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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 pervasively 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 100μM paraquat, 100mM ethanol or 100 mM ethanol + 100 μM paraquat.

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

All cell were cultured at 37ºC and in atmospheric pressure with 5% v/v CO_2.

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 500μL of phosphate buffered saline (PBS) solution. 400μL of serum-free, phenol red free DMEM containing 0.5mg/mL of MTT was added to each well. Samples were then incubated at 37ºC for 2 hr. The supernatant in each well was then removed. 500μL of Isopropanol/0.04M HCl solution was then added into each well. After 5 minutes of incubation at room temperature, each well was triturated and the media centrifuged at 13kg for 5 minutes. Absorbance of the supernatant was measured at 540nm.

Caspase 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 500μL of lysis buffer (25mM hepes, 1mM EGTA, 5mM EDTA, 5mM $MgCl_2$) was added on to 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 16kg for 20 minutes. An aliquot (400μL) of the supernatant from each tube was then mixed with 800μL of assay buffer (25 mM Hepes, 10mM DTT, 15% w/v sucrose, 0.15% chaps). We then took 300μL from each tube and mixed them with 5μL 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.

Nitric Oxide Assay for BV2 Cells

Cells were treated with serum-free DMEM (control) containing 33 μM paraquat alone, 100mM ethanol alone, 100mM ethanol + 33μM paraquat, 0.5μg/ml Lipopolysaccharide (LPS), or 0.5 μg/ml LPS + 100mM ethanol. 50μL of supernatant was combined with 50μL 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, 50μL of NED solution (0.1% N-1-napthylethylenediamine dihydrochloride) was added, and samples were incubated again at room temperature for 5 minutes, protected from light. Absorbance was then measured at 540nm.

Results

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

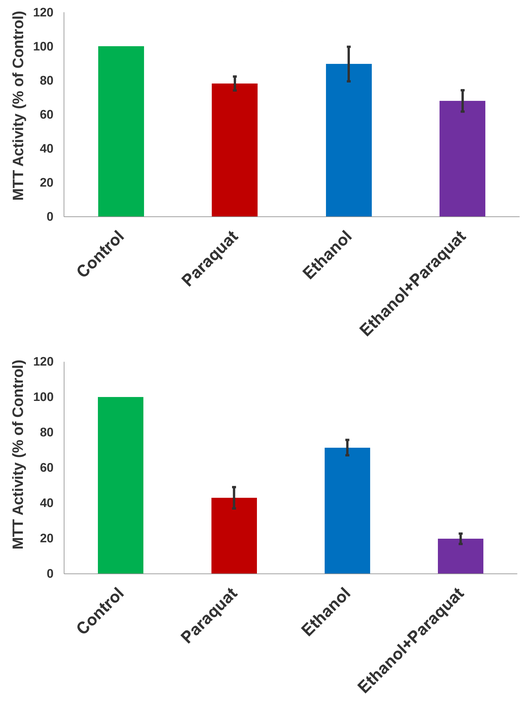

Figure 1. MTT activity in PC12 Cells treated with paraquat in the absence and presence of ethanol. Cells were treated with 100 μM paraquat, 100 mM ethanol, or 100 mM ethanol + 100 μM paraquat for 24 hr (top) or 48 hr. (bottom) Data are expressed as relative percentage of control and are expressed as mean ± S.D. (N=6-8). Low MTT reduction indicates low cell viability.

