The effects of antidepressant drug on ethanol-induced cell death

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ABSTRACT: Alcoholism is a serious health problem. Alcohol-dependent subjects have many health-related problems, such as severe cognitive impairments, alcoholic liver disease and coronary heart disease, resulting from ethanol-induced cell injury or cell death. Understanding the mechanisms underlying the cell death may provide clues for novel treatment strategies to prevent alcohol-induced cell damage. Prolonged ethanol consumption causes apoptotic activity in a host of cell types – more obviously affecting the liver, heart and surprisingly affecting the brain. This study uses four cell lines: neuronal cell line (SH-SY5Y), glia cell line (U-118 MG), liver cell line (E47) and heart cell line (the rat H9c2), and addresses that alcohol does, in fact, cause cell death in these four cell types, whether ethanol induced cell death is through apoptotic pathway, and whether an monoamine oxidase (MAO) inhibitor (*e.g.* deprenyl) protects cells from the effects of alcohol. We have found that ethanol exposure lowers cell proliferation in all cell types, but affects brain cell lines (neuron and glioma) the most, while ethanol and deprenyl exposure in unison increases cell viability largely in brain cells, and then in liver cells. Our results suggest that MAO-mediated apoptosis may contribute to ethanol-induced cell death. Individuals suffering from alcoholism or alcohol abuse may be treated with deprenyl to alleviate the apoptotic activity resulting from alcohol consumption and protect the body's cells from alcohol-induced death. In summary, this study demonstrates the effects of deprenyl as an anti-apoptotic agent against the detrimental effects of alcohol.

Key Words: Alcohol, neuroblastoma, glioma, cell culture, cell viability

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Introduction

Alcohol (ethanol) is one of the most commonly used drugs around the world and like all other drugs it has profound effects on the body. In particular, it affects the brain and alters its normal function (*1*). This includes altering the effects of neurotransmitters, suppressing nerve signals and causing cell death (*2*). In rodents, ethanol exposure during development significantly reduces the size of the brain as well as brain/body weight ratios (*3*). There are many adverse physical effects from long-term exposure to alcohol including the increased activity in the liver that cause cell death and hardening of the tissue (cirrhosis of the liver) (*4*) and an increase in the number of apoptotic cells in various brain areas (*5*). This study investigates the effects of ethanol on neuronal cell death, liver cell death and heart cell death. Furthermore, this investigation determines the effects of deprenyl on cell death.

Deprenyl (selegiline), an irreversible inhibitor of monoamine oxidase B (MAO B), was synthesized as an antidepressant and used to treat Parkinson's disease (*6*). Because MAO degrades serotonin and produces reactive oxygen that may cause cell death, an MAO inhibitor prevents cell apoptosis (*7-9*). Deprenyl or related compounds may be neuroprotective in general through the inhibition of "death" signal transduction-mediated by MAO, induced by endogenous and environmental factors (*10*). Deprenyl in low concentrations that induce MAO B inhibition (0.001 nM to 1.0 nM) potently inhibits serum withdrawal induced apoptosis in tissue cultures of neuro-ectodermal origin (*11*). These studies demonstrated that ethanol can induce apoptosis in both neuronal and non-neuronal cells and compared the effects of deprenyl on the protection of cells from the harmful effects of ethanol in cell lines-derived from neuron, glioma, liver and heart.

Materials and Methods

Cell lines

A human neuroblastoma cell line, SH-SY5Y, a human glioma cell line, U-118 MG, and a rat heart cell line,

H9c2, were purchased from The American Type Culture Collection (ATCC). A human liver cell line, E47, is a gift from Dr. Arthur Cederbaum, Mount Sinai School of Medicine, New York. E47 cells are HepG2 cells which were transfected with human P450 enzyme CYP2E1 cDNA in the sense orientation and constitutively express CYP2E1. CYP2E1 produces increased oxidative stress and liver cell toxicity, therefore, E47 cell line responds to ethanol in ways similar to liver parental cells (*12,13*).

