Synergistic Genotoxicity of Alcohol and Benzo[a]pyrene

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Abstract

Alcohol use accounts for about 6% of all cancers and 4% of all cancer deaths in the United States according to American Cancer Society; it has been linked with several cancers, including those of the mouth, liver, and breast. The underlying mechanisms for alcohol carcinogenesis are still unclear. Multiple factors can be at play: alcohol is metabolized into acetaldehyde, a genotoxic chemical that can cause cancer in laboratory animals; and/or that alcohol increases reactive oxygen species (ROS), leading to DNA damage and lipid peroxidation, increasing the risk of cancer. However, alcohol has not been proven genotoxic. Although synergistic effects of combining drugs and alcohol have been widely described, their combination relative to genotoxicity endpoints has not been previously reported. In this study, HepG2 cells were treated with different concentrations of alcohol and benzo[a]pyrene (B[a]P) separately or in combination. Genotoxicity was evaluated using the Micronucleus Assay, the Mouse Lymphoma Assay, and the measurement of ROS to explore possible synergistic genotoxicity when combining alcohol and genetic agents. Co-treatment of 50mM alcohol with 1 or 5uM B[a]P induced significantly higher ROS than either chemical alone. Micronucleus Assay results showed a higher genotoxic sensitivity of the combination, 25mM alcohol with 1uM B[a]P inducing significantly higher micronucleus formation than 1uM B[a]P alone. Increased concentrations of B[a]P and alcohol resulted in increases in MN in a dose-dependent manor. In the Mouse Lymphoma Assay, EtOH alone did not cause a 2-fold increase in total mutation frequency (MF) over control while B[a]P alone caused a 9.3-fold increase in MF. Co-treatment of 25mM EtOH and 5uM B[a]P increased MF by 11.4-fold and co-treatment of 50mM EtOH and 5uM B[a]P caused a 14.2-fold increase in MF above control. The same trend was observed when looking at both small colony and large colony MF individually for these groups. As in previous reports, the combination of alcohol and B[a]P caused a significant increase in lipid staining in HepG2 cells compared to alcohol alone further demonstrating a synergistic ROS effect. Currently, enzyme modified Comet Assays are being performed to demonstrate specifically the possible effect the combination of alcohol and B[a]P has on the formation of DNA adducts and DNA damage. These preliminary results suggest that there is a synergistic genotoxic effect of alcohol and B[a]P, possibly via increasing total oxidative stress.

Introduction

Alcohol consumption and abuse is prevalent in the United States, the 2019 National Survey on Drug Use and Health reported that nearly 70% of adults had consumed alcohol in the past year and over 25% of adults reported that they engaged in binge drinking in the past month. Though alcohol is linked to multiple types of cancer, most notably oral, esophageal, liver, breast, and colorectal, the mechanism(s) have not been fully resolved. Metabolism through alcohol-dehydrogenase to the known genotoxic substance, acetaldehyde, may play a role, and that alcohol metabolism increases intracellular reactive oxygen species (ROS) which can cause DNA damage, along with protein and lipid peroxidation are also factors. Benzo[z]pyrene (B[a]P) is a carcinogenic polyaromatic hydrocarbon that results from combustion, commonly found in tobacco smoke and grilled/charred meats. Both of which are often consumed frequently with alcohol, B[a]P can also create DNA adducts as well as generate ROS to damage cells internally. The combination usage of alcohol and various other drugs, like cigarettes, has been widely reported upon, as have synergistic effects created from simultaneous usage. Specifically, genotoxicity endpoints have not been reported. Here we assessed the combination of alcohol and B[a]P in the flow-cytometry Micronucleus Assay and the Mouse Lymphoma Assay. We also measured ROS and examined Oil Red-O staining in HepG2 cells. Enzyme modified Comet Assays and Next Generation Sequencing are being currently performed to gain a more complete understanding of the combined effect of alcohol and B[a]P on the formation of DNA adducts, DNA damage, and mutagenesis.

Materials and Methods

ROS Measurement
Treated HepG2 cells were preincubated with DCF-DA based on manufacturer instructions (Invitrogen, Carlsbad CA, USA) and then analyzed using a BioTek Synergy2 plate reader (Winooski VT, USA) at 520nm.

Micronucleus Assay
Treated HepG2 cells were cleaved from their 96-well plates and processed with the In Vitro MicroFlow Kit (Litron Laboratories, Rochester NY, USA) per manufacturer instructions. Dyed treated cells were then processed through FACSAria (BioRad, Hercules CA, USA) Using FlowJo (Ashland OR, USA) software. All performed according to OECD-487 guidelines. Relative Survival is indicated by a red dashed line at 40% RS.

Oil Red-O staining
Treated HepG2 cells were rinsed with PBS then fixed in 4% formalin for 30 minutes. After fixation cells were covered in Oil Red Working solution (MilliporeSigma, St. Louis MO, USA) then rinsed twice with water. Counterstaining was performed after plates were dry with Gil's Hematoxylin Solution 2 (Santa Cruz Biotech, Dallas TX, USA).

Mouse Lymphoma Assay
Mouse Lymphoma cells were treated for 4 hours in the presences of rat S9 mixture then cultured in 96 well plates for 14 days. Small and Large colony mutant fractions were counted, and MF was determined. All conditions and calculations were performed using OECD-490 guidelines

Statistical analysis
Statistical analysis was performed using ANOVA, and $p < 0.05$ was used to identify statistically significant differences.

