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Title: Binge alcohol is more injurious to liver in female than male rats: histopathological, pharmacological, and epigenetic profiles.

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Running title: Binge alcohol is more injurious to female

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Discussion: 1485 words

Abbreviations used

ALT: Alanine aminotransferase
BAC: Blood alcohol concentration
CREB: cyclic AMP response element binding protein
CYP2E1: Cytochrome P4502E1
DGKα: Diacylglycerol kinase α
ERK: Extracellular signal-regulated kinase
ETOH: Ethanol
H3AcK9: Histone H3 acetylated at Lys 9
H3AcK9PS10: Histone H3 acetylated at lys9, phosphorylated at ser10
H3PS10: Histone H3 phosphorylated at Ser10
H3PS28: Histone H3 phosphorylated at ser28
JNK: C-jun N-terminal kinase
MAPK: Mitogen activated protein kinase
PA: Phosphatidic acid
PEth: Phosphatidylethanol
PGC1α: Peroxisome proliferator-activated receptor gamma coactivator 1α

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Abstract

Binge alcohol consumption is a health problem but differences between sexes remain poorly defined. We have examined the in vivo effects of acute three repeat binge alcohol administration on liver in male and female rats. Sprague-Dawley rats were gavaged with alcohol (5 gm /kg body weight) 3 times at 12 hourly intervals. Blood and liver tissues were collected 4 hours after the last binge ethanol. Subsequently, a number of variables were analyzed. Compared to male, female had higher levels of blood alcohol, alanine aminotransferase, and triglycerides. Liver histology showed increased lipid vesicles with larger sizes in females. Protein levels of liver CYP2E1 were higher in female than male liver after binge. Hepatic phospho-ERK1/2 and phosph-p38MAPK protein levels were lower in female compared to male after binge alcohol with no differences in the phospho-JNK levels. PGC1α and CREB protein levels increased more in female than male liver. However, increases in phospho-CREB levels were lower in females. Remarkably, c-fos was reduced substantially in female liver with no differences in c-myc protein. Binge ethanol caused elevation in acetylated (H3AcK9) and phospho-acetylated (H3AcK9PS10) histone H3 in both sexes but without any difference. Binge alcohol caused differential alterations in the levels of various species of phosphatidylethanol (PEth) and a larger increase in the diacylglycerol kinase-α protein levels in female compared to male livers. These data demonstrate for the first time similarities and differences in the gender-specific responses to repeat binge alcohol leading to the increased susceptibility of female liver to injury in vivo.

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Significance

This study examines the molecular responses of male and female rat liver to acute binge alcohol in vivo and demonstrates significant differences in the susceptibility between genders.

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Introduction

Alcohol consumption is a major cause of chronic liver disease in the United States and around the globe (Axley et al., 2019). Development of fatty liver disease is seen in more than 90% of alcoholics but only 20 to 40% of alcoholics develop further liver injury characterized by hepatitis and fibrosis (Kourkoumpetis and Sood, 2019; Taylor and Miloh, 2019). Among the factors contributing to increased susceptibility to alcohol, gender difference is considered as one important factor contributing to increased alcoholic liver injury. Females are more susceptible to alcoholic liver injury than males both in clinical studies and preclinical models (Sato et al., 2001; Frezza et al., 1990; Becker et al., 1996; Iimuro et al., 1997; Kono et al., 2000; Yin et al., 2000; Fulham and Mandrekar 2016).

In addition to sex differences, binge pattern of alcohol drinking is emerging as a significant determinant of alcoholic liver injury (Shukla et al., 2013a; Ghosh et al., 2018). Binge drinking by humans is on the rise in many countries. Over 90% of US adults who drink excessively report binge drinking in the past 30 days. About 17% of US adults binge drink 4 times a month. There is epidemiological evidence that females are more sensitive to alcohol and experience alcoholic liver injury at lowers levels of drinking than men and for this reason NIAAA defines binge drinking about 5 or more drinks in male and about 4 or more drinks in female in a span of 4 hrs (see White et al, 2018). Recent studies have reported increased incidence of binge drinking in women underscoring the significance of understanding sex differences in alcoholic liver injury (Kirpich et al., 2017, Breslow et al., 2017, Fernandes et al., 2018). Despite these

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differences the pharmacological mechanisms of the acute binge effect of alcohol on liver of male and female have not been examined. We and others have shown changes in MAPK signaling components (Aroor and Shukla, 2004), alterations in transcriptional factors, and epigenetic histone modifications (Shukla and Aroor, 2006; Shukla and Zakhari, 2013b) associated with binge mode of alcohol intake (Shukla et al., 2013a) in male rat (Aroor et al., 2010; 2011) and mouse models (Shukla et al., 2015b) of alcoholic liver injury (Bardag-Gorce et al., 2010; Kim and Shukla 2006; Kirpich et al., 2012; Abdelmegeed et al., 2013; Aroor et al., 2012a; Aroor et al., 2014; Shukla et al., 2015b). We have used here an animal model that mimics the human acute binge scenario wherein 3 repeat doses of binge alcohol were administered by oral route and their effects were compared in male and female rats.

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Materials and Methods

All chemicals and reagents used were of the highest analytical grade available. Cell Signaling Technology supplied antibodies for CREB protein (cat # 9192), phospho-CREB (Cat # 9198B), ERK protein (Cat # 4595), phospho-ERK (cat # 9101), p38 protein (Cat # 9212), phospho-p38 (cat # 4511), JNK protein (cat # 9552), phospho-JNK (cat # 9255), c-fos (cat # 2250), and c-caspase 3 (Cat # 9961). Antibodies for c-MYC (cat # sc 764), catalase (cat # sc 34280), and PGC1α (cat # sc 13067) were acquired from Santa Cruz Biotech. DGKα antibody (cat # PA5-44058) was purchased from Thermo Fisher Scientific. CYP2E1 antibody (cat# ab 28146) was from ABCAM. Antibodies for Histone H3 AcK9 (cat # 06-942), H3PS10 (cat # 05-817), H3PS28 (cat # 07-145) were from Upstate Biotechnology. Antibody for histone H3AcK9PS10 (cat # 9101) was from Cell Signaling Technology.