SH-SY5Y was cultured in Minimum Essential Medium (MEM) containing Earle salts, fetal bovine serum, 1.0 mM sodium pyruvate, and 1.5 g/L sodium bicarbonate. U-118 MG and H9c2 were cultured in a Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/L glucose, fetal bovine serum and 200 mM glutamine. E47 was cultured in Minimum Essential Medium (MEM) containing Earle salts and fetal bovine serum.

Cell culture and treatments with ethanol and deprenyl

Before treatments, SH-SY5Y, U-118 MG, E47 and H9c2 cells were seeded on 6-well plates. After overnight culture in medium, the medium was replaced with new medium containing different concentrations of ethanol and deprenyl, daily, for three days. The ethanol concentration we used (200 mM for examining the effect of deprenyl) was within the standard range of in vitro study (*14*). This concentration was also decreased to ~1/4 after one day by evaporation (data not shown), thus the medium with ethanol was changed every 24 h. When a heavy drinker's ethanol concentration in blood reaches ~50 mM, he probably shows slurred speech and unsteadiness (*15*). Thus, the ethanol concentration for this study is around the physiological effect of ethanol in alcoholics.

MTT assay for proliferation rate/cell viability evaluation

Cell viability and proliferation was measured by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (*9*). The medium in excess of 2 mL (6-well plates) was removed and 40 μL of MTT dye (5 mg/mL) in sterile PBS was added to 360 μL of medium or PBS depending on cell confluence. More confluent cell lines used PBS and plates with fewer cells used medium. Plates were incubated for 4 to 5 h, during which time the mitochondria in living cells converted the soluble yellow dye (MTT) into an insoluble purple formazan crystal. Cells and dye were then solubilized by the addition of 800-1000 μL of DMSO to the 6-well plates. Optical density of each well at 572 nm was determined using the NanoDrop Spectrophotometer.

Western blot analysis

Cells were cultured in medium with ethanol (0, 200 and 400 mM) for 2 d, washed by PBS (pH 7.4), and sonicated in 500 μL of RIPA lysis buffer (10 mM Tris·HCl, pH 7.4/160 mM NaCl/1% Triton/1% Na dexycholate/0.1% SDS/1 mM EDTA/1 mM EGTA) supplemented with protease inhibitors (Sigma, Japan). Thirty micrograms (for MAO B assay) of total proteins were separated by 10.5% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After the transfer, membranes were blocked at room temperature for 2 h with 5% nonfat dry milk in TTBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.05% Tween 20). The membranes were then incubated with mouse anti-caspase 3 antibody (1:500) or mouse anti-actin antibody (1:1000) overnight at 4°C. After incubation with respective secondary antibody at room temperature for 2 h, the bands were visualized by hoseradish peroxidase (HRP) reaction using SuperSignal West Pico Chemiluminescent Substrate (PIERCE).

Statistical analysis

The statistical significance was evaluated using a Student's *t* test for two-group comparisons when needed. A value of $P < 0.05$ was considered to be significant.

Results

Ethanol induces cell death in a concentration dependent manner

SH-SY5Y, U-118 MG, E47 and H9c2 cells were treated with different concentrations of ethanol (0, 100, 200, 300 and 400 mM) for three days. Then the cell viability was determined by MTT assay. The results showed that ethanol induced cell death in a concentration dependent manner as determined by MTT assay (Figure 1). In addition, ethanol affected the brain cell lines (SH-SY5Y and U-118 MG) more than liver (E47) and heart cell (H9c2) lines at the concentration of 200 mM (Figure 1).

