were added to respective samples individually and incubated at room temperature for 1 hour. Additional isopropanol was added to samples to ensure organic composition > 60% prior to incubation.

2.5. Strong Cation Exchange (SCX)-based Fractionation

SCX Microspin columns (Nest Group, #SEM-HIL-SCX, Southborough, MA) were used according to manufacturer's instructions. The column was conditioned sequentially with 2 × 100 μL methanol, 2 × 100 μL water, 2 ×100 μL 1 M ammonium acetate in 5% acetonitrile/0.1% formic acid (buffer B) and finally 2× 100 μL 5% acetonitrile/0.1% formic acid (buffer A). Half the sample (100 μL containing 100 μg peptide) was loaded on-column. The flow through was saved but not utilized. Samples were then washed with 1 × 100 μL buffer A. Flow through was saved but not utilized. Samples were step-eluted into 10 fractions using increasing salt concentration. Each elution was 100 μL. The 10 salt concentrations were: 5, 25, 30, 40, 50, 80, 125, 250, 400 and 1000 mM ammonium acetate in 5% acetonitrile/0.1% formic acid. Fractions were frozen and taken to dryness using vacuum centrifugation. Peptides were reconstituted in 300 μL 0.1% formic acid for direct injection and LC MS/MS analysis.

2.6. Mass Spectrometry

Each SCX fraction was analyzed by nano LC MS/MS with a Waters NanoAcquity HPLC system interfaced to a ThermoFisher LTQ Orbitrap Velos. Peptides were loaded on a trapping column and eluted over a 75μm analytical column at 350 nL/min; both columns were packed with Jupiter Proteo resin (Phenomenex, Torrance, CA). A 2h gradient per fraction was employed. The mass spectrometer was operated in data dependent mode, with MS performed in the Orbitrap at 60,000 full width at half maximum resolution and MS/MS performed using higher energy collisionally activated dissociation and product ions detected in the Orbitrap at 7,500 FWHM resolution. The eight most abundant ions were selected for MS/MS.

2.7. Data Processing and Analyses

Data were searched using a local copy of Mascot with the following parameters: Enzyme: Trypsin; Database: Uniprot Mammalia (concatenated forward and reverse plus common contaminants); fixed modification: methylthio (C); variable modifications: oxidation (M), acetyl (N-term), pyro-glu (N-term Q), deamidation (N,Q), iTRAQ 8-plex (N-term, K, Y); mass values: monoisotopic peptide mass tolerance: 25 ppm; fragment mass tolerance: 0.02 Da; maximum missed cleavages: 2. Scaffold Q+ (v3_00_08, Proteome Software Inc.) was used to quantitate Isobaric Tag peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 50.0% probability as specified by the Peptide Prophet algorithm [39]. Protein identifications were accepted if they could be established at greater than 90.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Peptides were quantitated using the centroided reporter ion peak intensity. Intra-sample channels were normalized based on the

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median ratio for each channel across all proteins. Inter-sample, protein reference, and spectrum normalizations were performed. Protein quantitative values were derived from only uniquely assigned peptides. The minimum quantitative value for each spectrum was calculated as 5.0% percent of the highest peak. Differential expression was then presented as Log_2 fold change of reference. Thus, the fold change for each individual reporter ion is based on referencing a reporter channel which is then log transformed to the base 2. For instance, when the measures are transformed to a log2 scale, a value of 1.0 in this scale corresponds to a two-fold change. Student's t test was performed using Scaffold Q+ to compare the Control and Alcohol groups. α level of significance was established *a priori* at $P < 0.05$.

3.0 RESULTS

Independent of treatment, a total of 363 proteins were detected with >2 unique peptides/protein (see supplementary Information 1). A reverse decoy strategy was used for the calculation of false discovery rate (FDR) [40]. As per Molecular & Cellular Proteomics (MCP) Paris guidelines, at 90% protein, 50% peptide prophet algorithm settings, and 2 unique peptides, the FDR was 0%. Representative MS/MS spectra with the Y and B ion series for 78 kDa glucose-regulated protein (parent mass error, −0.17 ppm) and alpha-2-HS glycoprotein (parent mass error, 0.4 ppm) are depicted in Figure 1. Among these, specifically, 31 proteins were significantly ($P < 0.05$) altered by alcohol (Figure 2); 14 were upregulated (↑) and 17 downregulated (↓). These proteins included those related to cell structure (eg. ↓ tubulin β, $P = 0.002$; ↓ vimentin, $P < 0.001$), transcription & translation regulation (eg. ↓ elongation factor 1α, $P < 0.001$; ↑ 40S ribosomal protein S19, $P = 0.002$; ↓calreticulin, $P = 0.004$), histones (eg. ↑ histone 4, $P < 0.001$; ↑ histone 2B-1k, $P = 0.036$), Ca+2/nitric oxide (NO) (eg. ↓ heat shock protein (HSP)90 β, $P = 0.014$; ↑ calmodulin, $P < 0.001$), and redox balance (eg. ↓ thioredoxin, $P = 0.017$).