Animals and binge alcohol administration: Male and female Sprague–Dawley rats, were purchased from Harlan Laboratories (Indianapolis, IN). When procured, they were seven weeks old and weighed between 250-300 g. They were housed under a 12-h/12-h light/dark cycle and were permitted ad libitum consumption of standard laboratory rat chow and water. The animal care and protocol for their use was approved (protocol # 9092) by the University of Missouri Animal Care & Use Committee. Rats were used for binge alcohol studies after a 1-week equilibration period. We have selected three binge mode of ethanol administration as a model of repeat binge administration that is commonly seen in alcohol abusers and binge drinkers (Aroor et

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al., 2014; White et al., 2018). For binge ethanol administration, ethanol was diluted to 32% (v/v) in sterile water. Using an 18 gauge stainless steel blunt tipped needle, an average volume of 7.5 ml ethanol (5g/kg body weight) was administered by intragastric administration. Ethanol was administered three times at 12 hr intervals. Water replaced ethanol in control group. Four hours after the last binge ethanol administration, blood samples were collected and liver was rapidly perfused with cold phosphate buffered saline containing phosphatase inhibitors. A small portion of the liver tissue was fixed in formalin and remaining liver tissue was quickly frozen in liquid nitrogen and stored at -70^{0}C for further analysis. We have used 4 male and 4 female rats for the treatments and data are presented as male control (MC), male ethanol binge (ME), female control (FC), and female ethanol binge (FE).

The alcohol treatment model used here has been applicable to both rat (Aroor et al, 2011; 2014) and mice (Shukla et al, 2015a). NIAAA definition of binge refers to human but mimicking that situation of heavy binge in small animal models is challenging and requires appropriate modifications. In the rat model used here the blood alcohol concentration were in the range of 50-100 mM (i.e. about 0.25-0.5 %). This range of alcohol has been observed in human chronic alcohol abusers taking binge alcohol (discussed in Shukla et al., 2013a and references cited therein). Catabolism of ethanol is up to 5 times higher in rodents and thus BAC levels cannot be directly compared with human. The ethanol dose used here is a commonly used dose for rat model subjected to binge in many other laboratories. Rats were still conscious and responsive to toe and tail pinch.

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Determination of ALT levels: Serum ALT levels were measured by kinetic ALT assay in an automated analyzer (University of Missouri Research Animal Diagnostic Laboratory, Columbia, MO, USA) as used earlier by us (Aroor et al., 2011).

Determination of BAC levels: Blood alcohol was determined by alcohol dehydrogenase assay kit from Genzyme Diagnostics Framingham, MA. Serum (100 µl) were used for the analysis.

Preparation of cell extracts and western immunoblot: The whole cell extract (WCE) were prepared at 4^0C as described (Aroor et al., 2014; Shukla et al., 2015a). After homogenizing the frozen liver tissue in lysis buffer (containing 50mM Tris HCl, pH7.4, 25 mM KCl, 5 mM MgCl2, 5 mM glycerophosphate,1 mM EDTA, 1 mM Na-ortho vanadate, 1 mM EGTA, 1 mM DTT, and Sigma protease inhibitor cocktail p8340) a small portion of the extract was saved. Bio-Rad DC protein assay was used to determine the protein concentrations in the whole cell extracts.

The whole liver lysate protein (40µg) was separated by 10% or 15% SDS-PAGE followed by transfer onto nitrocellulose membrane using Bio-Rad Trans Blot apparatus. Membrane was washed with 20 mM Tris HCL, pH 7.5 containing 0.1% Tween 20 and 150 mM NaCl (TBST) and blocked with TBST containing 5% nonfat dry milk for 2 h at room temperature. Next, membrane was incubated with primary antibody overnight at 4^0C in 3% BSA in TBST and washed with TBST followed by incubation with corresponding secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature. Immunoblots were visualized using chemiluminescent reagent

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(Pierce Chemicals, Rockford, IL, USA). LAS-3000 imaging system (Fujifilm Life Sciences, Stamford, Connecticut, USA) was used to capture chemiluminescence and Multi Gauge^{tm} software (Fujifilm Life Sciences) was used for quantitation of immunoblots. The chemiluminescence intensity was within the linear range of the detection. Beta actin and/or histone H1 protein were used for equalizing protein loading. Digitalized data were normalized to controls and statistically analyzed using GraphPad Prism 4.

Histology: Formalin fixed liver tissue was embedded in paraffin, sectioned and stained with hematoxylin and eosin (H & E) at the University of Missouri Research Animal Diagnostic Laboratory, Columbia, MO, USA as described earlier (Aroor et al., 2011). Microscopic images of slides were taken using a Nikon Eclipse E600 with Nikon Digital Camera, DXM 1200 and Nikon ACT-1 v2.70.

Lipid vesicle determinations: For a semiquantitative determination of the vesicle numbers in the images, a section of histology images was selected and divided into 4 quadrants; vesicles were counted and recorded. Data are from 4 male control (MC), 4 male ETOH (ME), 4 female Control (FC) and 4 female ETOH (FE) samples. Data from each group were pooled together for calculations.

Hepatic triglyceride assay: Liver triglyceride levels were determined essentially as reported earlier (Aroor et al., 2011). Liver tissue (30 mg) was homogenized in 0.5 ml hypotonic buffer containing 20 mM Tris, 2% Triton X-100, and Sigma protease inhibitor cocktail (p8340) in a 2 ml capacity Dounce homogenizer with pestle A. The sample was heated to 60°C followed by centrifugation at 13,000xg for 5 min. The supernatant was used for triglyceride

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estimation using the assay kit and protocol provided by the supplier (Sigma-Aldrich Company, St. Louis, MO, USA; Cat #F6428). Results are reported as mg/gm of liver tissue.

Analysis of phosphatidylethanol (PEth): The frozen liver tissues from rats were homogenized in saline on ice. The homogenates were then subjected to a modified Bligh-Dyer extraction (Bligh and Dyer, 1959) with the addition of internal standard 14:0/14:0 (sn-1/sn-2 aliphatic groups indicated as number of carbons:number of double bonds) PEth. PEth internal standard was synthesized using phospholipase D as previously described (Omodeo et al,1989) yielding >90% conversion of 14:0/14:0 PC (Avanti Polar Lipids, Alabaster, AL) to 14:0/14:0 PEth as determined by ESI/MS. Unreacted 14:0/14:0 PC (Avanti Polar Lipids, Alabaster, AL) and phosphatidic acid were separated from product ,14:0/14:0 PEth (Rf = 0.56), using thin-layer chromatography with chloroform/methanol/water (16/6/1, v/v/v), and purity confirmed to be > 95% by ESI/MS. 14:0/14:0 PEth was subsequently quantified by both ESI-MS with comparisons to known amounts of commercially available 16:0/18:1 PEth (Avanti Polar Lipids, Alabaster, AL) and quantification was confirmed using GC-FID of the fatty acid methyl ester derivatives of 14:0/14:0 PEth (e.g., tetradecanoyl methyl ester) compared to the derivative of the internal standard heptadecanoic acid (Gross,1984).