Ethanol induced cell death is through apoptotic pathway

To test whether cell death induced by ethanol is through apoptotic cascade, the levels of apoptotic protein, caspase 3, was determined in each cell line treated without or with ethanol, because caspase 3 mediated apoptotic pathway has been found to participate in MAO involved apoptotic signaling pathway (*9*). As determined by western blot (Figure 2A), the relative intensity of each casapase 3 protein band was quantified by a PhosphorImager system (Figure 2B) and expressed as -fold control, in which the cells without ethanol treatment was taken as 1. These quantitative data showed that the levels of caspase 3 was increased significantly by ethanol treatment (200 and 400 mM for

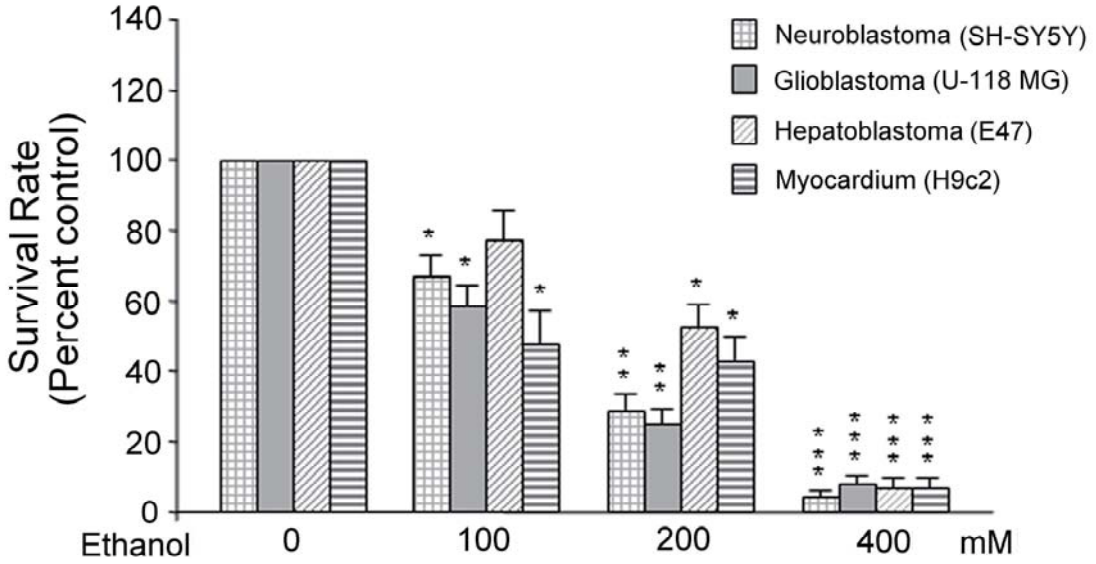

Figure 1. Effects of different concentrations of ethanol on cell survival rate. SH-SY5Y, U-118 MG, E47 and H9c2 cells were treated with different concentrations of ethanol (0, 100, 200, 300 and 400 mM) for three days. Then the cell viability was determined by MTT assay. Controls were untreated cells (0 mM) which were taken as 100%. Data represent the mean ± S.D. of three independent experiments (**P* < 0.05, ***P* < 0.02, ****P* < 0.005 versus respective control cells without ethanol treatment).