The Gene Ontology (GO) terms of the human equivalent of all the protein identifications that were significantly altered by alcohol except for the uncharacterized protein were classified using Software Tool for Researching Annotations of Proteins (STRAP) as described in detail by Bhatia and Coworkers [41]. Annotation is the process of assigning GO terms to proteins and is described in detail at http://www.geneontology.org/GO.annotation.SOP.shtml. The categories for classification are described elsewhere [41]. Among the biological processes regulated by the altered proteins, the highest number of annotations was identified for the category of general "cellular processes" and "regulation" (Figure 3). Among the cellular components associated with the proteins that were altered by alcohol, the highest number of annotations was identified for the nuclear sub-cellular compartment followed by those localized in the cytoplasm (Figure 4). Among the major molecular functions of proteins altered by alcohol, the highest number of annotations was noted for "binding" followed by "catalysis" (Figure 5).

We then utilized the human equivalent of the 31 proteins except for the uncharacterized protein and analyzed the pathways using ArrayTrack software (US Food and Drug Administration, Table 1) by referencing the proteins against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [42, 43] (Table 1). The ArrayTrack analysis identified biological pathways regulated by proteins altered by alcohol (Figure 2). The analyses showed specific map titles that were significantly altered including protein processing in endoplasmic reticulum ($P = 0.002$), antigen processing and presentation ($P = 0.002$), ribosome ($P = 0.003$), nucleotide oligomerization domain (NOD)-like receptor signaling pathway ($P = 0.01$), glycolysis and gluconeogenesis ($P = 0.02$), and prostate cancer ($P = 0.03$) belonging to the categories of folding, sorting, degradation/genetic

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information processing, immune system/organismal systems, translation/genetic information processing, carbohydrate metabolism/metabolism, and cancers/human diseases.

4. DISCUSSION

The following six findings can be gleaned from this study. This is the first study to utilize the well established quantitative iTRAQ label-based technology for proteomic analyses on any cell type in FASD field. Second, chronic binge-like alcohol exposure has specific direct and substantial effects on the maternal uterine vascular compartment at the level of the endothelial proteome. Third, these data support alcohol effects on proteins regulating processes at multiple levels including epigenetic, transcriptional and translational. Fourth, alcohol has major effects on proteins related to nitrosative and oxidative stress. Fifth, gene ontology-based classification of molecular functions altered by alcohol showed the highest number of annotations for protein binding. Finally, specific pathways were altered by alcohol including protein processing, immune system function, carbohydrate homeostasis, and cancer.

Few studies have been conducted utilizing proteomic methodologies in an effort to identify candidate FASD biomarkers and to understand the underlying mechanistic perspectives [9, 14–18]. This is the first time a study has utilized iTRAQ labels, one of the most robust quantitative tools available to investigate alcohol-induced proteomic alterations in the uterine artery endothelial cells obtained from pregnant sheep. The current study is also in agreement with previous gel-based studies using 2-D DIGE; the iTRAQ analysis herein showed specific categories of proteins that were altered including those related to cell structure, protein translation, and redox balance [9]. Although 2-D DIGE and iTRAQ both have their advantages, with both techniques yielding complimentary information, studies have suggested that iTRAQ was more sensitive than other techniques including isotope-coded affinity tags (ICAT), and DIGE [12].