The lipid extracts were stored under nitrogen in chloroform until they were dried under nitrogen and suspended in methanol/chloroform (4/1, v/v) to be quantified by ESI-MS/MS on a triple quadrupole instrument (Thermo Fisher Quantum Ultra) using shotgun lipidomics methodology (Han and Gross, 2005). PEth species were quantified in negative-ion mode. PEth molecular species were detected by product ion scanning of

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m/z 181.2 and aliphatic group assignments confirmed by product ion scanning of fatty acids associated with phospholipid species. Individual molecular species were quantified by comparing their ion intensities to the respective lipid class internal standards, with the addition of type I and II [^{13}C] isotope corrections (Han and Gross, 2005; Rong et al., 2017).

Statistical analysis: Graph Pad Prism 4 was used for calculations. Data were analyzed by ANOVA and P values less than 0.05 ($P < 0.05$) were considered as statistically significant.

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Results

In this in vivo study we compared the effects of acute binge alcohol on histopathological and biochemical changes in male and female rat livers. A diverse set of parameters were examined, including blood alcohol concentration (BAC), histology, pathological markers (ALT, Cyp2E1, catalase, caspase 3), steatosis (fat vesicles, triglyceride), MAPK signaling components (ERK1/2, p38 MAPK, JNK), transcription related proteins (CREB, PGC1α, c-fos, c-myc), and epigenetic markers (site specific histone modifications). We also determined the levels of PEth, a phospholipid exclusively generated after ethanol exposure, and the protein levels of DGKα. The goal was to establish the differences due to binge alcohol treatment between male and female using diverse set of parameters of liver function and pathology.

Our groups of 4 rats provided statistically significant differences in many parameters between the two sexes. Data showed low variability, were consistent, and reliable. This can be considered as the robustness of the responses in female that are distinct from male. It is possible that additional differences may be found if more animals are used, but we are not suggesting that other parameters where we have not observed statistically significant differences are 'sex-independent'. The details of the data are described below.

Histological and pathological changes: Three doses of acute binge alcohol (5g/kg) by intragastric route 12 hr apart, caused both histological and pathological alterations in the liver of the two sexes with dramatic differences. The BAC levels in female were about twice than measured in male (Fig. 1A). For the calculation of the fold changes we used gender matches; i.e. male control for male ethanol, and female control for female ethanol,

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throughout this manuscript. Data are presented as fold change over control of the respective sex. The histological changes were also more pronounced in female than in male samples (Fig. 1B). For example, although both sexes showed binge alcohol induced fatty vesicle increase, the female liver contained larger size fatty vesicles (macrosteatosis). Females had about 4 times higher number of vesicles than the males (Fig.1C). The liver triglyceride values were also consistent with this pattern (Fig. 1D) wherein TG values were increased by about 2 fold in male and 4 fold in female samples, compared to their control levels. The ALT levels, a marker of necrosis, was relatively unchanged in male but increased about 50% over control in female (Fig. 1E). In contrast, the apoptotic marker caspase 3 levels were decreased by binge alcohol in both male and female. Their levels in female liver were slightly higher than in male after binge ethanol (Fig. 1F). Binge alcohol administration might be favoring necrotic injury in lieu of apoptotic changes, but female rat livers could still be more prone to apoptotic damage than male livers.

Of other pathological markers we measured, CYP2E1 protein levels were increased by binge alcohol in both male (2 fold) and female (2.5 fold) with higher levels in female (Fig. 2A). Compared to their controls, catalase protein levels appeared to decrease in male after binge but were unaltered in female liver (Fig. 2B).

Changes in MAPK signaling components by binge alcohol: It has been shown that acute and chronic alcohol causes alterations in protein kinases in the liver (Aroor and Shukla, 2010). We examined the levels of various MAP Kinases, their phosphorylated levels, and the ratio of phospho- to protein levels in the two sexes. Following binge ethanol administration, the levels of ERK protein were increased to similar extents in male and female liver but the phospho-ERK while increased in both

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sexes was much less robust in female compared to male (Fig. 3A). This was reflected in a decrease in the ratio of P-ERK/ERK in ethanol treated female vs male. The protein levels of p38 MAPK were not changed in male or female after binge alcohol (Fig. 3B). However the phospho-p38 levels increased in both male and female but more so in male. This was in agreement with the phospho-p38/p38 protein ratio (Fig. 3B). In the case of JNK there was an insignificant increase in female JNK protein (Fig. 3C) but their phospho- levels were similar in male and female. There was a concomitant decrease in the ratio of P-JNK/JNK (Fig. 3C).

Changes in transcription related proteins: Compared to control values, the CREB protein level was elevated about 75% in female but was unchanged in male (Fig. 4A). The increase in phospho-CREB, surprisingly, was higher in male than female after binge (Fig. 4B). Interestingly, the ratio of phospho-CREB/CREB increased about 2 fold (over control) in male but decreased by 20% in female, compared to control (Fig. 4C). The levels of PGC1α protein were not affected by binge alcohol in male but were elevated in female liver by about 30% (Fig. 4D). The c-fos protein levels after binge alcohol were elevated by 50% over control in male, but was reduced substantially to about 20% of the control in female liver (Fig. 4E). The levels of c-MYC protein did not change much by acute binge in either sex (Fig. 4F).

Binge alcohol induced changes in epigenetic markers: We next investigated the binge ethanol induced changes in the site specific histone modifications. Levels of acetylated H3AcK9 increased in both male and female, but without any difference between the two sexes (Fig. 5A). A similar profile was seen with dually modified phospho-acetylated histone H3 (i.e. H3AcK9PS10; Fig. 5B). On the other hand, the

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levels of H3PS10, and H3PS28 were not altered by binge alcohol in male or female (Fig. 5C and Fig. 5D).

