NEUROPROTECTIVE EFFECT OF VITAMIN C AGAINST PTZ INDUCED APOPTOTIC NEURODEGENERATION IN ADULT RAT BRAIN

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ABSTRACT

The present study was designed to observe the effect of PTZ on expression of caspsae-3, and to evaluate the neuroprotective role of vitamin C (vit-C) against PTZ-induced apoptotic neurodegeneration in adult rat brain. We observed that administration of a single conclusive dose of pentylenetetrazol (PTZ 50mg/kg) in adults rats induced epileptic seizure and increased activation of caspase-3 and caused neuronal death. Further, rats were injected with vit-C (250 mg/kg) 30 min before PTZ injection. The protective effect of vit-C against PTZ-induced apoptotic neurodegeneration in adult rat brain was observed using Western blot analysis and Nissl staining. The results showed that conclusive dose of PTZ-induced seizure, increased expression of caspase-3 and neuronal apoptosis in adult rat brain. Whereas, the pretreatment of vit-C along with PTZ showed significantly decreased expression of caspase-3 as compare to control group. Finally, our results indicated that vit-C can prevent some of the deleterious effect of seizure and neuronal degeneration induced by PTZ in adult rat brain.

Keywords: PTZ, neurodegeneration, vitamin C, adult rat brain.

INTRODUCTION

Epilepsy is one of the most prevalent neurological disorders have significant influences on brain structure and are able to induce neuronal death, primarily the hippocampal part of the brain (Covolan *et al.,* 2000). Though epilepsy does not belong to the group of "neurodegenerative diseases", but some evidence showed that seizures induced cell death(Duncan, 2002; Heinemann, 2004; Holmes, 2002). It was shown that cognitive disorders (Becker *et al.,* 1994a), changes in emotional behavior (Duncan, 2002) and neuronal loss in the hippocampus (Franke and Kittner 2001; Pavlova *et al.,* 2003; Pohle *et al.,* 1997) appear after PTZ kindling.

Kindling has been demonstrated to be able to induce neuron death. Despite discussion of this phenomenon (McEachern and Shaw, 1999), however the nature of the seizure-induced death remains unknown. Activation of caspase-3 is known to be a key event in apoptosis in the brain (Nicotera, 2000; Porter and Janicke, 1999). Data obtained in recent years have provided evidence for a possible role for caspase-3 in changes in brain plasticity (Kolasa and Harrell, 2000, Kudryashov *et al.,* 2001, Shimohama *et al.,* 2001). In this study we used single conclusive dose of PTZ (50mg/kg) to induced neurodegneration. PTZ is a convulsive drug widely used to induce seizures experimentally. Though the mechanism of action of PTZ is not fully understood, it is generally accepted that part of its action is due to its antagonist binding to the picrotoxin-binding site of the postsynaptic $GABA_A$ receptor (Macdonald and Barker, 1977).

Vitamin C or l-ascorbate is an essential nutrient required for a range of essential metabolic reactions in all animals and plants. The pharmacophore of vit-C is the ascorbate ion. In living organisms, ascorbate is an antioxidant, which protects the body against oxidative stress (Sebrel and Harris, 1967; Padayatti *et al.,* 2003). Previously, it is known that antioxidant nutrients, like vit-C, are important for neurological function (Daskalopoulos *et al.,* 2002, Rice, 2000; Siushansian and Wilson, 1995). Recently we have also reported the neuroprotective effect of vit-C against ethanol and PTZ induced apoptotic neurodegeneration in prenatal and postnatal rat hippocampal and cortical neurons (Naseer *et al.,* 2009a, 2011). This study was designed to assess the protective effect of vit-C against the PTZ mediated toxic effects relevant to neuronal apoptosis.

Our results suggest that PTZ-induced apoptotic neurodegeneration, while the pretreatment of vit-C, may effectively protects against the deleterious effects of PTZ-induced apoptotic neurodegeneration, which may be use along with therapeutic approaches towards PTZ-induced seizure in adults rat brain.

