VITAMIN-C PROTECT ETHANOL INDUCED APOPTOTIC NEURODEGENERATION IN POSTNATAL RAT BRAIN

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ABSTRACT

Objective: To evaluate ethanol effects to induced activation of caspase-3, and to observe the protective effects of Vitamin C (vit-C) on ethanol-induced apoptotic neurodegeneration in rat cortical area of brain.

Methodology: Administration of a single dose of ethanol in 7-d postnatal (P7) rats triggers activation of caspase-3 and widespread apoptotic neuronal death. Western blot analysis, cells counting and Nissl staining were used to elucidate possible protective effect of vit-C against ethanol-induced apoptotic neurodegeneration in brain.

Results: The results showed that ethanol significantly increased caspase-3 expression and neuronal apoptosis. Furthermore, the co-treatment of vit-C along with ethanol showed significantly decreased expression of caspase-3 as compare to control group.

Conclusion: Our findings indicate that vit-C can prevent some of the deleterious effect of ethanol on developing rat brain when given after ethanol exposure and can be used as an effective protective agent for Fetal Alcohol Syndrome (FAS).

KEY WORDS: Ethanol, Neurodegeneration, Vitamin C, Cortex.

INTRODUCTION

Ethanol is the most common human teratogen. Excessive alcohol consumption during pregnancy can result in fetal alcohol syndrome (FAS). It is characterized by abnormalities in the central nervous system (CNS). These are reduced brain size (microencephaly), growth retardation, and facial dysmorphology in the newborn children.⁶ Ethanol may damage the developing brain by affecting neurogenesis, migration, or survival of cells.² Neurons are more susceptible to ethanol-induced apoptotic cell death during synaptogenesis, also known as the brain growth spurt.³⁴

Several studies have documented specific ethanol-induced damage to the hippocampus. The hippocampal formation and number of
neurons are adversely affected by exposure to high doses of ethanol in neonatal rats.\(^5\) Cell migration and differentiation are important processes for a developing fetal central nervous system (CNS), and apoptosis is an indispensable mechanism for these processes. Apoptosis discriminates the cell type, population, and function in various regions.\(^6\) Previous studies have also shown that ethanol induces widespread apoptotic neurodegeneration in the developing rat forebrain.\(^3\)

Ascorbic acid concentration in brain is highly regulated. The brain normally contains high concentrations of vit-C, which is actively taken through the choroids plexus. However, its specific functions in the CNS are only beginning to be elucidated. Vit-C acts as part of the intracellular antioxidant network, and as such is an important neuroprotective constituent. Recently, it has become clear that antioxidant nutrients, including vit-C, are important for neurological function.\(^9\)\(^12\) High intake of vitamin E and C has been found to be associated with lower risk of Alzheimer’s disease.\(^13\) Therefore, the objective of this study was to assess the protective effect of vit-C against the ethanol mediated toxic effects relevant to neurological damage.

Our results suggested that ethanol induced apoptotic neurodegeneration, while the vit-C, may effectively protects against the deleterious effects of ethanol-induced abnormalities, which may be used as a therapeutic approach towards FAS-associated brain damage during early developmental stages.

**METHODOLOGY**

*Animals and drug treatment:* P7 Sparague-Dawley rats of 15g (Gyeongsang National University, Neurobiology Laboratory, Chinju, South Korea) were injected subcutaneously with 20% ethanol in saline solution delivering 5g/kg body weight and 200mg/kg of vit-C and scarified after 4 h, while during co treatment of vit-C plus ethanol, vit-C was injected after 30 min of ethanol treatment, whereas control group were treated with saline.

*Cell Counting:* Cells were counted one section out of every six (240 µm apart from each other) throughout the brain by using a Nikon Eclipse E600 with a Nikon 40× objective. Two to four sections per brain for the LDN (lateral dorsal nucleus) and six to ten sections per brain for the cingulate cortex were counted in five to six animals of each group. Cells were counted in both hemispheres. Contours of Cingulate CX (Bregma from 1.34 to “0.82”)\(^14\) were traced using a personal computer, and the area was calculated with Stereo Investigator software (MBF Bioscience, Williston, Vermont, United States).

*Western blotting:* Animals were killed at four hours after ethanol and vit-C administration. Brains were dissected out and cortical part was removed carefully and tissue was frozen in dry ice. For each treatment group, three to four pups from 6 different litters were analyzed. Caspase-3 analysis was done as previously described with some modification.\(^15\) Western blots were analyzed by densitometry using the computer-based Sigma Gel (SPSS Inc. Chicago, USA) system. Density values were expressed as mean ± SEM. One-way ANOVA analysis followed by Tukey-Kramer multiple-comparisons test was performed to determine the significance of differences between relevant treatment groups. In every case, the acceptance level for statistical significance was *P < 0.05.

*Nissl staining:* Nissl staining was done as previously described with some modification.\(^16\) Morphology of cells and presence of apoptotic and necrotic bodies was assessed on microscope slides mounted 12µm thick brain sections. Cresyl violet, recognize all structure particular nucleus and nucleic acids appear violet, neurons faintly blue. Neuronal damage was then estimated as a rate of the number of degenerated neurons to that of both surviving and degenerated in three distinct areas of the cingulate cortex in coronal sections for each animal.\(^17\)

*Data analysis and statistics:* The object band from Western blot were scanned and analyzed by densitometry using a computer based on the Sigma Gel System (SPSS Inc., Chicago, IL). Density values were expressed as mean ± SEM. One-way ANOVA analysis followed by Tukey–Kramer multiple-comparisons test was per-
formed to determine the significance of differences between relevant treatment groups. The acceptance level for statistical significance was \( *P < 0.05 \).