Binge alcohol induced changes in PEth & DGKα: Phosphatidylethanol (PEth) is a phospholipid that is exclusively produced in the presence of ethanol (Aroor et al., 2002; Aradottir et al., 2006; Viel et al., 2012). We examined the profile of various PEth species using ESI-MS/MS on a triple quadruple instrument using shotgun lipidomics methodology. The total basal amount of PEth is about 2 fold higher in the liver of female compared to male. The small basal PEth level is intriguing and may arise due to endogenous metabolic reactions including that in gut. However, the increase in PEth after binge ethanol was 10 fold over control in male versus 5 fold in female. The total quantity of PEth after binge ethanol was not much different in male from female liver (Fig 6). When individual PEth species were evaluated then differences were seen. PEth species 34:2, 36:4 and 38:5 were reduced in female compared to male after binge ethanol treatment. On the other hand, the species 36:2 was elevated in female compared to male. Other species (34:1, 36:3, 36:5, 38:6, and 38:4) showed no differences (Fig. 6).

Diacylglycerol is produced in cells from various lipid turnover and remodelling pathways including that via triglyceride lipase, phosphatidate phosphohydrolase, and phospholipase C (Van der Veen et al, 2017). Diacylglycerol kinase-α (DGKα) is a lipid metabolizing enzyme and converts DG to phosphatidic acid (PA) which can be further converted to lyso-PA and other metabolites. DGKα is proposed to have role(s) in neoplasm and in promotion of cancer (see Purow 2015) and its gene levels were recently shown to be elevated in brain of mice after binge alcohol (Finn et al., 2018). We

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therefore determined the protein levels of DGKα and found that in female there was a large increase, about 95%, in this protein compared to only about 20% in male, after binge alcohol treatment (Fig. 7).

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Discussion

This is the first report demonstrating differences in the pattern and profile of the commonly known indices of liver physiology and pathology between male and female rats exposed to three repeat acute binge alcohol treatments in vivo. A diverse set of parameters were examined related to the pathophysiology of liver, epigenetics, and cell signaling responses. In brief, three categories of data were observed: (1) data that showed significant differences in male and female, (2) that showed alterations by binge ethanol levels but with negligible differences in male and female, and (3) data showing no alterations in response to ethanol and no difference between sexes (Table 1).

Liver injury, characterized by hepatic steatosis, was seen in both male and female rats but the magnitude of steatosis was more in female rats. This increase in steatosis was also associated with increased serum transaminase suggesting more hepatic injury in females. The caspase-3 levels were decreased in both male and female mice suggesting differential regulation of necrosis and apoptosis after binge. Binge alcohol increased blood alcohol levels in both male and female rats with significantly higher levels in female rats. Body weight, fat, and age can influence alcohol metabolism. Variations in them can result in different levels of BAC. Since male and female rats used here were of same age and weight, and administered same amount of alcohol, therefore it can be ruled out that differences in BAC are due to body size differences. It suggests that differences in BAC are due to sexes. It has been reported that gastric alcohol dehydrogenase activity and first-pass metabolism is decreased in women resulting in higher blood alcohol levels (Frezza et al., 1990). As can be seen

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from our results, although a higher alcohol was seen in animals with more steatosis, other markers of injury or signaling pathways that are known to be modulated by alcohol were variably affected in males versus females, thereby supporting the conclusion that BAC is not solely responsible for all the changes observed. Increased alcohol concentration in blood may contribute to increased liver injury, although others have suggested that liver injury may be related to altered gut microbiome and independent of blood alcohol levels (Kirpich and McClain, 2017). Enhanced ethanol metabolism and associated oxidative stress have also been considered as factors contributing to hepatic injury (Shukla and Aroor, 2006; Shukla et al., 2013a). An increase in CYP2E1 in female compared to males may contribute to the increased injury in females. With higher levels of ethanol in female it is likely that higher levels of acetaldehyde and acetate will be present. This, together with the roles of CYP2E1 and catalase in the increased susceptibility of female to alcohol remain to be studied.

Changes in mitogen activated protein kinases (MAPKs) comprising extracellular regulated kinases 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 MAPK related to alcoholic liver injury after acute, chronic and binge alcohol administration have been shown earlier in male rats (Aroor and Shukla, 2004; Lee and Shukla, 2005; Venugopal et al., 2007; Apte et al., 2007; Mandrekar and Szabo, 2009). It has also been proposed that acute effects of ethanol may be related to the prolongation of ERK1/2 activation (Nguyen and Gao, 2002; Hsu et al., 2006). However, sex differences in the pattern of the activation of all three MAPKs during alcohol binge administration have not been examined until now. In this study, the notable finding in relation to MAP kinase signaling is the highly significant increase in ERK1/2 and p38 MAPK phosphorylation in male rats

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after alcohol binge compared to a more moderate increase in phosphorylation of ERK1/2 and p38MPAK in female rats. A role of ERK1/2 activation in hepatic steatosis and necrosis is gradually being appreciated (Nguyen and Gao, 2002; Kaizu et al., 2008). Administration of MEK inhibitor U-0126 was shown to suppress endotoxin induced liver injury after sensitization induced by acute administration of ethanol in mice (Beier et al., 2009) and ERK1/2 activation has been shown to mediate arachidonic acid induced hepatocyte necrosis in oxidatively stressed CYP2E1 overexpressing rat hepatocytes (Schattenberg et al., 2004). Our previous proteome studies on acute alcohol binge administration in male rats showed increased expression of CYP2E1 together with microvascular steatosis (Aroor et al., 2011). It is therefore somewhat surprising to see that the magnitude of ERK1/2 phosphorylation was higher in male rats compared to female rats but steatosis was more marked in female rats. This finding may be reconciled by noting that enhanced phosphorylation of p38 MAPK and ERK1/2 in male rats coincided with a concomitant larger increase in phosphorylation of CREB in male rats. In this regard, CREB is known to be phosphorylated by both p38 MAPK and ERK1/2, and has been implicated in regulation of hepatic triglyceride accumulation (Aroor et al., 2012b). Impairment of CREB signaling using dominant negative CREB in vivo promotes development of steatosis (Hergiz et al., 2003; Puigserver and Speigelman, 2003; Shukla et al 2015a).Therefore, it is likely that impairment of CREB phosphorylation in female rats may interfere with CREB signaling and contribute in part to the development of increased hepatic steatosis after alcohol binge in female rats. In contrast to our earlier in vitro studies with hepatocytes (Lee and Shukla, 2005), we have not observed increased JNK phosphorylation in this study. Liver regeneration

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subsequent to hepatectomy, or chemical injury is associated with differential regulation of c-myc and c-fos (Ohri et al., 2002). However, c-fos, and c-myc were not affected during liver regeneration after chronic ethanol injury (Diehl at al., 1990). In the present study, female rat liver c-fos protein levels were significantly reduced after alcohol binge. This may suggest a role of c-fos in the susceptibility of liver to binge alcohol injury in female leading to impaired reparative responses.

