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A Neurotoxic Alcohol Exposure Paradigm Does Not Induce Hepatic Encephalopathy

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Abstract

Alcohol abuse is associated with neurological dysfunction, brain morphological deficits and frank neurotoxicity. Although these disruptions may be a secondary effect due to hepatic encephalopathy, no clear evidence of causality is available. This study examined whether a 72 h period of alcohol intoxication known to induce physical dependence, followed by a single withdrawal, was sufficient to induce signs of hepatic encephalopathy in male and female mice. Animals were continuously intoxicated via alcohol vapor inhalation, a procedure previously shown to induce significant neurotoxicity in female mice. At peak synchronized withdrawal (8 hours following the end of alcohol exposure), blood samples were taken and levels of several liver-regulated markers and brain swelling were characterized. Glutathione levels were also determined in the medial frontal cortex (mFC) and hippocampus. Results revealed elevated levels of cholesterol, albumin, alkaline phosphatase (ALP), alanine aminotransferase (ALT) and decreased levels of blood urea nitrogen and total bilirubin in alcohol-exposed male and female groups compared to controls. Brain water weight was not affected by alcohol exposure, though males tended to have slightly more water weight overall. Alcohol exposure led to reductions in tissue levels of glutathione in both the hippocampus and mFC which may indicate increased oxidative stress. Combined, these results suggest that hepatic encephalopathy does not appear to play a significant role in the neurotoxicity observed following alcohol exposure in this model.

Keywords

Vapor inhalation; hepatic encephalopathy; neurotoxicity; brain damage; oxidative stress

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1. Introduction

Alcohol use disorders (AUD) represent one of the most common and debilitating diseases world-wide. The lifetime prevalence of AUD in the United States of America is estimated at 29%, with fewer than one in five of these individuals ever seeking or receiving treatment (Grant et al., 2015). The apparent drive for the acquisition and consumption of alcohol causes significant upheaval for those with an AUD, however, damage to the various organ systems of the body may be the most debilitating aspect of the disease. Chronic alcohol abuse leads to widespread damage throughout the body, with the heart, liver, pancreas, lungs and brain disproportionately impacted (Gonzalez-Reimers et al., 2014). Mortality rates are also significantly increased among those with an AUD (John et al., 2013, Roerecke and Rehm, 2013). The effects of alcohol are extremely complicated, and the mechanisms of alcohol-induced damage are not fully understood.

To gain a greater understanding of how alcohol impacts various organ systems, several models of alcohol-induced damage have been developed (Tabakoff and Hoffman, 2000). Alcohol-induced organ damage typically requires substantial alcohol exposure for extended periods of time. This level of exposure generally exceeds the amount of alcohol most rodents will voluntarily consume. Therefore, models have been developed to achieve highly intoxicating doses of alcohol that include liquid diets (Lieber et al., 1965) and intragastric administration of alcohol (Majchrowicz, 1975, Tsukamoto et al., 1986, French et al., 1986) which are commonly used to examine alcohol-induced liver damage. Alcohol vapor administration is another widely used model of alcohol-induced damage that we and others have used to characterize alcohol-induced neuroadaptations (Hashimoto and Wiren, 2008, Wilhelm et al., 2015b, Wilhelm et al., 2015a, Wilhelm et al., 2014). In addition, sex-specific changes are observed with increased vulnerability to brain damage and neurotoxicity in females in this paradigm (Hashimoto and Wiren, 2008, Wilhelm et al., 2015b, Wilhelm et al., 2015a, Wilhelm et al., 2014). The various models of alcohol exposure each possess benefits and drawbacks, but as described by Gilpin and colleagues (Gilpin et al., 2008), alcohol vapor inhalation is a non-invasive method which provides tight control of alcohol dose, duration and pattern. This technique allows for the maintenance of relatively constant blood ethanol concentrations (BECs) over short or long durations, and can induce dependence and synchronized withdrawal to study the discrete effects of alcohol intoxication and acute and long-term withdrawal.