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MATERIALS AND METHODS

Animals and drug treatment

Spargue-dawley male rats of 250g (Gyeongsang National University, Neurobiology Laboratory, Chinju, South Korea) were injected i.p. with a single dose 50mg/kg of PTZ in saline solution. Vitamin C 250mg/kg was administered as pretreatment 30 min before PTZ and scarified after 4 h of PTZ injection, whereas control group were treated with saline. They were maintained in a 12-h on and 12-h off light/dark schedule with free access to food and water, except during experimental procedures. Animals were treated in accordance with standard guidelines for laboratory animal care. All the experimental procedures were approved by the local animal ethics committee of the Division of Applied Life Sciences, Department of Biology, Gyeongsang National University South Korea.

Western blotting

Animal were killed at 4 h after PTZ and vit-C treatments. Brains were dissected out and hippocampal part was removed carefully and tissue was frozen in dry ice. For each treatment group, three to four rats were analyzed. The brain tissues were homogenized in 0.2 M PBS with protease inhibitor cocktail. The protein concentration was measured using Bio-Rad protein assay solution. Equivalent amounts of protein (40 µg per sample) were electrophoresed on 10-15% SDS-PAGE gels under reducing conditions and transferred to a polyvinylidene difluoride (PVDF) membrane (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Caspase-3 analysis was done as previously described with some modification (Naseer *et al.,* 2009b). Western blots were analyzed by densitometry using the computer-based Sigma Gel (SPSS Inc. Chicago, USA) system. In every case, the acceptance level for statistical significance was $*P < 0.05$.

Tissue collection and sample preparation

Animals were sacrificed 4 h after drug treatment. Brain sections from control rats and rats subjected to PTZ followed by vit-C for 24 h were analyzed. For tissue analysis (n = 4-5 per group), adult rat were perfused transcardially with 4% ice-cold paraformaldehyde followed by 1×PBS; brains were post-fixed in 4% paraformaldehyde overnight and then transferred to 20% sucrose until they sank to the bottom of the tubes. Brains were frozen in O.C.T compound (A.O. USA) and 16 µm sections were made in the coronal planes using a Leica cryostat (CM 3050C, Germany). Sections were thaw-mounted on probe-on plus charged slides (Fisher).

Nissl staining

Nissl staining was done as previously described with some modification (Majid *et al*., 2008). Cresyl violet was used to stain tissue sections for histological examination and measurement of neuronal loss. Nissl histology of adult rat brain and the presence and absence of dead and injured neurons were analyzed on microscope slide-mounted 16-µm-thick brain sections. Sections from all investigated rat pups were defatted in ascending alcohols (70–100%), hydrated in descending alcohols (95-70%), washed in acetate buffer pH 5.0 and subsequently stained with 0.25% cresyl violet for approximately 15 min. Section were then washed with distilled water and dehydrated in graded ethanol. Images were viewed with a fluorescence light microscope.

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. Comparisons between treated groups and controls were done by Student's *t*-test to determine the significance of differences between relevant treatment groups. In every case, the acceptance level for statistical significance was $P < 0.05$.

RESULTS

PTZ-induced seizures

A single convulsive dose of PTZ (50 mg/kg, i.p.) was administered in adult male rats. After injection of PTZ, occurrence of central nervous system (CNS) excitation was noted for 10–15 min by observing the animals in a plexiglass chamber (30 cm × 24 cm × 22 cm).

Vitamin C inhibits PTZ-induced apoptotic neurodegeneration

Activation of caspases results in nuclear, plasma-membrane and mitochondrial changes. In the present study we observed that treatment of PTZ-induced seizure significantly increased the expression of caspase-3. Caspases are proteases which have a central role in the initiation and execution of apoptotic cell death (Le *et al*., 2002; Carloni *et al*., 2004). The increased caspase-3 disturbs homeostatic processes and initiate an orderly disassembly of cells including genomic DNA degradation (Zhu *et al*., 2000). Further, to determine whether vit-C can prevent PTZ-induced apoptosis different experiments was performed. The doses of vit-C 250 mg/kg were administered subcutaneously 30 min before PTZ treatment, Western blot results showed that the animals injected with convulsant dose of PTZ showed significant increase expression of caspase-3 compared with a saline control, while the pretreatment of vit-C with PTZ significantly decreased the expression of caspase-3 as compare to PTZ treated group (fig. 1). The high concentrations of conclusive dose of PTZ (50mg/kg) were used in the experiment to evaluate the effect of PTZ in adult rat brain and the effect of seizure induced by PTZ and vit-C plus PTZ is represented in table 1.