It has been shown that epigenetic histone modifications caused by acute, chronic, and acute on chronic binge alcohol administration results in alterations in the expression of gene related to hepatic steatosis, alcohol metabolism, and inflammatory response in gene selective manner (see Shukla and Zakhari, 2013b). However, gender differences in epigenetic histone modifications have not been investigated. We examined site specific modifications in histone H3. Although histone serine phosphorylation was not changed after alcohol binge, histone H3K9 acetylation and H3K9PS10-phospho-acetylation were increased after alcohol binge in both male and female rats to a similar magnitude. Previously we have reported role of p38 MAPK and ERK1/2 in the regulation of histone phosphorylation and acetylation (James et al., 2012). Absence of histone phosphorylation despite changes in p38MAPK, and similar increases in acetylation and phospho-acetylation of histone H3 in male and female despite differential activation of ERK1/2, suggests additional mechanisms that may be MAPK independent. For example, we have reported histone H3 acetylation can be affected by oxidative stress and acetate accumulation (Park et al., 2005; Choudhury et al., 2010; Choudhury et al., 2011; Shukla et al., 2015b). Increased histone H3 acetylation was considered to be associated with gene specific upregulation of fatty acid synthase gene

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and down regulation of carnitine palmitoyl transferase 1a (CPT1a) gene thereby contributing to increased synthesis of triglyceride and impaired oxidation of fatty acid (Kirpich et al., 2013).

This is the first report describing intriguing pattern and differences in the individual PEth species formed in male and female rat liver exposed to binge alcohol. Although total PEth levels after binge ethanol were rather similar in the two sexes (ME vs FE), the levels of PEth species 36:2 increased in female, and others decreased (e.g. 34:2, 36:4, 36:5), or did not change (e.g. 34:1, 36:3, 38:6, 38:4). Such approaches have therefore the potential to identify and examine the specific roles of individual PEth species uniquely generated after alcohol exposure. It is noteworthy in the present study that DGKα levels increased much higher in female than in male after binge ethanol. DGKα is known to be involved in the promotion of neoplastic growth (Dominguez et al, 2013), triglyceride metabolism (Van der Veen et al, 2017), T cell receptor signaling/immunological responses (Purrow, 2015), and in the development of fibrosis (Weigel et al, 2016). Whether females are at increased risk for the development of cancer with binge ethanol need to be further investigated, particularly in breast cancer where a strong relationship with alcohol use is known (Shield et al., 2016).

Based on this study it is suggested that acute binge ethanol effects are not ‘all or none’ responses in male and female. The fact that some indices changed but not others in male versus female, is reflective of selective susceptibilities. The higher accumulation of triglyceride and enhanced liver injury in female rats in conjunction with dysregulated MAPK signaling, oxidative stress related to alcohol metabolism, and specific alterations in PEth & DGKα may be involved in binge-induced increased injury

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in female. This sets the stage for additional studies to decipher the sex differences to binge alcohol. In conclusion, acute binge ethanol in vivo selectively modifies molecular pathways in male and female liver leading to increased susceptibility in female.

Authorship contributions:

Participation in research design: Shukla, SD, Aroor AR

Conducted experiments: Shukla SD, Aroor AR, Restrepo RJ, Liu X, Franke JD

Performed data analysis: Shukla SD, Aroor AR, Restrepo RJ, Lim RW, Franke, JD, Ford DA, Korthuis RJ

Wrote or contributed to writing of the manuscript: Shukla SD, Aroor AR, Restrepo RJ, Lim RW, Ford DA, Korthuis RJ

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Footnotes

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FIGURE LEGENDS

Figure 1. Histological and pathological changes in ethanol binge treated rats. Ethanol three repeat binge treatments (each 5g/kg, 12 hrs. apart) in male and female rats (groups of 4) were performed as described under Methods. Four hrs after the last binge blood samples and liver tissues were collected. A: Blood ethanol concentration, B: Histology image showing differences in lipid vesicles, C: Count of lipid vesicles, D: Triglyceride levels, E: Serum ALT levels, F: Cleaved caspase-3. Values are mean ± SEM (n = 4 rats). For figures A, C, D & E the values are shown on y-axis. For Fig. F values are presented as fold increase over respective sex controls. MC: male control; ME: male ethanol binge; FC: female control; FE: female ethanol binge. a: significant compared to control ($p < 0.05$); b: significant compared to the male ethanol binge group; ($p < 0.05$).

Figure 2. Levels of CYP2E1 (A) and Catalase (B). After binge treatments (as in Fig. 1) the whole cell extracts (WCE) were prepared (see Methods). The protein levels of these components were determined by western blotting. Fold change over respective male or female control values are presented as mean ± SEM (n = 4 rats). a: significant compared to control ($p < 0.05$); b: significant compared to the male ethanol binge group; ($p < 0.05$). MC: male control; ME: male ethanol binge; FC: female control; FE: female ethanol binge.

Figure 3. Changes in MAPK signaling components. Levels of total ERK ½, phospho- ERK ½, and ratio of phospho- to total ERK ½ (A); levels of total p38, phospho-p38, and ratio of phospho- to total p38 (B); and levels of Total JNK, phospho-

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JNK, and ratio of phospho- to total JNK (C) are presented. The experimental details were as described in Fig. 2. The protein levels of these components in WCE were determined by western blotting. Similar to Fig. 2, the values are mean ± SEM (n =4 rats). a: significant compared to control ($p < 0.05$); b: significant compared to the male ethanol binge group; ($p < 0.05$). MC: male control; ME: male ethanol binge; FC: female control; FE: female ethanol binge.

Figure 4. Changes in transcription related factors. Protein levels of total CREB (A); phospho-CREB (B); ratio of phospho- to total CREB (C); PGC-1α (D); c-FOS (E); and c-MYC (F) are shown. The protein levels of these components were determined by western blotting. Similar to Fig. 2, values are fold change over respective sex controls and are mean ± SEM (n = 4 rats). a: significant compared to control ($p < 0.05$); b: significant compared to the male ethanol binge group; ($p < 0.05$). MC: male control; ME: male ethanol binge; FC: female control; FE: female ethanol binge.

