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Brief communication

A neurotoxic alcohol exposure paradigm does not induce hepatic encephalopathy

Joel G. Hashimoto [a,b], Kristine M. Wiren [a,b], Clare J. Wilhelm [a,c,*]

[a] Research & Development Service, VA Portland Health Care System, Portland, OR, USA
[b] Department of Behavioral Neuroscience, Oregon Health & Science University, Portland, OR, USA
[c] Department of Psychiatry, Oregon Health & Science University, Portland, OR, USA

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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 h 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.

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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

* Corresponding author at: VA Portland Health Care System (R&D 17), 3710 SW US Veterans Hospital Rd., Portland, OR 97239, USA.
E-mail address: wilhelmc@ohsu.edu (C.J. Wilhelm).

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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 neuroadapations 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 ± 1 °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 μL 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 ≈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 were 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 ± standard error of the mean (SEM).