Short Communication

Acute ethanol exposure does not significantly alter cytoskeletal integrity in C6 glioma cells

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Abstract

Prolonged ethanol abuse has been associated with brain injury. In rodents, postnatal exposure to ethanol has been shown to be a major contributing factor to neurodegeneration in the hippocampus and cortex, leading to deficits in synaptic function and memory. Among the potential mechanisms involved in ethanol-induced brain damage, oxidative stress is considered a primary factor. However, the molecular mechanisms underlying ethanol-induced neurotoxicity remain incompletely understood. Evidence suggests that ethanol impairs the functions of both the cytoskeleton and cellular membranes, resulting in alterations to neuronal physiology. The plasma membrane of eukaryotic cells contains microdomains enriched in specific glycosphingolipids, gangliosides, and cholesterol, collectively forming membrane/lipid rafts (MLRs). As demonstrated in previous studies, MLRs function as scaffolds for a variety of molecular entities, including signaling receptors and ion channels. In addition, they mediate the organisation of the cytoskeleton. Indeed, numerous cytoskeletal components, their binding partners, and enzymes that regulate cytoskeletal dynamics localise to MLRs and contribute to the regulation of the lateral diffusion of membrane proteins and lipids in response to extracellular stimuli. The objective of the present research was to examine the effects of ethanol on the cytoskeleton, specifically actin and tubulin, in C6 glioma cells.

Keywords: Ethanol; neurotoxicity; cytoskeleton; C6 glioma cells; tubulin; oxidative stress

1. Introduction

Ethanol is one of the most widely abused psychoactive substances worldwide and represents a major cause of neurological impairment and neurodegeneration. As demonstrated in the seminal study by (Vallés et al., 2004), chronic alcohol consumption has been demonstrated to result in alterations in brain structure and function. Furthermore, (Weil et al., 2018) has shown that, in certain cases, these alterations can result in brain injury. Furthermore, the manner in which exposure to ethanol occurs is a pivotal factor. The harmful effects of binge drinking are significantly more pronounced than those resulting from continuous exposure to low concentrations (Reddy et al., 2013). It has also been demonstrated that the effects of ethanol are not confined to neurons but also extend to glial cells (Marshall, 2021). In this context, it has been demonstrated that ethanol can promote the formation of reactive oxygen species (ROS) and modulate nitric oxide synthase (NOS) activity. These processes have been shown to be closely linked to alcohol-induced oxidative damage and neurodegeneration. These effects have also been observed in glial cells (Vallés et al., 2004). Oxidative stress is regarded as one of the principal mechanisms involved in ethanol-induced neuronal damage (Alfonso-Loeches & Guerri, 2011; Nordmann et al., 1992). As demonstrated by numerous studies, the presence of ethanol has been shown to induce alterations in membrane fluidity, cytoskeletal organisation, and intracellular signalling pathways (Luo, 2012; Maturu et al., 2010). C6 glioma cells are frequently employed as an in vitro model for astrocyte-like glial cells, offering a valuable system for investigating the molecular mechanisms underlying neurotoxicity (Galland et al., 2019). The objective of this study was to assess the effects of acute ethanol exposure at varying concentrations on the organization of actin and tubulin in C6 glioma cells, employing Western blotting and Fluorescence recovery after photobleaching (FRAP) analysis.

2. Materails and Methods

C6 cells in flasks were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Hyclone Laboratories, Logan, UT, USA), at 37°C in humidified 5% CO2 atmosphere.

The cells were treated with different ethanol dilution (10μM, 20μM, 35μM, 50μM) (ethanol 200 proof, Decon Laboratories, Inc.) for 3 hours. Only for 20µM cells were treated at different time (1h, 3h, 6h, 24h). Following ethanol exposure, C6 cells were placed on

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37°C for 3h. C6 cells were scraped in PBS. The cellular material was homogenized and centrifuged at low speed (1000xg for 3 min at 4°C) to precipitate nuclear material. From supernatant was removed 300µl and sonicated (5% x10s). The sonicated material was centrifuged at low speed (1000xg for 3 min at 4°C). The sonicated resulting was used immediately for SDS-pages for cells treated with different dilution.

Equal samples quantity was loaded onto Stain-Free acrylamide gel for SDS-PAGE (Bio-Rad, Hercules, CA, USA). Only for 20μM cells, duplicates were made to treat with two different antibodies. Gels were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA USA) for western blotting. blocked with 5% non-fat dry milk diluted in TBS-T (10mM Tris-HCl, 159mM NaCl, and 0.1%, Tween 20, pH 7.4) for 1h. After incubation, the blot was washed twice for 5min with Tween-20/Ponceau Stain (T-TBS/Ponceau Stain) (Sigma) and then incubated with Anti-acetylated-tubulin mice (1:5000) (Sigma) and Anti-α-tubulin mice (1:5000) (Sigma) for 3h at 4°C. The blot was washed with TBS-T and incubated with a secondary antibody(1:20000).