Effects of alcohol vapor inhalation have not been thoroughly examined for their ability to cause dysregulation of the liver and the related condition, hepatic encephalopathy. In humans, hepatic encephalopathy may result from alcoholic hepatitis, which typically requires decades of heavy abuse to develop (Lucey et al., 2009). Hepatic encephalopathy and the resulting decline of brain function are associated with compromised liver function tests, oxidative stress and brain swelling (McMillin et al., 2014, Vogels et al., 1997). Serum Gamma-Glutamyltransferase (GGT) is a commonly used marker of alcohol abuse, may be an early indicator of liver damage and averaged 576 U/L (9 – 48 U/L reference range (Staff, 2015)) in subjects with alcoholic fatty liver disease (Nishimura and Teschke, 1983). Liver function tests were disrupted in a cross-section of patients with hepatic encephalopathy, levels of serum bilirubin averaged 5.5 mg/dl (0.1 – 1.2 mg/dl reference range (Staff, 2015)),

albumin levels of 29 g/L (3.5 – 5.0 g/L reference range (Staff, 2015)) blood urea nitrogen 26 mg/dl (7 – 20 mg/dl (Staff, 2013)), Alkaline phosphatase 202 U/L (reference range 45 – 115 U/L (Staff, 2015)) alanine aminotransferase (ALT) of 117 U/L (7 – 55 U/L reference range (Staff, 2015))(Bustamante et al., 1999, Malaguarnera et al., 2011, Behar et al., 1999) and cholesterol levels of 113 mg/dl (< 200 mg/dl for health subjects)(Akriviadis et al., 2000, Bustamante et al., 1999, Malaguarnera et al., 2011, Behar et al., 1999). Oxidative stress is also a common hallmark of hepatic encephalopathy and results in decreased levels of brain glutathione (Lemberg and Fernandez, 2009). Factors associated with poor prognosis in hepatic encephalopathy include increased serum bilirubin, ALP, blood urea nitrogen and decreased serum albumin (Bustamante et al., 1999). We know of no previous studies examining the potential contribution of liver damage to the neuroadaptations and neurotoxicity associated with alcohol vapor inhalation procedures. Therefore, the purpose of this study was to examine whether hepatic encephalopathy could contribute to the alcohol-induced neurotoxicity we have repeatedly observed in females following a 72 h vapor inhalation (Hashimoto and Wiren, 2008, Wilhelm et al., 2015b, Wilhelm et al., 2014) by comparing liver function results, brain swelling and brain glutathione levels to changes previously reported.

2. Methods

2.1 Animals

Withdrawal Seizure-Resistant (WSR) mice are selected lines produced from an 8-way cross of inbred mice and were provided by the laboratory of Dr. John Crabbe in Portland, OR (Kosobud and Crabbe, 1986). WSR mice were used because previous data indicated that sex, not genotype/phenotype was the strongest influence on gene expression at peak withdrawal (Hashimoto and Wiren, 2008), and because WSR mice are resistant to the potential confound of alcohol-withdrawal induced seizures. Mice were maintained in groups of 2 – 5 under a standard light/dark cycle with lights on between 0600 – 1800 h. All tissue and blood samples were collected at peak withdrawal (8 h following removal from the chambers; approximately 1400 h). Water and standard lab chow were available *ad libitum*. Room temperatures were maintained at $22 \pm 1^\circ\text{C}$. Animal procedures were approved by the VA Portland Health Care System Institutional Animal Care and Use Committee and followed US National Institutes of Health and animal welfare guidelines.