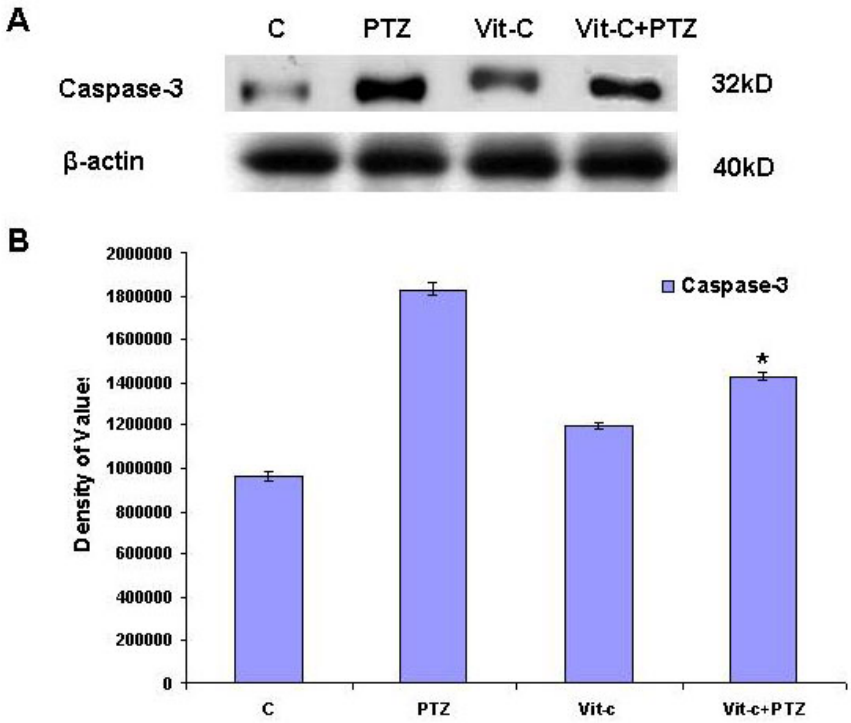

Fig. 1: Western blot analyses of the caspase-3 in the cortical brain. Adults rat of 250g were treated for PTZ 50mg/kg while vit-C was treated 30 min before PTZ injection. Saline was injected as control (C), rat treated with PTZ (PTZ), rat treated with vit-C (vit-C), and rat treated with vit-C 30 min before PTZ treatment (vit-C+PTZ). β-actin is taken as loading control. **A:** Immunoblots of caspase-3 of hippocampal 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. The density values on the Y-axis are expressed as arbitrary units (AU). **P* < 0.05 versus control group.

Table 1: Observations after PTZ and pretreatment of vit-Cp1us PTZ injection

	PTZ treated	vit-C plus PTZ treated
Number of seizures	7.1 ± 1.5	3.1 ± 1.2
Seizure time (min)	7.8 ± 0.9	4.8 ± 0.3
Hind limbs kicks	17 (90.2%)	11 (70.2%)
Immobility associated with tonic flexion of body	14 (89.4%)	10 (66.4%)
Tonic and colonic twistching of four limbs while lying down	15 (88.2%)	10 (58.2%)
Myoclonic jerks	12	7
Number of dead animals n=8	3	1

Histological findings

To further determine whether inhibition of caspase-3 activation by vit-C is sufficient to prevent PTZ-induced cell death, brains were analyzed to observe the neuronal loss. The Nissl staining results showed that PTZ-induced seizure significantly increased neuronal death, while the pretreatment of vit-C significantly inhibited the neuronal death compared to PTZ treated group (fig. 2). The results showed that that vit-C an antioxidant, have anticonvulsant effects against PTZ-induced seizure may effectively protects against the deleterious effects of PTZ-induced abnormalities by decreasing neuronal death in hippocampal brain of adult rat. Furthermore the cell counting under light microscope after Nissl stain also showed the same increased neurodegeneration upon PTZ treated group and decreased significantly upon pretreatment of vit-C as compare to alone PTZ treated group (fig. 3).