[peroxidase-conjugated rabbit anti-mouse IgG]. The blot was washed 3-times for 5 min with TBS-T. The blot was developed using a chemiluminescence ECL kit (Amersham, Oakville, Ontario). ECL Luminata Forte chemiluminescent reagent (Millipore, Billerica, MA, USA). Blots were imaged using a Chemidoc computerized densitometer (Bio-Rad, Hercules, CA, USA) and quantified by ImageLab 6.0 software (Bio-Rad, Hercules, CA, USA).

C6 glioma cells stably transfected with GFP-Gas were plated on glass microscopy dishes and treated with ethanol (10μM, 20μM, 35μM, 50μM) for 3 hours. On the time of imaging, ethanol was washed out 1 h prior to imaging and media was replaced with low serum (2.5% NCS) phenol red-free DMEM to reduce background fluorescence. Temperature was maintained at 37°C using a heated stage assembly during imaging on a Zeiss LSM 710 META at 512×512 resolution using an open pinhole to maximize signal but minimize photobleaching. Total of 150 data points 300 ms apart, including 10 pre-bleach values, were measured for each cell. Zeiss Zen software was used to calculate FRAP recovery half-time utilizing a one-phase association fit, correcting for total photobleaching of the analyzed regions.

3. Results

Western blot analysis demonstrated a modest decrease in acetylated α-tubulin following exposure to ethanol at varying concentrations (ranging from 10 to 50 μM). However, these alterations were not statistically significant (**Fig. 1**). Cells treated with 20 μM of ethanol at different times, although not statistically significant, demonstrated a slight decrease in tubulin at 3 hours, followed by an increase at 6 hours that remained stable at 24 hours (**Fig. 2**).

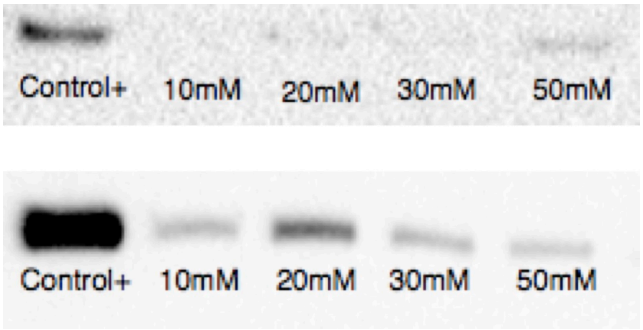

Figure 1 Effect of ethanol on α-tubulin (top) and acetylated α-tubulin (bottom) expression in C6 glioma cells.

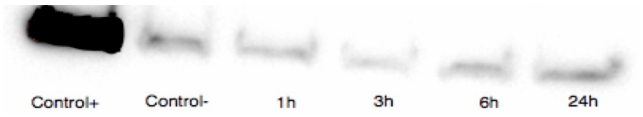

Figure 2 Time-course analysis of α-tubulin expression following 20 mM ethanol exposure.

Immunocytochemical analysis using an anti-GFAP antibody demonstrated that in cells exposed to ethanol for a maximum of three hours, the cytoskeletal network remained unaltered when compared to the control group (**Fig. 3**). This finding suggests a relative resistance of the cytoskeletal network to short-term ethanol treatment.

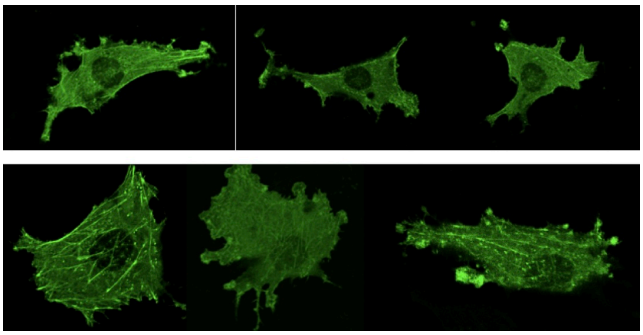

Figure 3 Immunofluorescence analysis of GFP-actin organization in C6 cells exposed to ethanol for 3 hours. [Actin-gfp control (top), Actin-gfp ethanol treatment (bottom)].

4. Discussion

The present study investigated whether acute ethanol exposure could induce detectable alterations in the cytoskeletal organization of C6 glioma cells. Although previous studies reported ethanol-induced cytoskeletal remodeling and oxidative stress in neural cells, our findings indicate that short-term exposure to ethanol concentrations up to 50 mM does not significantly disrupt tubulin organization or GFAP distribution (Alfonso-Loeches & Guerri, 2011; Guerri & Pascual, 2010; Luo, 2012). Previous studies reported actin

disorganization and altered signaling pathways following prolonged ethanol exposure (Gao et al., 2024; Romero et al., 2010). The discrepancy with our findings may depend on differences in exposure duration, ethanol concentration, or adaptive cellular responses (Crews & Nixon, 2009; He & Crews, 2008). Overall, our data suggest that the cytoskeleton of C6 glioma cells exhibits relative resistance to acute ethanol exposure, although subtle molecular changes cannot be excluded. Similar observations have been reported in studies showing that acute ethanol treatment often induces biochemical and signaling alterations before overt cytoskeletal disruption becomes detectable by conventional imaging approaches (Alfonso-Loeches & Guerri, 2011; Pandey et al., 2008).

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Conflict of Interest

The authors declare no competing interests.

Data Availability

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

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