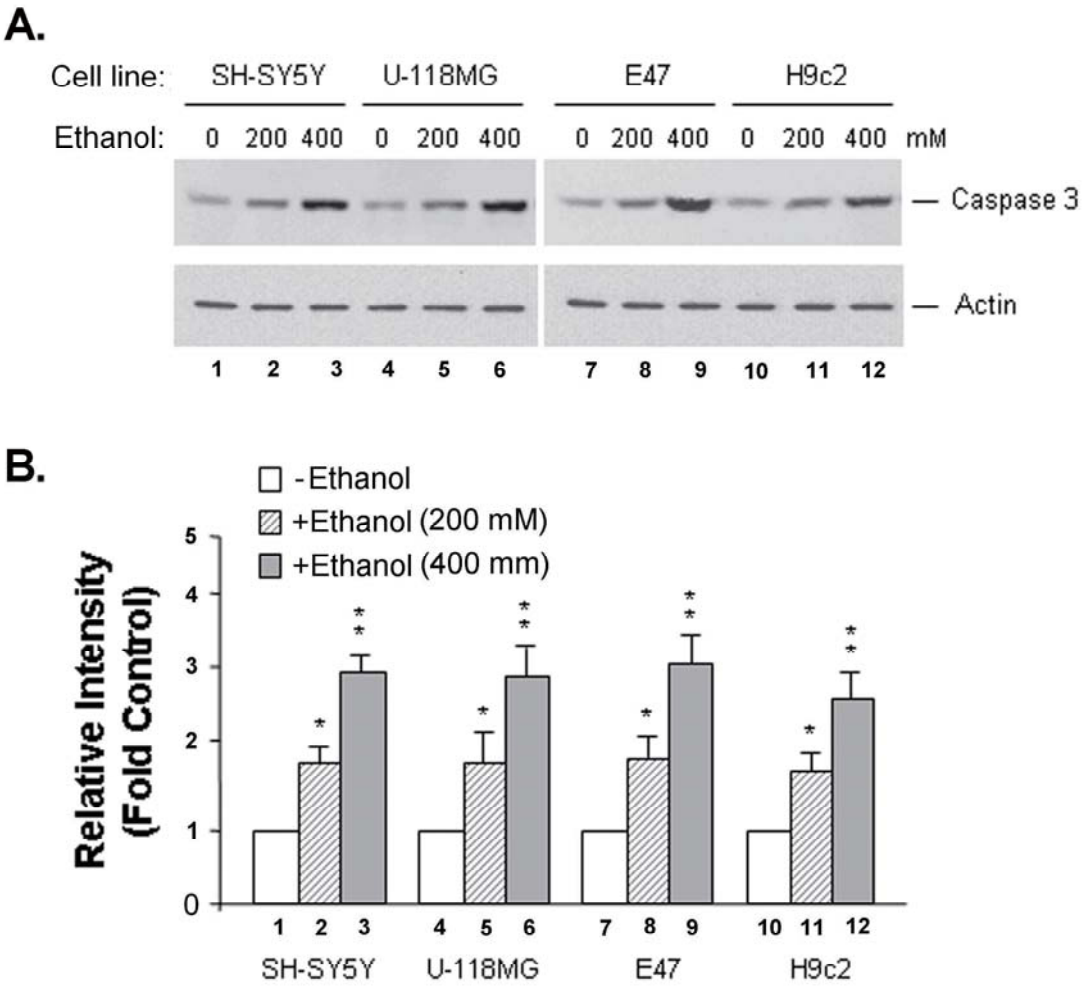

Figure 2. The expression of the apoptotic protein, caspase 3, after ethanol exposure. (A) Cells were exposed to 0, 200 and 400 mM ethanol for 48 h, and the cell lysates were subjected to the western blot analysis using anti-caspase 3 antibody. (B) Quantitative analysis. The relative intensity of each caspase 3 band was quantified by PhosphorImager. Values were expressed as fold of control, in which the cells without ethanol treatment was taken as 1. Data represent the mean ± S.D. of three independent experiments (**P* < 0.05, ***P* < 0.02 versus respective control cells without ethanol treatment).