Results and Discussion

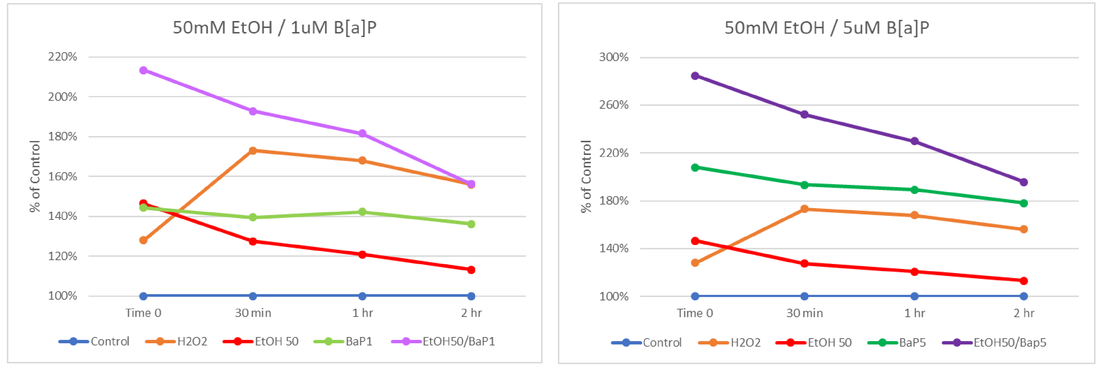

Figure 1. [Left] ROS measurement in HepG2 cells using DCF fluorescence Control, H2O2, B[a]P 1uM, EtOH 50mM, EtOH50mM/B[a]P1uM. [Right] ROS measurement using DCF fluorescence Control, H2O2, B[a]P 5uM, EtOH 50mM, EtOH50mM/B[a]P5uM

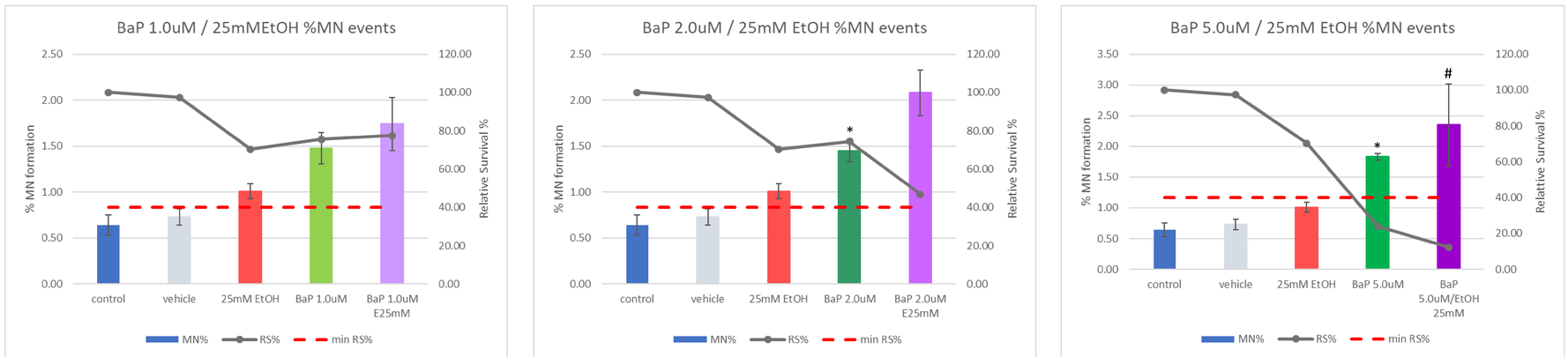

Figure 2. Flow cytometry-based Micronucleus Assay results from HepG2 cells treated for 24hrs with Vehicle, EtOH 25mM, B[a]P 1uM,2uM, or 5uM, and combination EtOH 25mM/B[a]P 1uM, 2uM, or 5uM.

Group	Total MF/10^{-6}	Small Colony MF	Large Colony MF
Control	87.5	13.3	74.0
EtOH 25mM	57.1	17.2	39.9
EtOH 50mM	141.9	46.3	95.6
B[a]P 5µM	817.2 *	442.2	375.0
EtOH 25mM/B[a]P 5µM	996.8	512.0	484.8
EtOH 50mM/B[a]P 5µM	1244.5#	678.3	566.2

Table 1. Mouse Lymphoma Assay results from ML cells treated with Vehicle, EtOH 25mM and 50mM, B[a]P 5uM, and combination EtOH 25mM or 50mM/B[a]P 5uM

Conclusion

Alone, alcohol and B[a]P caused an expected increase in ROS production over control. The combination of 50mM alcohol with either 1uM or 5uM B[a]P caused significant increases in ROS production over that chemical concentration alone. 25mM alcohol did not significantly increase formation of micronuclei, while concentrations of B[a]P as low as 1uM did. The co-treatment of 25mM alcohol and any concentration of B[a]P resulted in further significantly increased formation of micronuclei versus chemical alone. In the Mouse Lymphoma Assay, 5uM B[a]P treatment caused a 9.3-fold increase in mutation frequency, whereas co-treatment with 25mM alcohol caused an 11.4-fold increase and co-treatment with 50mM alcohol caused a 14.2-fold increase. These findings suggest that alcohol and B[a]P are acting synergistically to increase genotoxicity and findings from enzyme modified Comet Assays and Next Generation Sequencing will reveal further specifics about their combined impact on DNA.