We observed that chronic binge-like alcohol has specific effects on the uteroplacental compartment. Early work in placental biology show that alcohol perfusion in human umbilical veins decreases prostacyclin levels and increases thromboxane/prostacyclin levels [44]. In another study conducted in mice, acute alcohol exposure induced early onset of parturition and was attributed to increased uterine prostaglandin E and F2α levels[45]. Although nearly the entirety of the FASD literature primarily deals with alcohol-induced developmental neuro-anatomic deficits and behavioral problems in the offspring, studies have now begun to focus on the role of the maternal-fetal interface [9, 22, 23, 46]. The importance of the maternal-fetal interface in FASD pathogenesis has been suggested previously [19] and the current data reinforce the concept that it is important to not ignore the role of the utero-placental compartment [4, 31]. For instance, 1g alcohol/min administered over one hour decreased pregnant ovine uterine blood flow from 1477 ± 169 ml/min to 1180 ± 195 ml/min [8]. Another study in pregnant mice showed that chronic alcohol administration (gestational day 6–18, peak BAC, ~110 mg/dl) resulted in decreased mesenteric vascular response to methacholine by affecting the NO component [7]. In contrast to these reports, another study that utilized an intermittent alcohol exposure paradigm (acute four consecutive administrations), leading to higher BACs of 332 mg/dl and 538 mg/dl resulted in an increase in uterine blood flow though absolute blood flows were not reported [10]. This difference may be due to the pattern of alcohol exposure, adaptive change in downstream resistance or the perfusion pressure. In another study, in rats, microsphere analysis was utilized to show that chronic alcohol administration (10% and 20% V/V for a month before pregnancy, and 30% V/V during gestation) decreased placental blood by around 52% in the alcohol group compared to the controls [47]. Taken together, these data establish that alcohol produces alterations in the maternal uterine artery

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endothelium and may have implications for vessel remodeling, angiogenesis and vasodilation. Future studies will include a translational approach to test the effects of chronic binge alcohol drinking on uterine vascular adaptations *in vivo*.

Our data show effects of alcohol on proteins that regulate processes at multiple levels including epigenetic, transcriptional, and translational. We observed that histone 2B-type 1 and histone 4 were increased in response to alcohol. Although recent studies have reported alcohol-induced decreases in acetylation of histone 4 in the rodent cerebellum, a sensitive target to developmental alcohol exposure [48], we herein report alteration at the protein level. The current study also demonstrates decreases in numerous proteins related to transcription and translation including elongation factor 1α, 40S ribosomal protein S19, and calreticulin. Though Goodlett et al. have reviewed numerous transcription factors that are altered in response to developmental alcohol exposure [3], we herein show for the first time alcohol-induced alterations in proteins that are directly part of the transcription/translation machinery.

We also observed that alcohol has effects on the proteins associated with nitrosative and oxidative stress. Examples of proteins associated with nitrosative stress that were altered in this study include HSP90 and calmodulin, the two proteins that synergistically activate the endothelial NO synthase (eNOS) system. HSP90, a component of the NO activation pathway was decreased in the current study. HSP 90 facilitates eNOS activation thus influencing the balance between NO and superoxide [49]. In addition to the effects on the uterine NO system, HSP90 regulates multiple cellular processes [50] including protein folding, signal transduction, cell cycle regulation etc. [51]. In contrast, we observed that calmodulin was increased. Calcium mobilizing agonists lead to increases in endothelial intracellular Ca^{+2} transients followed by Ca^{+2}/calmodulin-dependent regulation of eNOS [33]. Thus these data demonstrate the possible effects of alcohol on calmodulin-facilitated movement of eNOS from the membrane into cytosolic compartment, a step that is important for nitric oxide homeostasis [49]. We also detected proteins associated with oxidative stress like thioredoxin decreased with alcohol exposure, a finding that is in agreement with earlier studies, where thioredoxin pathways were significantly altered [9].

The GO terms of the proteins that were significantly altered by alcohol were classified. Though classification based on biological processes revealed that most annotations were related to general cellular processes and regulation, classification based on molecular functions yielded more specific potential mechanistic information pertaining to these processes. Most annotations were related to protein binding. For example, one of the proteins related to binding was alpha fetoprotein that binds to heavy metals, fatty acids, estradiol-17β, and estrogen receptors and previously suggested as a potential candidate biomarker for FASD [15, 52, 53]. Interestingly, we also observed that most of these annotations were associated with the nucleus and the cytoplasmic fractions of the cell. However, in conjunction with this data, we also noted that a pathway significantly altered by alcohol using ArrayTrack analyses was protein processing by the endoplasmic reticulum. The ArrayTrack analyses showed significant alterations to proteins associated with protein processing, translation, glucose metabolism and cancer. These novel findings on the effects of alcohol on carbohydrate homeostasis are consistent with previous reports on alcohol-induced deficits in maternal and/or fetal glucose-associated biochemical pathways [8, 54]. Although alcohol consumption among women is reported to have a direct relationship with incidence of breast cancer [55], gastrointestinal cancers [56], and as such cancer in general [56], to our knowledge we do not know of any report on fetal alcohol programming of cancer.

