The Neurotoxic Effects of Ethanol and Pesticides in the Central Nervous System

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Introduction

In our society, there is a high prevalence of alcohol (ethanol) consumption and abuse. In addition, paraquat, a herbicide, is also perversely used throughout the world. Therefore, it is very likely for people who drinks to be co-exposed to both ethanol and paraquat. There have been studies demonstrating that paraquat is associated with the neuronal loss in Parkinson’s disease [1]. This study aims to investigate the possible mechanisms of how ethanol and paraquat induce neurotoxicity and how ethanol and paraquat modulate the pro-inflammatory activation state (M1 state) of microglia, the primary innate immune cells in the brain. We chose PC12 cell line as the neuronal cell model, and BV2 cell line as the microglial cell model. Our hypotheses are:

1. Ethanol and paraquat interaction may decrease the viability of neuronal cells.
2. Ethanol and paraquat interaction may lead to significant neuronal apoptosis.
3. Ethanol and paraquat interaction may decrease the viability of microglia, compromising normal inflammatory responses.
4. Ethanol and paraquat may enhance M1 microglia activation resulting in an increased generation of pro-inflammatory cytokines and toxic intermediates.

Methods

Cell Culture and Treatments

PC12 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum and 5% heat-inactivated horse serum. Cells were then plated overnight and treated in the next day with serum-containing DMEM (control) and 100 mM paraquat, 100 mM ethanol or 100 mM ethanol + 100 μM paraquat.

BV2 cells were cultured in DMEM with 10% fetal bovine serum with 100 μg/ml Penicillin, 100 μg/ml Streptomycin and 250 μg/ml Amphotericin. Cells were then plated overnight and treated in the next day with phenol-red-free, serum-free DMEM (control) and 33 mM paraquat, 100 mM ethanol or 100 mM ethanol + 33 mM paraquat.

All cells were cultured at 37°C and in atmospheric pressure with 5% CO2.

Tetrazolium Dye (MTT) Assays for PC12 and BV2 Cells

Cell viability was measured by MTT assays. All samples were measured in duplicates. Cell media were first removed from wells and rinsed with 500 μl of phosphate buffered saline (PBS) solution. 40 μl of serum-free, phenol red-free DMEM containing 0.5 mg/ml of MTT was added to each well. Samples were then incubated at 37°C for 2 hr. The supernatant in each well was then removed. 500 μl of isopropanol, 0.4 M HCl solution was then added into each well. After 5 minutes of incubation at room temperature, each well was titrated and the media centrifuged at 13,000 g for 5 minutes. Absorbance of the supernatant was measured at 540 nm.

Casparase Assays for PC12 Cells

Caspase activity was determined by measuring the hydrolysis of DEVD-Amc. All samples were measured in duplicate. Cell media were first removed and the plates were then gently rinsed with PBS solution.

(MTT assay cont.) After 500 μl of lysis buffer (25 mM Heps, 1 mM EGTA, 5 mM EDTA, 5 mM MgCl2) was added on each plate, all contents of each plate were transferred to separate Eppendorf centrifuge tubes, sonicated for 10 seconds and then incubated in ice for 20 minutes. The tubes were then spun at 16,000 g for 20 minutes. An aliquot (400 μl) of the supernatant from each tube was then mixed with 800 μl of assay buffer (25 mM Heps, 10 mM DTT, 15% w/v sucrose, 0.15% chaps). We then took 300 μl from each tube and mixed them with 5 μl of DEVD substrate for fluorescence measurement. Fluorescence in each sample was measured every 10 minutes for 60 minutes (Excitation: 360nm, Emission:460nm). The results were expressed as fluorescence/min/mg of protein.

Nicotine Oxide Assay for BV2 Cells

Cells were treated with serum-free DMEM (control) containing 33 μM paraquat alone, 100 mM ethanol alone, 100 mM ethanol + 33 μM paraquat, 0.5 μg/ml Lipopolysaccharide (LPS), or 0.5 μg/ml LPS + 100 mM ethanol. 50 μl of supernatant was combined with 50 μl of sulfanilamide solution (1% sulfanilamide in 5% phosphoric acid), and samples were then incubated at room temperature for 5 minutes, protected from light. Following the incubation, 50 μl of NED solution (0.1% N-1-ethyl-3-(3-dimethylaminopropyl)carbodimide hydrochloride) was added, and samples were incubated again at room temperature for 5 minutes, protected from light. Absorbance was then measured at 540 nm.

Figure 1. Ethanol Enhances Paraquat-induced Neurotoxicity at 48 hour (bottom panel) but not 24 hour (top panel).

Figure 2. Paraquat-induced Increase in Caspase Activity is Enhanced by Ethanol at 48 hour (bottom) but not 24 hour (top).

Figure 3. Ethanol Does not Alter Paraquat-induced Toxicity.

Figure 4. Paraoquat Decreases BV2 Cell Number.

Figure 5. Microglia M1 activation level measured by nitric oxide assay. Cells were treated for 24 hours prior to experiment. LPS-treated cells served as positive control; ethanol reduces LPS-stimulated M1 microglial activation. Data are representative of four separate experiments. Nitrite concentration is directly related to the nitric oxide concentration in each well. Higher nitrite level indicates higher level of microglia M1 activation.

Conclusions

- Although both ethanol and paraquat reduce PC12 cell viability, the temporal responses differ.
- At 48 hours, ethanol and paraquat synergistically interact to decrease the viability of the neuronal-like PC12 cells.
- Ethanol and paraquat synergistically interact to increase neuronal apoptosis as indicated by caspase activity.
- Although paraquat reduces the viability of BV2 microglia, there was no synergistic interaction of ethanol and paraquat on viability of the microglia.
- Ethanol and paraquat alone do not trigger M1 activation of microglia.
- Ethanol impairs the LPS-induced M1 activation, possibly compromising microglia’s normal immune response to pathogens.

References