2.2 Ethanol Exposure and Blood Chemistry

Mice were made dependent on alcohol using a 72 h vapor inhalation method as described previously (Beadles-Bohling and Wiren, 2006), employing the alcohol dehydrogenase inhibitor pyrazole. Briefly, on day 1 animals in the alcohol exposure group were weighed, injected i.p. with alcohol (20% v/v) at 1.5 g/kg with 1 mmol/kg pyrazole to reduce variations in blood alcohol concentration (BEC) and placed into vapor inhalation chambers. Alcohol (ethyl alcohol, 200 proof) for use in vapor chambers and injections was purchased from Pharmco Products Inc. (Brookfield, CT), while other chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) or other commercial sources. On all days for control (Con) animals and days 2 and 3 for animals in the alcohol group, mice were weighed, given i.p. injections of pyrazole (1 mmol/kg) dissolved in 0.9% saline and placed in vapor

inhalation chambers. A saline-only control was not included because previous analysis using an unbiased PCR differential display screening method that included WSR mice failed to identify significant differences in gene expression across a broad spectrum of genes between saline and pyrazole-treated animals in this paradigm (Schafer et al., 1998). Alcohol-exposed animals had tail blood drawn on days 2, 3, and 4 for BEC determination as previously described (Beadles-Bohling and Wiren, 2006).

Blood samples for liver function tests were collected via cardiac puncture from animals that were deeply anesthetized using isoflurane as previously described (Wiren et al., 2004). Samples were rapidly transferred to heparinized tubes, mixed briefly and then 100 μ l of sample loaded onto VetScan Mammalian Liver Profile rotors and analyzed using a VetScan VS2 chemistry, electrolyte, immunoassay and blood gas analyzer (Abaxis, Union City, CA, USA).

2.3 Assessment of Brain Water Weight

Brains were harvested from animals following cervical dislocation, the cerebellum was removed and the remainder of the brain was hemisphered, with each half placed into a piece of tin foil and then weighed. The brains were heated overnight in a 100° C oven (approximately 20 h) and then re-weighed. The loss in brain weight divided by the starting weight of the brain was transformed to a percentage to determine the fraction of the brain that was water (Thal et al., 2013).

2.4 Glutathione measurement

Brains were removed and briefly placed in ice-cold isotonic buffer. The medial frontal cortex (mFC) was dissected with a coronal slice $\approx$ 2 mm deep to include tissue +2.0 mm from Bregma to the front of the brain (after removal of olfactory bulb and tubercle) and 2.0 mm lateral from midline. The dissection includes: frontal association cortex, prelimbic cortex, infralimbic cortex, rostral portions of secondary motor cortex, anterior cingulate cortex, primary motor cortex, medial, ventral and lateral orbital cortex, agranular insular cortex and dorsolateral orbital cortex. The hippocampus was exposed by gently peeling back the cortex to reveal the midbrain. The hippocampus and all sub-regions including CA1, CA2, CA3 and the dentate gyrus was collected using tweezers. Glutathione was measured in mFC and hippocampus including all sub-regions noted above using a plate based assay (Cayman Chemical #703002; Ann Arbor, MI). Tissue was deproteinated and processed as described in the manufacturer's protocol.

2.5 Statistical Analysis

Prism v6.04 (Graphpad Software, Inc., La Jolla, CA) or IBM SPSS Statistics 22 (IBM Corporation, Armonk, NY, USA) was used for ANOVA, and for unpaired *t*-tests with Sidak corrections for multiple comparisons. Huynh-Feldt corrections were used in repeated measures ANOVAs to correct for violations of sphericity. Data is presented as mean $\pm$ standard error of the mean (SEM).

3. Results

3.1 Alcohol Exposure and body weight

BECs were measured daily throughout the three-day alcohol exposure (Figure 1) and are presented for the animals used in the blood chemistry tests. The aggregate BEC values for all animals are presented in supplemental figure S1. A mixed-model ANOVA with day as a within-subject factor and sex as a between subject factor indicated a main effect of sex ($F_{1,8} = 2.40$, $p < 0.05$), with no day or day $\times$ sex interaction (p 's > 0.10). Over all treatment days males had an average BEC of 2.64 ± 0.18 mg/dl ($n = 5$) and females had an average BEC of 2.11 ± 0.07 mg/dl ($n = 6$).