Figure 5. Histone H3 acetylation and phosphorylations. Levels of H3AcK9 (A); H3AcK9PS10 (B); H3PS10 (C); and H3PS28 (D) are presented. The experimental details were as described in Fig. 2. The levels of these components were determined by western blotting. Values are fold change over respective sex control and are mean ± SEM (n = 4 rats). a: significant compared to control ($p < 0.05$); b: significant compared to the male ethanol binge group; ($p < 0.05$). MC: male control; ME: male ethanol binge; FC: female control; FE: female ethanol binge.

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Figure 6. Analysis of phosphatidylethanol (PEth) species. Lipids were extracted from liver samples and PEth analyzed by ESI-MS/MS on a triple quadrupole instrument using shotgun lipidomics method (see Materials & Methods). Values (mean ± SD) are from 4 male or female rats and are presented as pmole/mg tissue.

Figure 7. Diacylglycerol kinase α (DGKα) protein levels. DGKα protein levels in WCE were determined by western blotting followed by densitometric quantification. Increases in the protein levels after binge ethanol are presented as fold increases over respective sex controls. The experimental details were as described in Fig. 2. Values are mean ± SEM (n = 4 rats). a: significant compared to control ($p < 0.05$); b: significant compared to the male ethanol binge group; ($p < 0.05$). MC: male control; ME: male ethanol binge; FC: female control; FE: female ethanol binge.

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Table 1. The three categories of data are summarized in the table.

Category	Parameter
Show significant differences in sexes	
	Histology
	BAC, ALT, TG, Lipid vesicles
	Catalase
	TOTAL CREB, Phospho-CREB,
	c-fos, PGC1α,
	Phospho-ERK 1/2, Phospho-p38
	Diacylglycerol kinase α
Show change but negligible differences in sexes	
	Histone H3AcK9, H3AcK9PS10
	CYP2E1, c- Caspase 3
	Total ERK 1/2, Total JNK
	Phosphatidylethanol
Show no change and no differences in sexes	
	Histone H3SP10, H3SP28
	c-MYC
	Phospho-JNK, Total p38 MAPK

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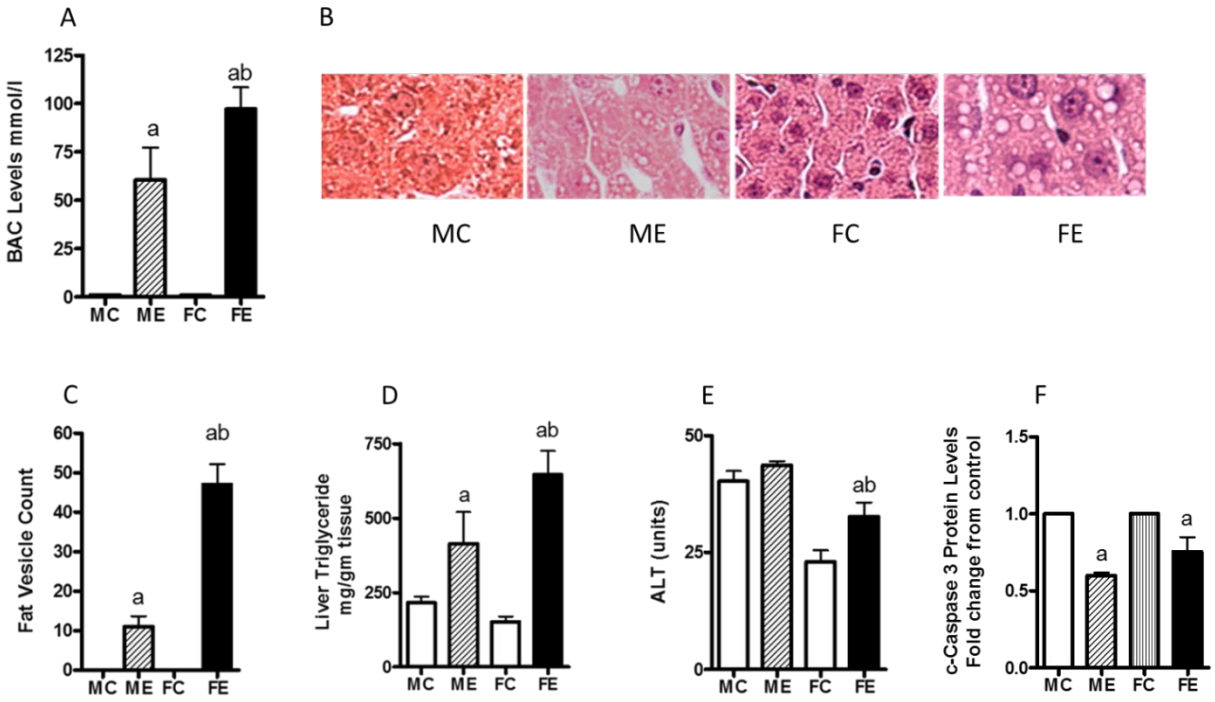

Figure 1

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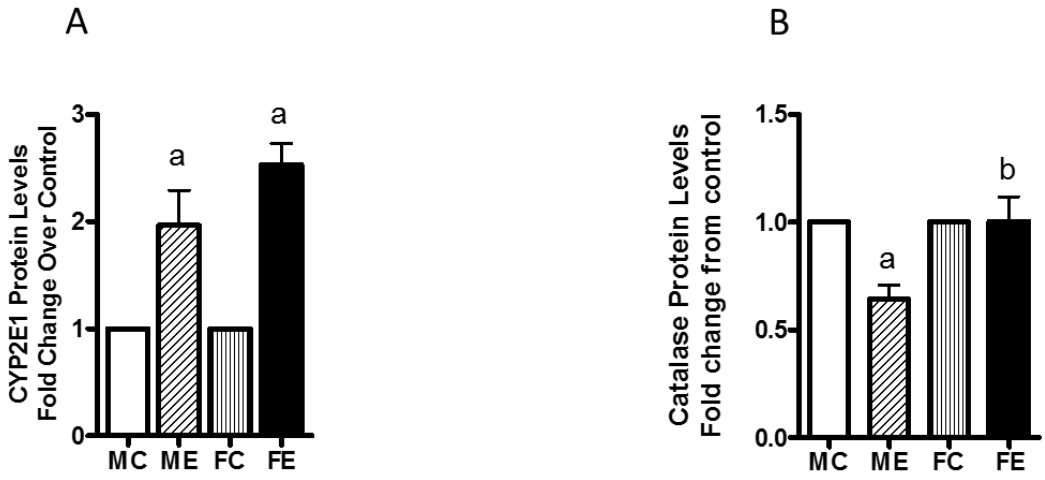