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

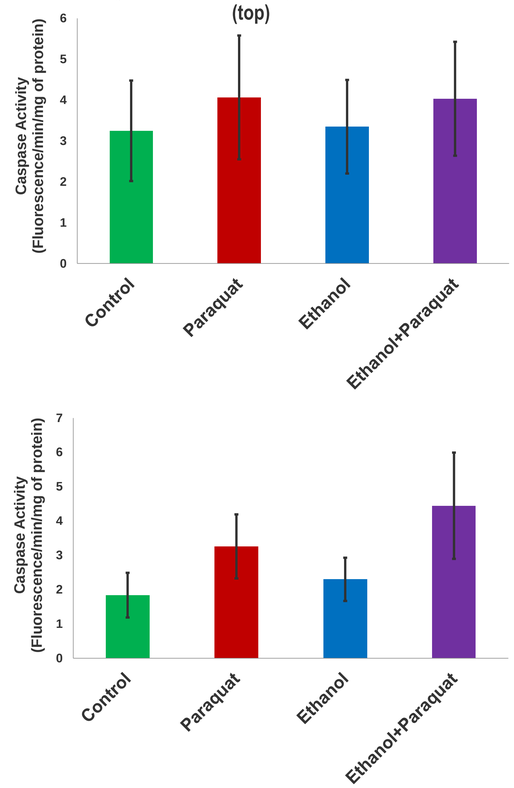

Figure 2. Caspase Activity in PC12 Cells After 24-hour (top) and 48-hour (bottom) of Treatment. Cells were treated with 100 μM paraquat, 100 mM ethanol, or 100 mM ethanol + 100 μM paraquat for 24 hr (top) or 48 hr (bottom). Results are expressed as mean ± S.D. (N=4-7). Higher fluorescence activity indicates higher caspase activity in the samples.

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

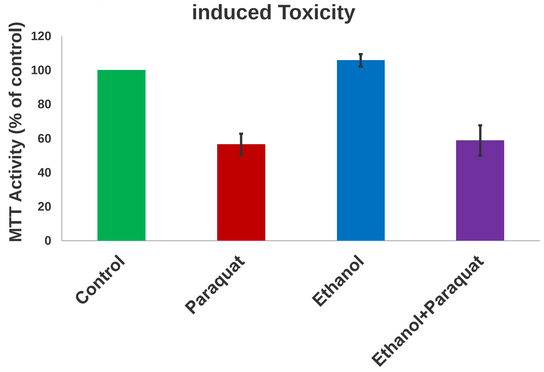

Figure 3. MTT activity in BV2 Cells treated with paraquat in the absence and presence of ethanol. Cells were treated with 33 μM paraquat, 100 mM ethanol, or 100 mM ethanol +33 μM paraquat for 24 hours. Data are presented as relative percentage of control and are expressed as mean ± S.D. (N=4-8). Low MTT reduction represents low cell viability.

Fig.4. Paraquat Decreases BV2 Cell Number

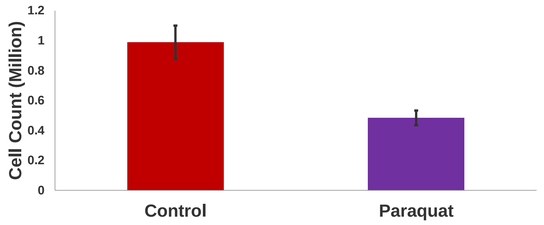

Figure 4. Cell Count of BV2 Cells treated with 33 μM paraquat for 24 hours. Cells were counted in hemocytometer and expressed as mean ± S.D. (N=6). This figure confirmed the observation found in MTT assays.

Fig. 5. Neither Ethanol nor Paraquat Alter NO in BV2 Cells

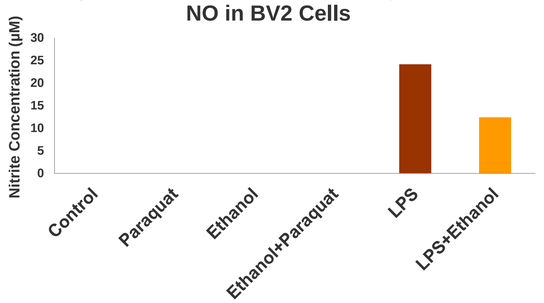

Figure 5. Microglia M1 activation level measured by nitric oxide assay. Cells were treated for 24 hours prior to experiment. LPS-treated cells served as positive control; ethanol reduces LPS-stimulated M1 microglial activation. Data are representative of four separate experiments. Nitrite concentration 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

1. Berry, C. et al. Paraquat and Parkinson's Disease. *Cell Death and Differentiation* (2010) **17,** 1115–1125