Body weights ranged from 17.9 – 30.3 g at initiation of the experiment, with a mean body weight of 23.2 ± 0.3 g for the female control group, 26.7 ± 0.6 g for the male control group, 23.9 ± 0.3 g for the female alcohol group, and 26.1 ± 0.8 g for the male alcohol group. Body weights were not different between the control and alcohol-treated groups for either males ($n = 17$ control, $n = 19$ EtOH-treated) or females ($n = 18$ control, $n = 21$ EtOH-exposed; t-tests, p 's > 0.05). Changes in body weights were analyzed over the course of the alcohol exposure paradigm (Supplemental Figure S2). A mixed effects ANOVA with day as a within-subject factor and alcohol treatment and sex as between subject factors indicated significant effects of day ($F_{1,88,133.70} = 114.24$, $p < 0.001$), sex ($F_{1,71} = 8.00$, $p < 0.001$), a day $\times$ sex interaction ($F_{1,88,133.70} = 10.04$, $p < 0.001$), and a day $\times$ sex $\times$ alcohol treatment interaction ($F_{1,88,133.70} = 3.25$, $p < 0.05$) on body weight as a percent of starting weight. In general, males weighed more than females and weights decreased throughout the course of the experiment. Post-hoc t-tests of alcohol effects by day independently in males and females indicated no significant effects of alcohol (p 's > 0.05).

3.2 No evidence of brain swelling in females following alcohol exposure

One symptom of hepatic encephalopathy is brain swelling, or increased water content. A two-way ANOVA of brain water weight indicated no treatment or sex $\times$ treatment interactions (p 's > 0.05), but a significant main effect of sex ($F_{1,26} = 7.80$, $p = 0.01$). Average water content was higher in males regardless of treatment condition (all females: $78.3 \pm 0.1\%$; $n = 7$ control, $n = 9$ EtOH-exposed, all males: $78.7 \pm 0.1\%$; $n = 6$ control, $n = 8$ EtOH-exposed). The apparent difference in water content between males and females was the result of very low variability in the samples.

3.3 Modest changes in blood factors in both females and males following alcohol exposure

As there was no evidence of brain swelling, a liver function analysis of blood factors was then carried out to examine the impact of alcohol vapor inhalation on measures of liver function and damage. These panels included tests across a wide range of blood factors known to be influenced by liver dysfunction or damage (Gonzalez et al., 2011). Two-way ANOVAs with sex and alcohol treatment group as within subject factors were used to analyze blood factors. Blood levels of cholesterol (Fig. 2A.) were increased following alcohol exposure and withdrawal as there was a significant main effect of alcohol treatment group ($F_{1,17} = 12.55$, $p < 0.01$; female $n = 5$ control, $n = 6$ EtOH-exposed; male $n = 5$

control, $n = 5$ EtOH-exposed), with no effect of sex or sex $\times$ alcohol treatment group interaction (p 's > 0.10). Albumin levels (Fig. 2B) were also increased in alcohol-exposed animals ($F_{1,17} = 14.90$, $p < 0.01$), with no effect of sex ($F_{1,17} = 3.15$, $p = 0.09$), or sex $\times$ alcohol treatment group interaction ($F_{1,17} = 3.15$, $p = 0.09$). Factors that are commonly associated with alcohol metabolism or liver cirrhosis were next examined. For ALP (Fig. 3A), there was a significant main effect of alcohol treatment group ($F_{1,17} = 19.90$, $p < 0.001$), with no effect of sex or sex $\times$ alcohol treatment interaction (p 's > 0.10). Blood levels of ALT (Fig. 3B) in the alcohol-exposed animals were increased (main effect of alcohol treatment group $F_{1,17} = 4.80$, $p < 0.05$), with no effect of sex or sex $\times$ alcohol treatment interaction (p 's > 0.10). Total bilirubin levels (Fig. 3C) were reduced following alcohol exposure as evidenced by a significant main effect of alcohol treatment group ($F_{1,17} = 8.36$, $p < 0.05$) with no effect of sex ($F_{1,17} = 4.26$, $p = 0.05$) or sex $\times$ alcohol treatment group interaction ($p > 0.10$). Blood urea nitrogen (Fig. 3D) was reduced following alcohol as indicated a significant main effect of alcohol treatment ($F_{1,17} = 11.99$, $p < 0.01$) on blood urea nitrogen concentration, but no effect of sex or sex by alcohol treatment interaction (p 's > 0.10). Also measured was gamma glutamyl transferase (GGT) and bile acids, however, all measurements were either near, or below the level of detection for these analytes.