Figure 2

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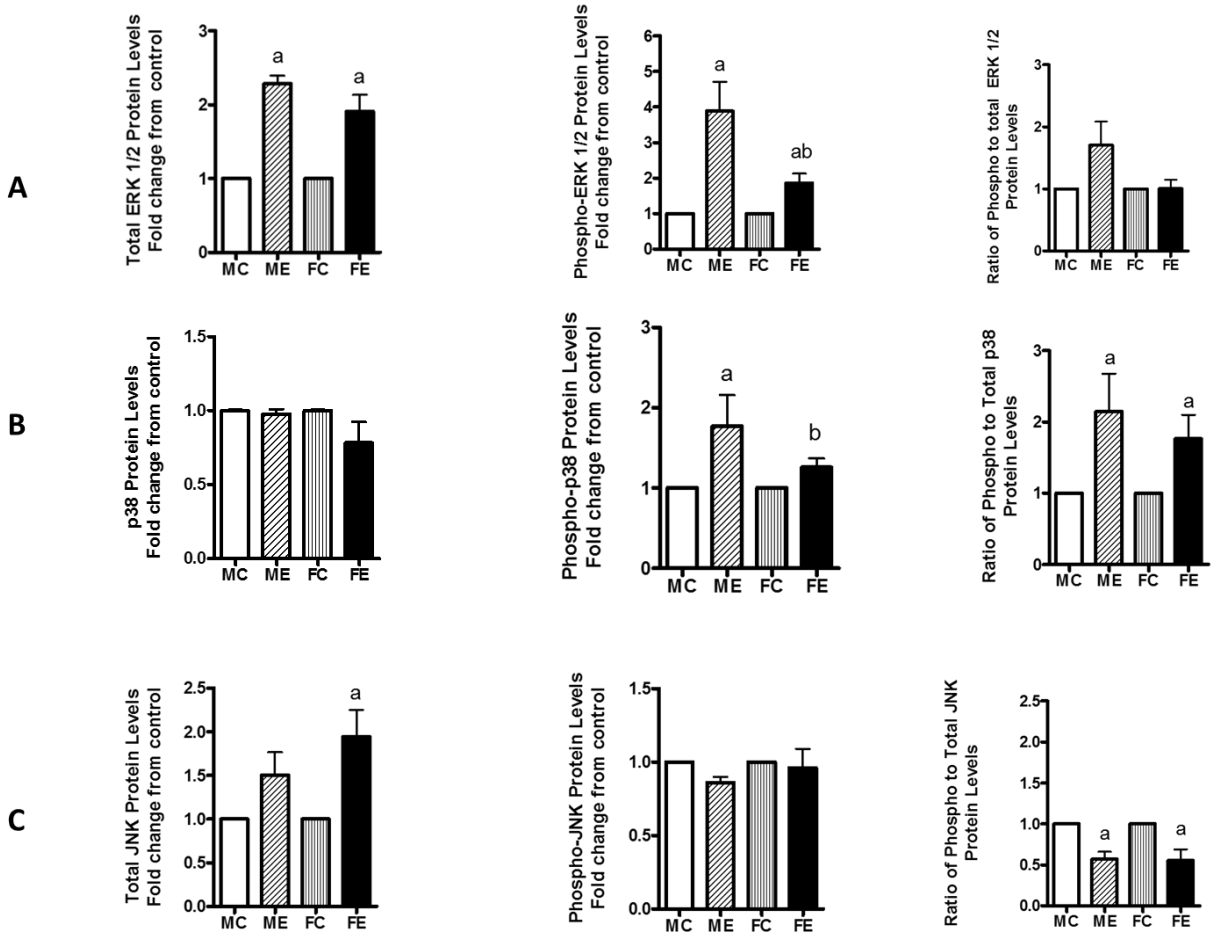

Figure 3

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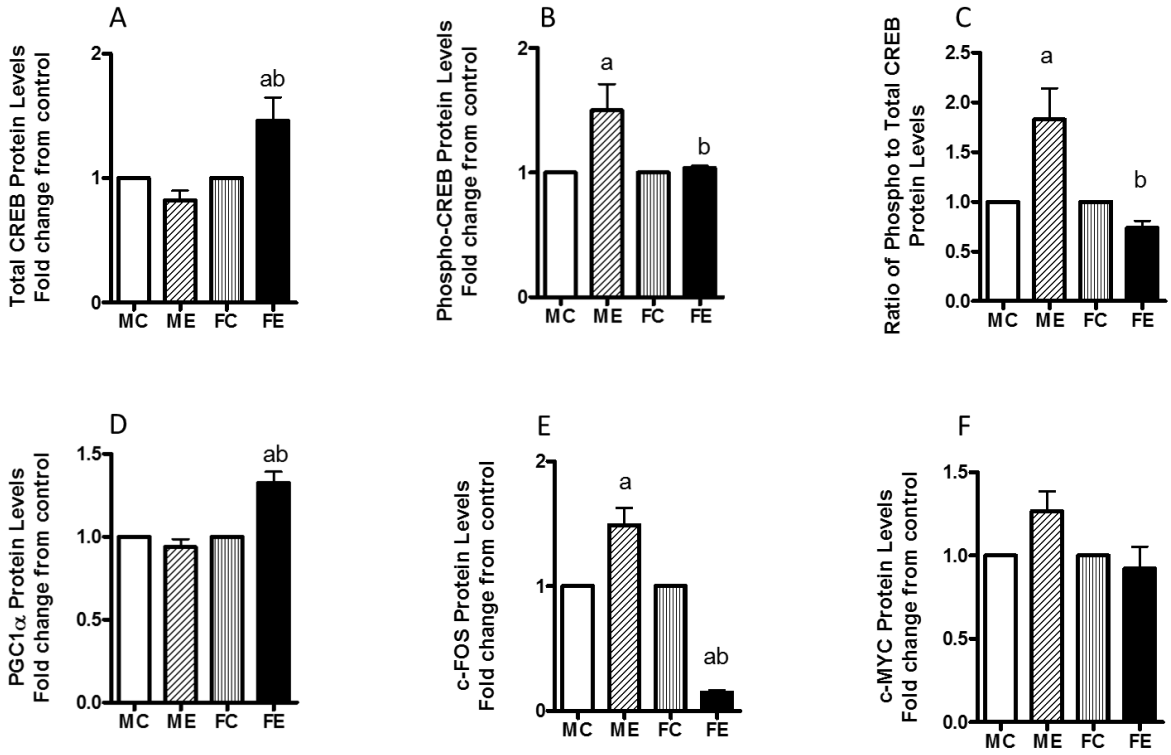