In conclusion, we report for the first time quantitative data using iTRAQ labeling in the field of FASD. Further, utilization of rapid and high throughput quantitative platforms may be very helpful for identifying targets of maternal alcohol exposure and in future enable to develop appropriate therapeutic strategies for FASD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

iTRAQ	Isobaric Tag for Relative and Absolute Quantification
MALDI TOF	Matrix-assisted laser desorption/ionization Time of Flight
LC MS/MS	Liquid Chromatography Mass Spectrometry
SCX	Strong Cation Exchange
FWHM	full width at half maximum
DIGE	Differential gel electrophoresis

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Highlights

- First study to utilize quantitative iTRAQ nano LC MS/MS analyses for maternal binge alcohol exposure.
- Specific effects on the maternal uterus at the level of the endothelium.
- Direct effects on transcriptional, translational and epigenetic protein machinery.
- Perturbation in NO/redox balance, protein binding and nutrient homeostasis.
- Major role for the maternal-fetal interface in Fetal Alcohol Spectrum Disorders.

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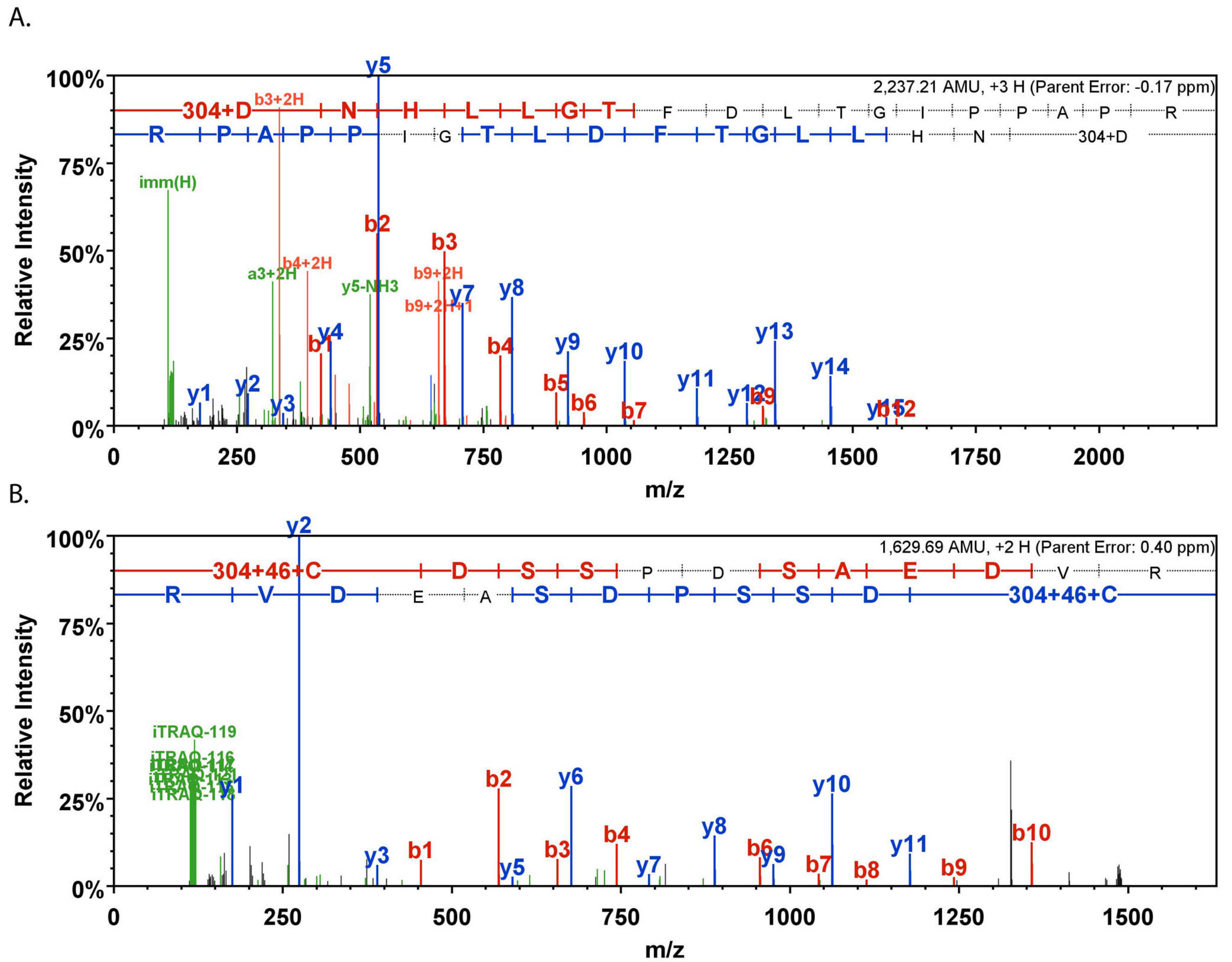