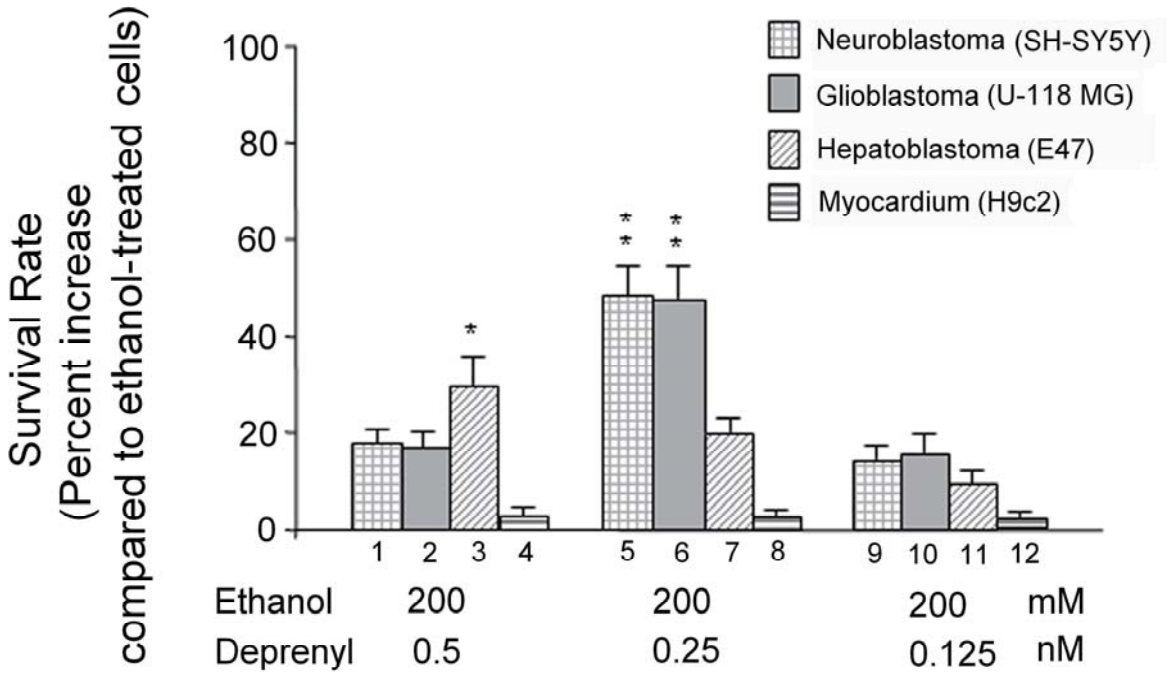

Figure 3. Effects of different dosage of deprenyl (MAO inhibitor) on the protection of ethanol induced cell death. SH-SY5Y, U-118 MG, E47 and H9c2 cells were treated with or without 200 mM ethanol in the conjunction with 0.5, 0.25 or 0.125 nM of deprenyl as indicated in the figure for three days. Then the cell viability (survival rate) was determined by MTT assay. The values of first group (+ ethanol) were obtained by comparing ethanol-treated group with normal control group which was taken as 100%. The values of second group (+ ethanol and deprenyl) were obtained by comparing ethanol/deprenyl-treated group with deprenyl-treated group which were taken as 100%. Then the percent increase in the survival rate by deprenyl is expressed by comparing the values of second group (+ ethanol and deprenyl) with the values of first group (+ ethanol) which was taken as 100%. All data are presented as the mean ± S.D. of at least three independent experiments (*$P < 0.05$, **$P < 0.02$ versus respective control cells with ethanol treatment but without deprenyl).

48 h) compared to control group (ethanol 0 mM) in a concentration dependent manner.

The inhibitor of MAO, deprenyl, against ethanol induced cell death

The physiologically relevant concentration of ethanol has been found to increase the MAO B gene expression and catalytic activity in the human glioma 1242-MG cells (*16*). Therefore we studied whether the MAO inhibitor (deprenyl) could reduce the ethanol-induced cell death. First, the different concentrations (0.125, 0.25 and 0.5 nM) of deprenyl were used in order to establish an ideal concentration for the cellular protection (Figure 3). The results showed that 200 mM ethanol treatment in conjunction with 0.25 nM deprenyl provided the most protection against apoptotic activity for brain cells SH-SY5Y and U-118 MG (Figure 3, compare column 5 and 6 to column 1, 2, 9 and 10). For the liver cells, the best protection was at 0.5 nM deprenyl (Figure 3, compare column 3 to column 7 and 11). However, for the heart cells, there was no protection for deprenyl at any concentrations (Figure 3, column 4, 8 and 12).