3.4 Reduced brain glutathione levels in both females and males following alcohol exposure

Alcohol is also known to induce oxidative stress throughout the body, including in the brain (Tiwari and Chopra, 2012). Therefore, levels of glutathione were examined in the mFC and hippocampus of males and females to determine whether oxidative stress was observed in the brain following alcohol exposure. Glutathione is present in high concentrations in all body organs, but particularly so in the brain, where it is a primary defense against reactive oxygen species (Dringen, 2000). A two-way ANOVA indicated alcohol exposure reduced glutathione levels in the mFC (Fig. 4A) as indicated by a significant main effect of alcohol treatment ($F_{1,12} = 30.96$, $p < 0.001$) with no effect of sex or sex $\times$ alcohol treatment interaction ($n = 4$ control females, $n = 4$ EtOH-exposed females, $n = 4$ control males, $n = 4$ EtOH-exposed males). Glutathione levels were also reduced in the hippocampus (Fig. 4B) following alcohol as evidenced by a significant main effect of alcohol treatment ($F_{1,20} = 15.45$, $p < 0.001$) with no effect of sex or sex $\times$ alcohol treatment interaction ($n = 6$ control females, $n = 6$ EtOH-exposed females, $n = 6$ control males, $n = 6$ EtOH-exposed males).

4. Discussion

Hepatic encephalopathy is a severe and potentially deadly disorder associated with liver dysfunction sometimes due to chronic alcohol abuse. The contribution of hepatic encephalopathy to alcohol-induced brain damage in the alcohol vapor inhalation model has not been examined despite previous studies using this paradigm demonstrating damage and behavioral alterations (Hashimoto and Wiren, 2008, Wilhelm et al., 2015b, Kliethermes et al., 2004). The results presented here indicate that hepatic encephalopathy is not a likely contributor to alcohol-induced neurodamage in a 72 hr vapor intoxication model of alcohol exposure that is commonly used to rapidly induce alcohol dependence.

Animal models of hepatic encephalopathy report very large increases of roughly an order of magnitude in serum levels of the liver factors ALT and total bilirubin as well as significant brain swelling (McMillin et al., 2014). In the present study, ALT levels were elevated but marginal effects were observed when males or females were analyzed separately. Alanine aminotransferases are released into the circulation in proportion to the level of liver damage (Daxboeck et al., 2005), thus this model of alcohol exposure appears to exert relatively mild effects on the liver. Furthermore, in contrast to a rise in total bilirubin with hepatic encephalopathy, males exhibited a significant reduction in total bilirubin. Similarly, humans with hepatic encephalopathy exhibit significant increases in blood urea nitrogen (Morgan et al., 1995), but blood urea nitrogen levels were lower following alcohol exposure. Females had elevated blood levels of ALP following alcohol exposure, while levels in males did not achieve a statistically significant increase, despite higher blood levels of alcohol attained in males. Finally, changes in brain water weight were not associated with neurotoxicity as brain weight was unchanged following alcohol exposure (Hashimoto and Wiren, 2008, Wilhelm et al., 2015b). Taken together, several measures associated with encephalopathy indicate a very modest, or lack of effect in this model of alcohol exposure.

Despite a lack of evidence of hepatic encephalopathy, levels of the antioxidant glutathione were reduced in the brains of both male and female mice following alcohol exposure. This likely indicates increases in oxidative stress following alcohol exposure, although other explanations are also possible. Treatment of neonatal rats with the antioxidant vitamin E attenuates alcohol-induced damage to the hippocampus (Heaton et al., 2000), suggesting that oxidative stress may be a primary mechanism driving alcohol-induced brain damage. The specific source of reactive oxygen species generation is currently unknown, however, astrocytes, microglia, neurons and even endothelial cells may contribute to alcohol-induced oxidative stress in the brain (Qin and Crews, 2012, Montoliu et al., 1995, Haorah et al., 2005, Haorah et al., 2008). To maintain consistency across dissections, a single experimenter performed all brain dissections, however because multiple sub-regions of the mFC and hippocampus were included in glutathione analyses, some differences in glutathione content may be due to variability among dissections. Increased understanding of the direct effects of alcohol on glutathione levels and oxidative stress in the brain will be an important step toward prevention of alcohol-induced neurotoxicity.