Figure 1.
Representative MS/MS spectra showing the Y and B ion series for (A) 78 kDa glucose-regulated protein (parent mass error, −0.17 ppm) and (B) alpha-2-HS glycoprotein (parent mass error, 0.4 ppm).

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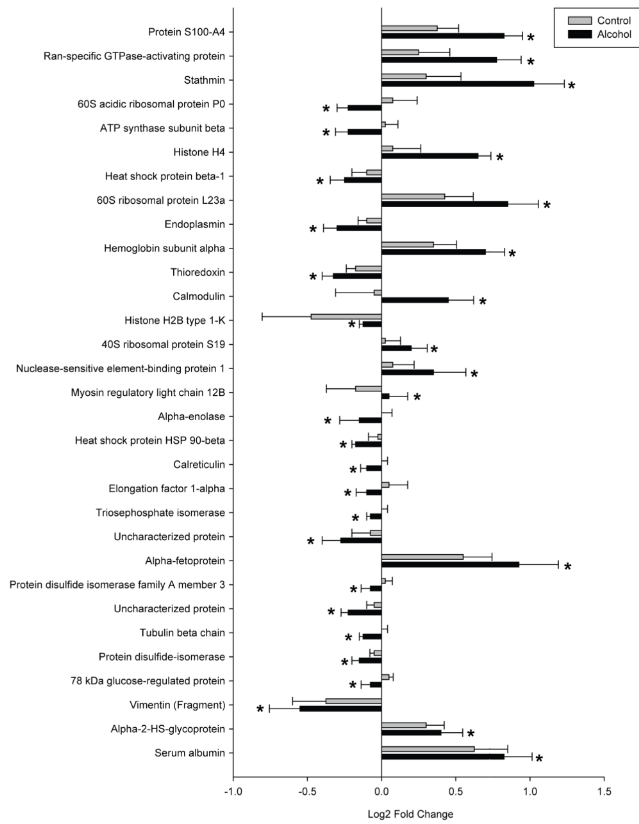

Figure 2.

iTRAQ LC MS/MS quantitation of alcohol-induced alterations in pregnant uterine artery endothelial proteome. Chronic binge-like alcohol significantly (Student's t test; *, $P < 0.05$) altered 31 proteins with 14 being upregulated and 17 downregulated. These included those related to cell structure, transcription & translation regulation, histones, Ca^{+2}/nitric oxide (NO), and redox balance. Data (mean ± SEM) are represented as log 2 fold change from reference iTRAQ sample.