RESULTS

**Vitamin C inhibits Ethanol-induced apoptotic neurodegeneration:** Activation of caspases resulted in nuclear, plasma-membrane and mitochondrial changes. In the present study exposure of ethanol significantly increased the expression of caspase-3. Increased caspase-3 can disturb essential homeostatic processes and initiate an orderly disassembly of cells including degradation of genomic DNA.\(^{18}\) In order to determine that vit-C can inhibit ethanol-induced caspase-3 activation, a series of different experiments was performed. The doses of vit-C 200mg/kg were administered subcutaneously 30 minutes after ethanol treatment. Western blot results showed that the animals injected with ethanol alone showed a 10-fold to 20-fold increase of caspase-3 compared with a saline control, while the co treatment of vit-C with ethanol significantly decreased the expression of caspase-3 as compare to ethanol treated group (Figure 1). The high concentrations of ethanol were used in the experiment to evaluate the effect of ethanol on developing brain (Fig- 1).

**Histological findings:** To determine whether inhibition of caspase-3 activation by vit-c is sufficient to prevent ethanol-induced cell death, brains were histologically analyzed for evidence of neurodegeneration. The Nissl staining results showed that ethanol significantly increased neuronal death, while the co treatment of vit-C with ethanol significantly inhibited the neuronal death compared to ethanol treated group (Fig- 2), which suggest that vit-C an antioxidant, may effectively protects against the deleterious effects of ethanol-induced abnormalities by decreasing neuronal death in cortical rat brain, furthermore the cell counting under light microscope after Nissl stain also showed the same increased neurodegeneration upon ethanol treated group and decreased significantly when treated with vit-c as compare to alone ethanol treated group (Fig- 3).

![Fig-1: Western blot analyses of the caspase-3 in the cortical brain. P7 rat were treated for 4 h with saline as control (C), rat treated with ethanol (E), rat treated with vit-C (vit-C), and rat treated with vit-c 30 min before ethanol treatment (vit-C+E). β-actin is taken as loading control. A: Immunoblots of caspase-3 of cortical area under different treatment conditions. The immunoblots were labeled with an anti caspase-3 antibody. B: Density values were expressed as mean ± SEM (n = 4) of the corresponding protein of caspase-3 are presented. *P < 0.05 versus control group.](image1)

![Fig-2: Histopathological changes in cortical area of brain treated (A) controls (B) ethanol group (C) vit-C (D) vit-C plus ethanol treated groups. Nissl staining using light microscopy. Magnification X 40.](image2)
DISCUSSION

Brain damage due to ethanol exposure is a cardinal feature in (FAS) in alcoholism. Enhanced neurodegeneration in combination with oxidative stress outcomes probably lead to the neurodevelopmental deficiencies that are encountered in FAS. It has already been established that ethanol decreases the population of neurons in the brain and causes malformation including the loss of brain mass. Present study indicate the apoptotic neurodegeneration induced by ethanol and a protective effect of antioxidants vit-C during early developmental stage.

Cell proliferation and differentiation are two critical processes in a developing fetal brain. Apoptosis Bcl-2 family proteins, plays decisive role. Moreover, the normal population of neurons is controlled by a balance of apoptosis and stem cell activities. In the present study, the activation of caspase-3 in response to ethanol exposure was investigated using Western blot analysis. It revealed increase expression of caspase-3 proteins. Furthermore, we have also demonstrated that the co administration of vit-C with ethanol treatment in early postnatal development prevents alcohol-induced apoptotic neurodegeneration. Administration of vit-C protected against this ethanol-induced apoptosis, suggests a link between neuronal loss during brain development and behavioral disturbances observed in the adult rat.

Ethanol exposure during prenatal development causes wide range of structural and functional brain abnormalities resulting in the condition of fetal alcohol effect (FAE) and alcohol related neurodevelopmental disorders (ARNDS) including cytoarchitectual abnormalities. In this study, we showed that the antioxidant vit-C can effectively reduce the severity of ethanol-induced brain injury and growth retardation during early brain development. The antioxidant vit-C can effectively reduce the severity of ethanol-induced brain injury and growth retardation. It is conceivable that vit-C can exert its neuroprotective role as a potent scavenger of oxygen free radicals. Second, it restores the expression of important neural markers including NCAM and Pax6. Third, it may also interact with ethanol extracellularly and hence alleviate the overall teratogenic effect of ethanol. The mechanisms underlying the vit-c neuroprotective effects are not fully understood, however, our results showed that co treatment of vit-C plus ethanol are in agreement with neuroprotective actions of vit-c reported in previous studies by pilocarpine.

In summary, this study showed that ethanol induced apoptosis neurodegeneration and the co treatment of vit-C with ethanol decreased ethanol-induced apoptotic neurodegeneration in developing brain. Although additional studies are necessary, we suggest, that vit-C a readily available and safe agent, could be used for the treatment and prevention of the FAS.

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