Figure 4

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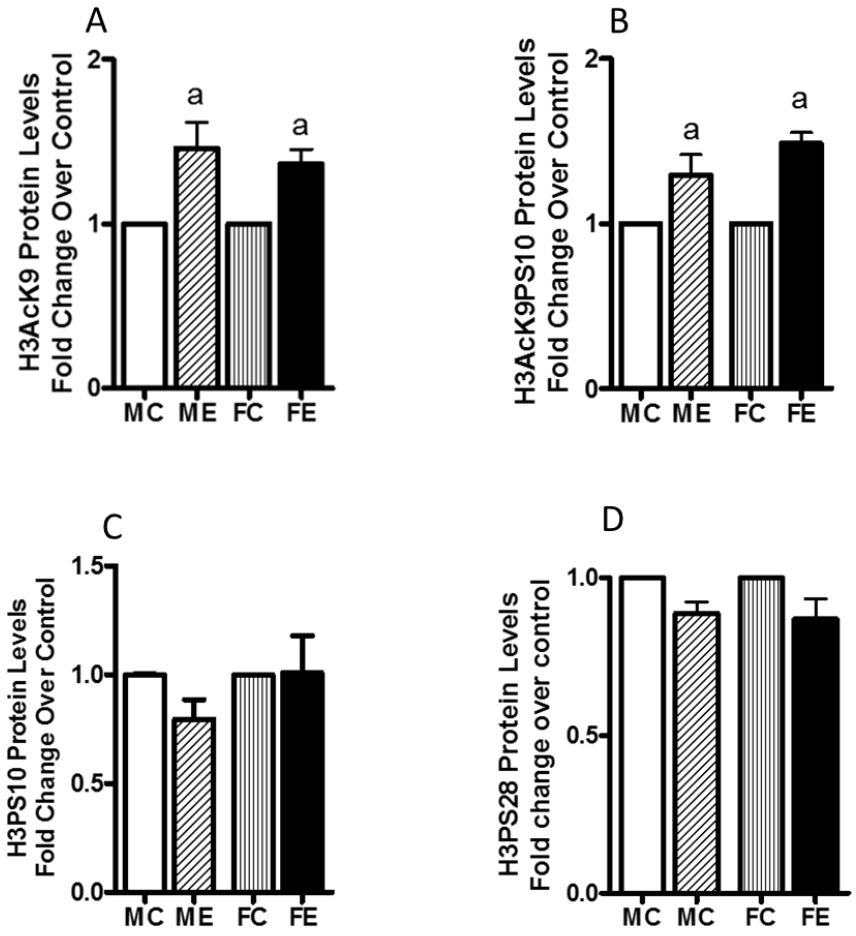

Figure 5

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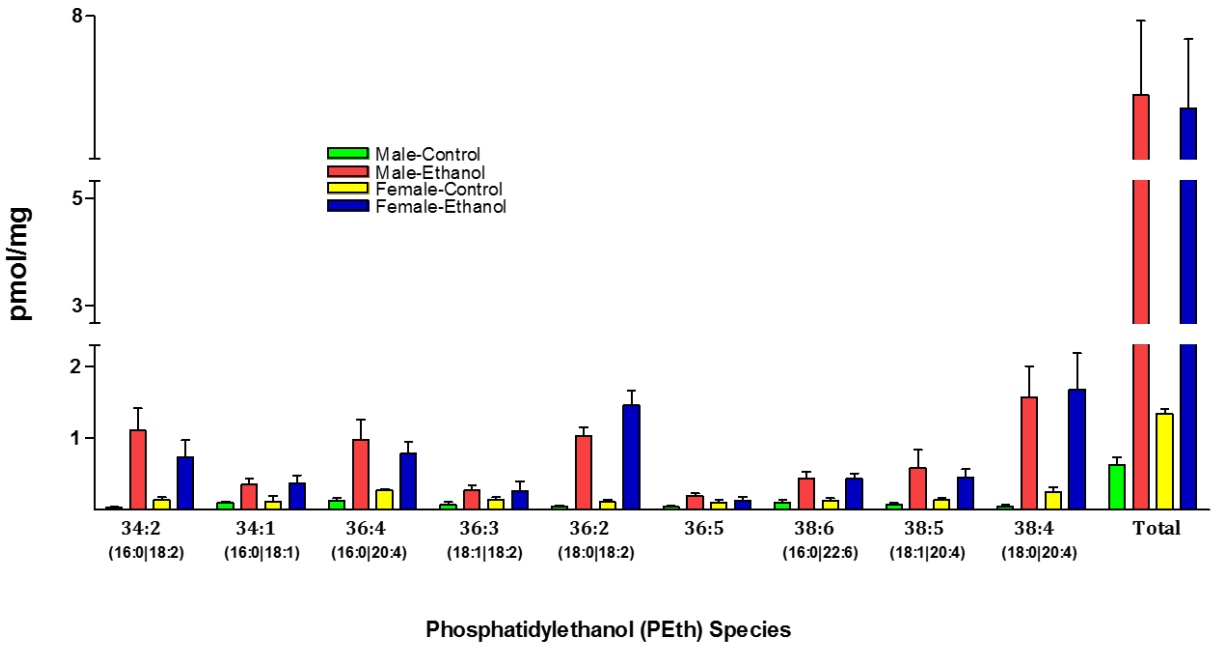

Figure 6

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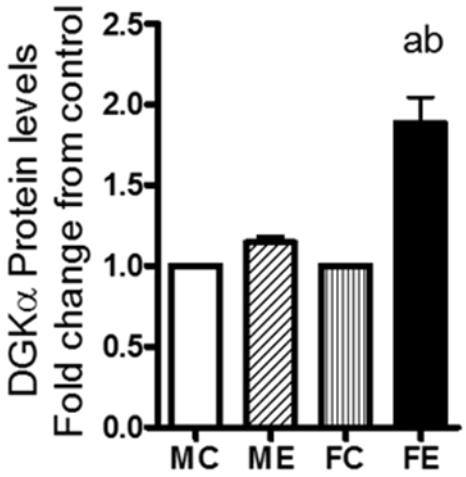

Figure 7

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