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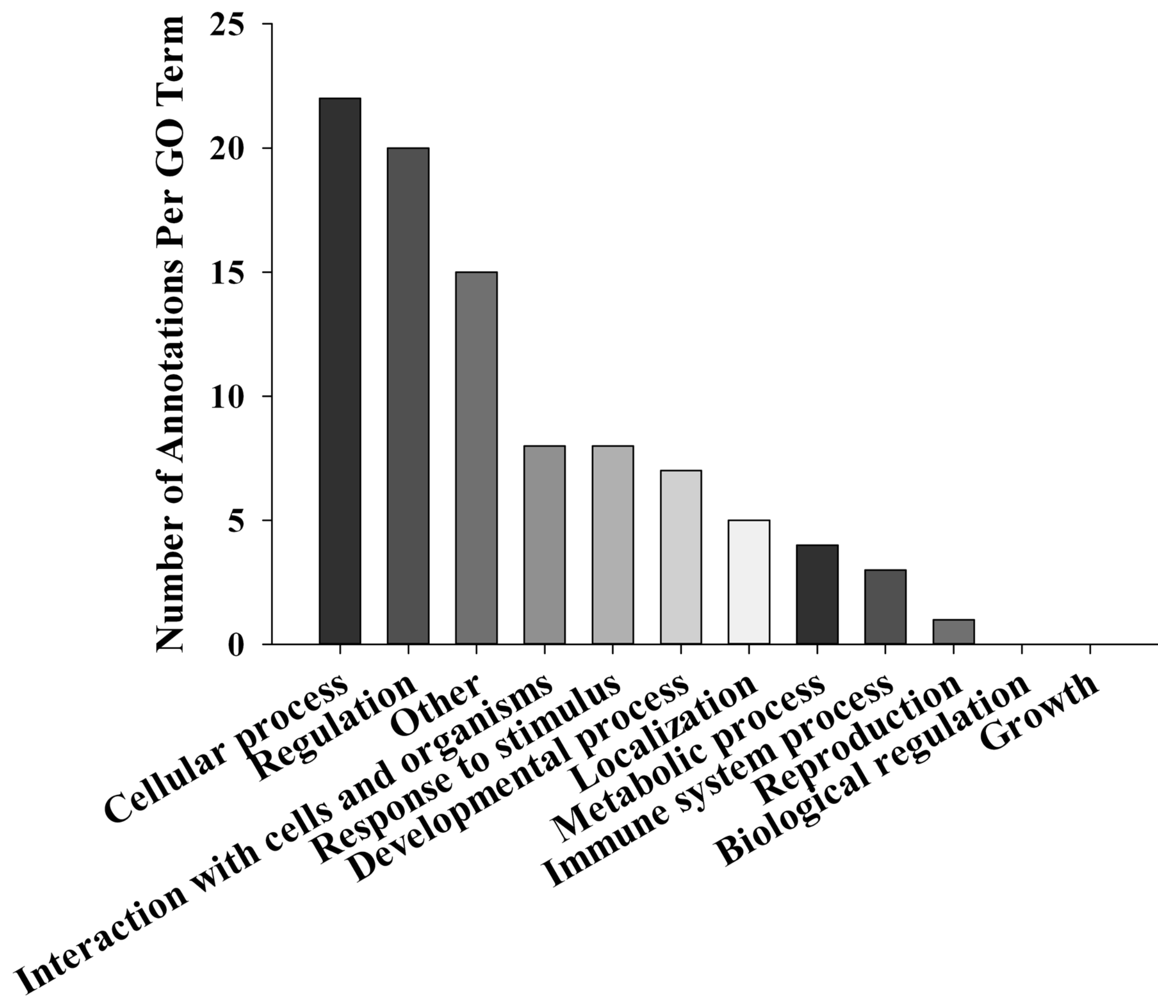

Figure 3.
The Gene Ontology (GO) terms of all the proteins that were significantly altered by alcohol except for the uncharacterized protein were classified according to their associated biological processes using Software Tool for Researching Annotations of Proteins (STRAP). The highest number of annotation terms was related to general cellular processes and regulation.