Based on these combined observations, our data suggest that the alcohol vapor inhalation paradigm does induce some hepatic damage, but there was no evidence to suggest the level of hepatotoxicity was sufficient to induce encephalopathy in alcohol-induced neurodamage following a three-day alcohol vapor inhalation paradigm. It should be noted, however, that long-term administration of pyrazole is hepatotoxic, particularly when combined with alcohol (Kalant et al., 1972). Thus, elevations in liver factors may be due to the combined effects of alcohol and pyrazole. The increased vulnerability of females to neurotoxicity observed in this paradigm after 72 h of exposure and a single withdrawal is thus more likely a result of direct alcohol action on cell types in the brain. We have previously demonstrated increased proinflammatory pathways (Hashimoto and Wiren, 2008, Wilhelm et al., 2015a, Wilhelm et al., 2014) and altered glucocorticoid signaling and glutamate homeostasis (Wilhelm et al., 2015b) in female mFC and primary astrocyte cultures following alcohol exposure. These results highlight important sex-specific pathways regarding mechanisms

underlying the detrimental health consequences associated with alcohol abuse. Given the increased rate of death of female alcohol abusers relative to male alcohol abusers (John et al., 2013, Roerecke and Rehm, 2013), further characterization of sex-specific responses are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Symptoms of hepatic encephalopathy were examined in mice following a 72 hour alcohol vapor inhalation procedure.
- Elevated blood levels of cholesterol, albumin, alkaline phosphatase and alanine aminotransferase were observed in alcohol-exposed mice.
- Reduced blood levels of blood urea nitrogen and total bilirubin were observed in alcohol-exposed mice.
- Reduced hippocampal and medial frontal cortex glutathione levels were observed following alcohol exposure.
- Alcohol-exposed mice exhibited signs consistent with liver damage, but not hepatic encephalopathy.

Figure 1.

Blood alcohol levels by day. Blood alcohol concentrations (BECs) were measured daily during the alcohol vapor exposure paradigm ($n = 6$ female EtOH, $n = 5$ male EtOH). Males generally had higher BECs than females. * $p < 0.05$ (main effect of sex) compared to female EtOH.

Figure 2.

Modest changes in blood levels of cholesterol and albumin following alcohol (EtOH).

Alcohol exposure lead to significant increases in blood cholesterol and albumin levels. ** p < 0.001 (main effect of alcohol treatment) compared to control; (n = 5 female Con; n = 6 female EtOH, n = 5 male Con; n = 5 male EtOH)

Figure 3.

Changes in blood liver factors following alcohol (EtOH) exposure. ANOVAs indicated significant increases in alkaline phosphatase (AP), alanine aminotransferase (ALT) and reductions in total bilirubin and blood urea nitrogen following alcohol (EtOH) treatment. No error bar is apparent for the female alcohol group total bilirubin measure because all samples in this group were at the 0.2 mg/dl level. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (main effect of alcohol treatment) compared to control; (n = 5 female Con; n = 6 female EtOH, n = 5 male Con; n = 5 male EtOH).

Figure 4. Alcohol- (EtOH) induced reductions in mFC and hippocampal glutathione. Levels of the key brain antioxidant glutathione were reduced in the mFC (n = 4 female Con; n = 4 female EtOH, n = 4 male Con; n = 4 male EtOH) and hippocampus (n = 6 female Con; n = 6 female EtOH, n = 6 male Con; n = 6 male EtOH) following alcohol exposure. *** p < 0.001 (main effect of alcohol treatment) compared to control.