According to above optimal condition, cells were exposed to 200 mM ethanol in conjunction with 0.25 nM (for brain cell lines) or 0.5 nM (for liver cell line) deprenyl for three days, and the cell viability were in survival rate was observed in both SH-SY5Y and U-118 MG cells with 200 mM ethanol and 0.25 nM deprenyl treatment compared to those of cells treated with 200 mM ethanol alone (Figure 4, column 2 and 4 *vs.* column 1 and 3). In addition, ~1.5-fold increase in survival rate was found in liver cells, E47 (Figure 4, column 6 *vs.* column 5). However, heart cells H9c2 were not protected at 200 mM ethanol treatment with 0.5 nM deprenyl (Figure 4, column 8 *vs.* column 7).

These data suggested that lower concentration of deprenyl (0.25 nM) protects brain cells, higher concentration (0.5 nM) protects liver, but deprenyl at either concentration does not protect heart cells.

Discussion

An aberrant increase of MAO B activity has been implicated in several psychiatric and neurodegenerative disorders (*17-19*). The MAO B gene is located on the X chromosome (*20*) and its activity increases progressively in the brain throughout adult life (*21,22*). MAO B is the enzyme that deaminates a number of biogenic amines and produces reactive oxygen (H_2O_2) which causes toxicity to cells. Thus one predicted mechanism for cell death is an abnormal increase in monoamine oxidase (*23*). Previously, the physiologically relevant concentration of ethanol has been found to increase the MAO gene expression and catalytic activity in the human glioma 1242-MG cells (*16*). The increased activity of MAO may thereby increase production of hydrogen peroxide (H_2O_2, a