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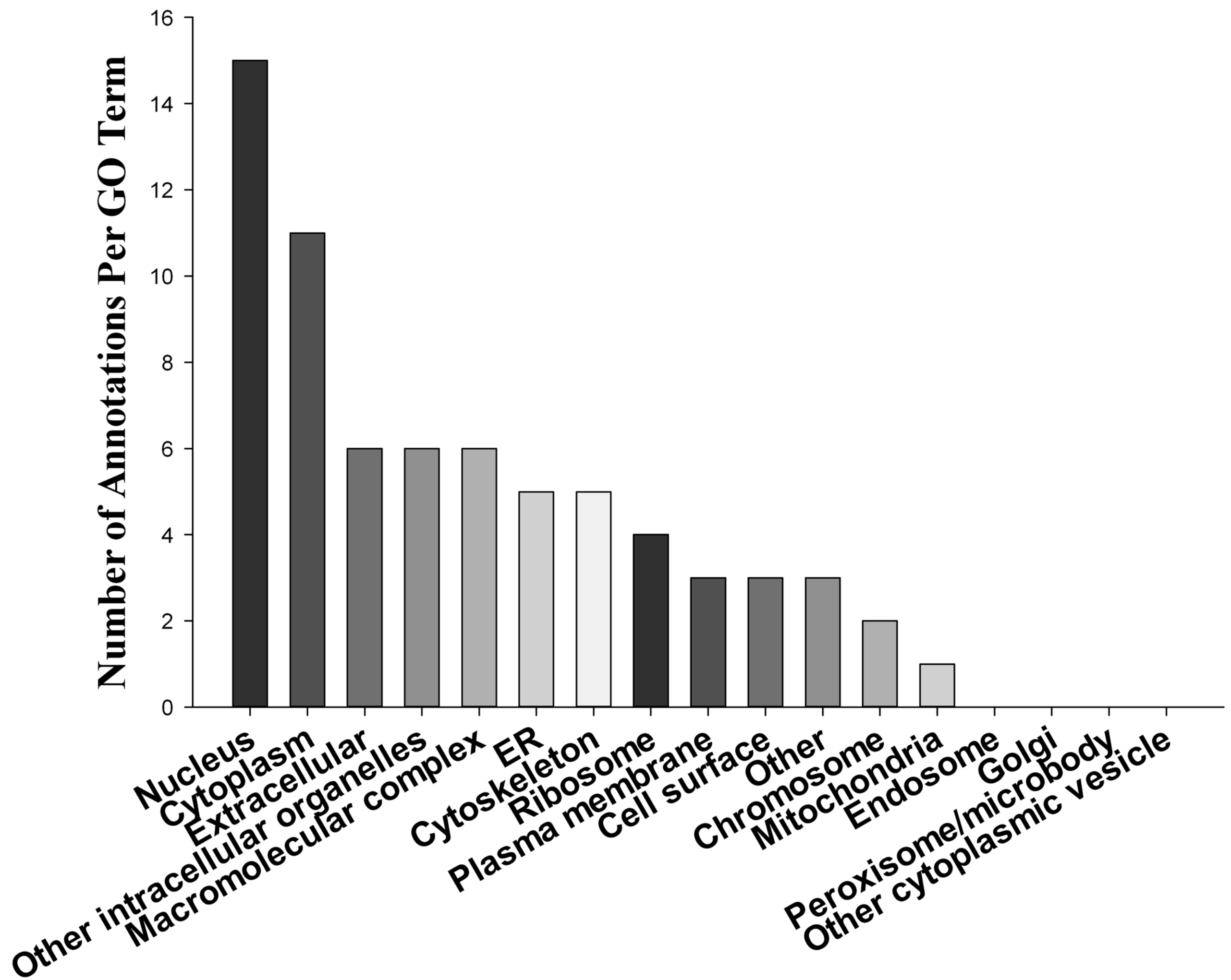

Figure 4.
The Gene Ontology (GO) terms of all the proteins that were significantly altered by alcohol except for the uncharacterized protein were classified according to their associated cellular components. The highest number of annotation terms was related to nucleus and cytoplasmic sub-cellular compartments.

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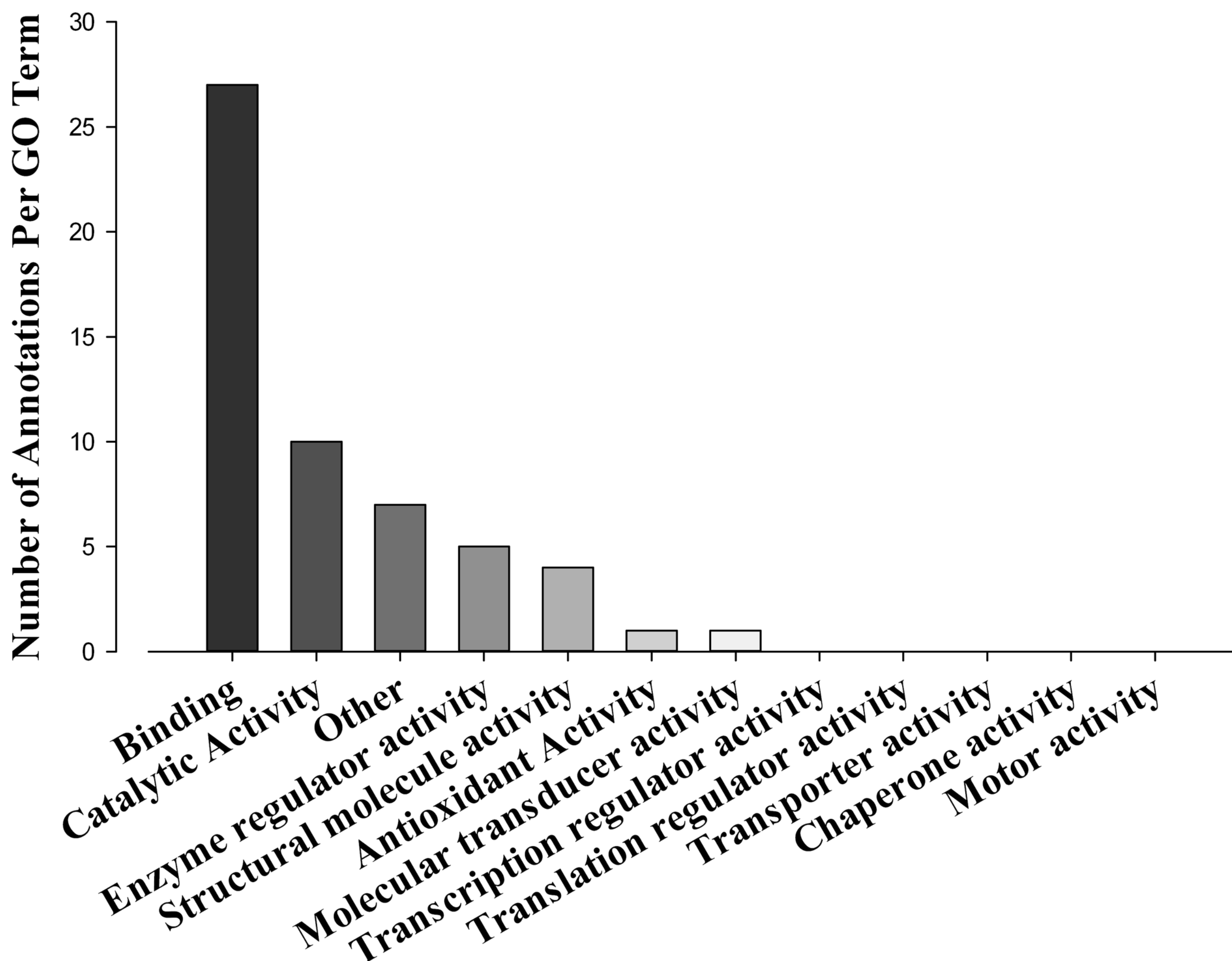