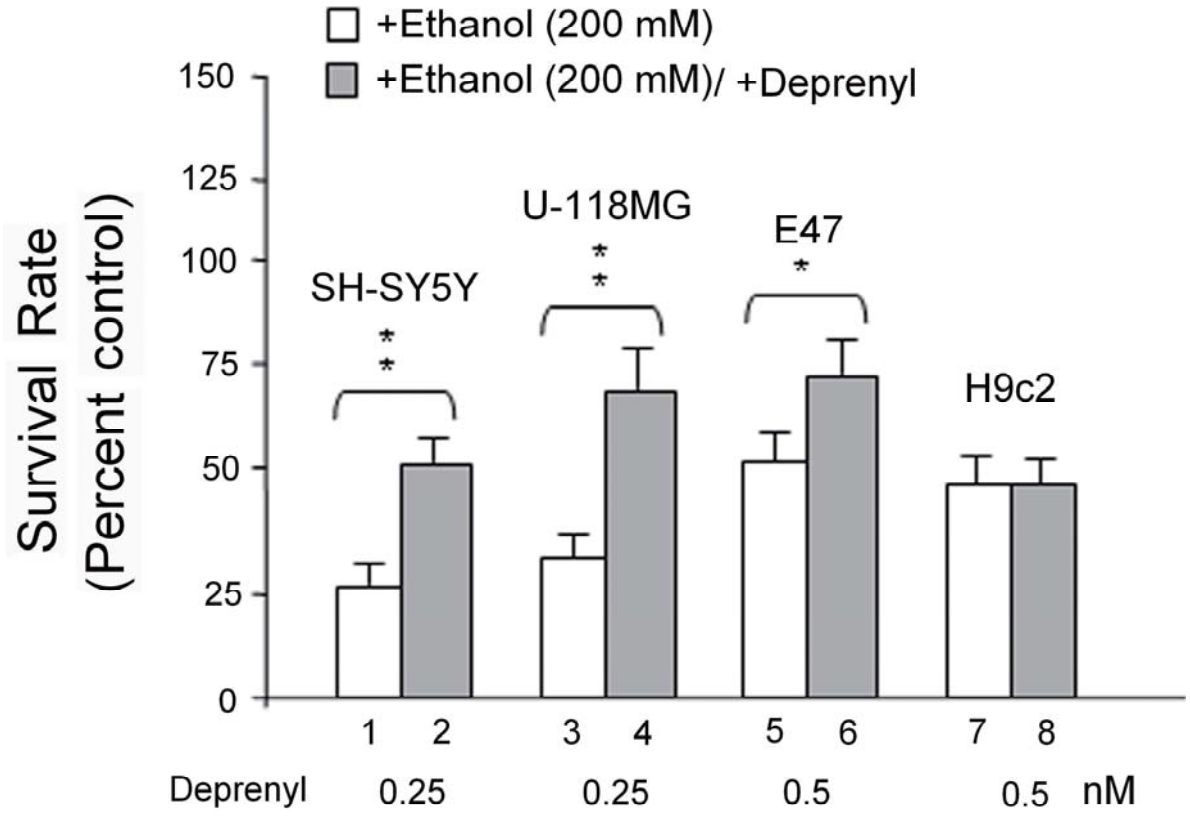

Figure 4. Effects of deprenyl on the protection of ethanol induced cell death. SH-SY5Y, U-118 MG, E47 and H9c2 cells were treated with or without 200 mM ethanol in the conjunction with 0.25 or 0.5 nM of deprenyl as indicated in the figure for three days. Then the cell viability was determined by MTT assay. The values of first group (+ ethanol) were obtained by comparing ethanol-treated group with normal control group which was taken as 100%. The values of second group (+ ethanol and deprenyl) were obtained by comparing ethanol/deprenyl-treated group with deprenyl-treated group which were taken as 100%. Then the percentages of both groups were shown in the figure. All data are presented as the mean ± S.D. of at least three independent experiments (*$P < 0.05$, **$P < 0.02$).

major source for oxidative stress) and cause apoptosis (*24*). Our present study shows that the levels of caspase 3, an apoptotic mark protein, were increased significantly by ethanol, suggesting that ethanol-induced cell death is mediated by apoptotic pathway.

Based on previous publications, deprenyl provides cell protection against apoptosis even in minute doses (*11,25*). Therefore we studied whether the MAO inhibitor (deprenyl) could protect the ethanol-induced cell death. Our results show that deprenyl specifically protects brain cells from ethanol treatment at a lower dose (0.25 nM) which is consistent with previous discovery. Further, it is interesting that deprenyl at a higher dose (0.5 nM) is able to protect liver cells from the harmful effect of ethanol, although the protection is less than that in the brain cells. One of anti-apoptotic mechanisms of deprenyl has been found to elevate the expression of antiapoptotic Bcl-2 protein for protecting against a toxic metabolite 1-methyl-4-phenylpyridinium ion (MPP+)-induced neurotoxicity (*26*). Therefore, the protective effect of deprenyl on ethanol-induced cell death might be through the disruption of MAO-mediated apoptotic pathway.

Based on our results, deprenyl may not protect heart cells. Our unpublished data show that the MAO catalytic activity has been increased upon ethanol treatment in SH-SY5Y, U-118 MG and E47, but not changed significantly in the rat heat cell line (H9c2). Therefore, the MAO-mediated apoptotic pathway might not be important in this rat heart cell line compared to human brain- and liver-derived cell lines. However, the heart cell line we used is a rat cell line (because a human heart cell line is not available commercially at present). Therefore, whether deprenyl can protect heart cells need to be further studied.

In summary, alcohol abuse causes a decrease in cell proliferation rates as a result of ethanol-induced cell death. Based on the findings, deprenyl, at the correct dosage, consistently provides cellular protection against the apoptotic effects of alcohol consumption, especially for the brain, and may also protect other organs or tissues, such as the liver. MAO inhibitors/anti-depressants such as deprenyl may be used as a means of treatment for individuals suffering from alcoholism to counteract the detrimental effects of alcohol abuse.

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