Figure 5.

The Gene Ontology (GO) terms of all the proteins that were significantly altered by alcohol except for the uncharacterized protein were classified for their associated molecular functions. The highest number of annotation terms was related to protein-binding.

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Table 1

ArrayTrack pathway analysis for proteins significantly altered in response to chronic binge-like alcohol. Gene Name refers to the Entrez Gene and the number in parenthesis is the Entrez Gene ID from the National Center for Biotechnology database. Map Title is the pathway title derived from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

Gene Name	Map Title	Category	Fisher P value
HSP90AB1(3326)	Protein processing in endoplasmic reticulum(hsa04141)	Folding, Sorting and Degradation/Genetic Information Processing	*0.00162086
HSP90B1(7184)	Protein processing in endoplasmic reticulum(hsa04141)	Folding, Sorting and Degradation/Genetic Information Processing	*0.00162086
HSPA5(3309)	Protein processing in endoplasmic reticulum(hsa04141)	Folding, Sorting and Degradation/Genetic Information Processing	*0.00162086
PDIA3(2923)	Protein processing in endoplasmic reticulum(hsa04141)	Folding, Sorting and Degradation/Genetic Information Processing	*0.00162086
HSP90AB1(3326)	Antigen processing and presentation(hsa04612)	Immune System/Organismal Systems	*0.00176551
HSPA5(3309)	Antigen processing and presentation(hsa04612)	Immune System/Organismal Systems	*0.00176551
PDIA3(2923)	Antigen processing and presentation(hsa04612)	Immune System/Organismal Systems	*0.00176551
RPL23A(6147)	Ribosome(hsa03010)	Translation/Genetic Information Processing	*0.00283376
RPLP0(6175)	Ribosome(hsa03010)	Translation/Genetic Information Processing	*0.00283376
RPS19(6223)	Ribosome(hsa03010)	Translation/Genetic Information Processing	*0.00283376
HSP90AB1(3326)	NOD-like receptor signaling pathway(hsa04621)	Immune System/Organismal Systems	*0.01461469
HSP90B1(7184)	NOD-like receptor signaling pathway(hsa04621)	Immune System/Organismal Systems	*0.01461469
ENO1(2023)	Glycolysis/Gluconeogenesis(hsa00010)	Carbohydrate Metabolism/Metabolism	*0.01756737
TPI1(7167)	Glycolysis/Gluconeogenesis(hsa00010)	Carbohydrate Metabolism/Metabolism	*0.01756737
HSP90AB1(3326)	Prostate cancer(hsa05215)	Cancers/Human Diseases	*0.03161828
HSP90B1(7184)	Prostate cancer(hsa05215)	Cancers/Human Diseases	*0.03161828

Category refers to the entry on the classification of pathways from the KEGG database.

Fisher's P value indicates if the input genes are significantly associated with a specific pathway.

* $P < 0.05$.