DISCUSSION

In the present work, the administration of vitamin C showed neuroprotection against PTZ-induced seizures in adult rats were studied. Previously it is known that PTZ

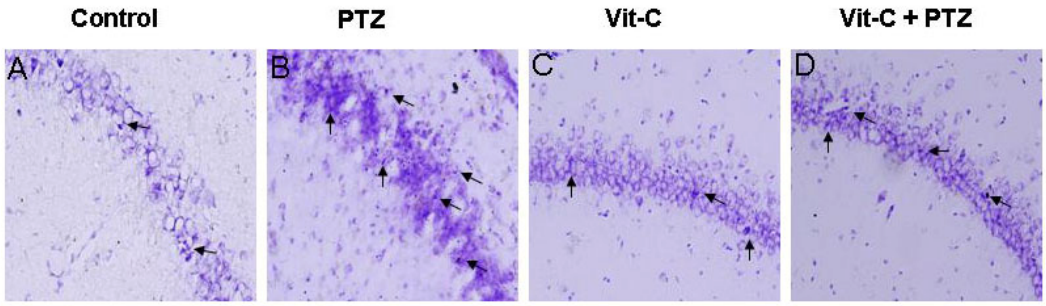

Fig. 2: Histopathological changes in hippocampal area of brain treated (A) controls (B) PTZ group (C) vit-C (D) vit-C plus PTZ treated groups. The arrows indicate shrunken and damaged neurons. Nissl-stained brain tissue at higher magnification with 40× objective field, Scale bar = 20 µm.

induced epileptic seizures and brain damage while the consequences of status epilepticus in the developing brain differ from those of the mature brain (Blennow *et al.,* 1978, Rauca *et al.,* 1999, Eracovic *et al.,* 2003).

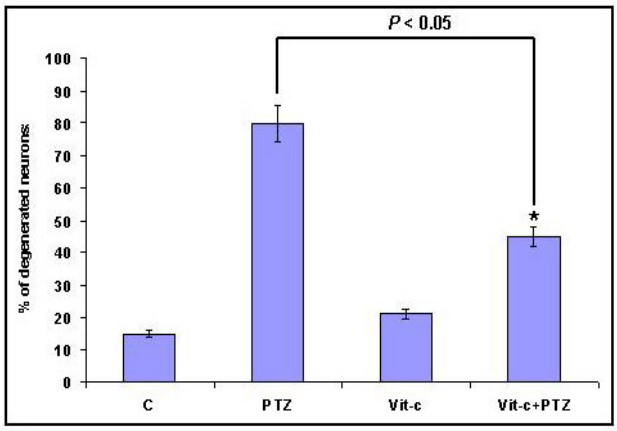

Fig. 3: Number of shrunken and damaged neurons were counted under light microscopy after Nissl staining. Graphic representation of neuroprotective effect of vit-C against PTZ-induced apoptotic neurodegeneration in hippocampal part of adult rat brain. Significance *$P <$ 0.05 versus control group.

PTZ-induced epilepsy that elicited cell death in the brain of experimental adult rats was examined using Western blot, Nasil stain for detection of neurodegeneration. PTZ-induced seizure is associated with an imbalance between excitatory and inhibitory neurotransmissions (Rocha *et al.,* 1996, Corda and Biggio, 1986). PTZ produces proconflict and convulsant effects in rodents (Becker *et al.,* 1994b), but cognitive deficits have also been noted (Howe *et al.,* 1987).

In the present study, the activation of caspase-3 in response to PTZ-induced seizures was investigated using Western blot analysis, which revealed increase expression of caspase-3 proteins. Furthermore, we have also demonstrated that the pretreatment of vit-C with PTZ treatment in adult rats prevents PTZ-induced apoptotic neurodegeneration. Our results showed that administration of vit-C protected against PTZ-induced apoptotic cell death, suggesting a link between neuronal loss and behavioral disturbances observed in the adult rat.

In this study, we showed that the antioxidant vit-C can effectively reduce the severity of PTZ-induced brain injury in adult rats. Previously it is reported that vit-c, as compared to others antioxidant drugs, exert potent anticonvulsant and neuroprotective effects (Xavier *et al.,* 2007). It is feasible that vit-C can exert its neuroprotective role as a potent scavenger of oxygen free radicals (Penga *et al.,* 2005). The exact mechanisms through which vit-C show its neuroprotective effects are not fully understood, however, our results showed that pretreatment of vit-C plus PTZ are in agreement with neuroprotective actions of vit-C reported in previous studies that vit-C pretreatment decrease hippocampal lipid peroxidation content by increasing catalase activity, indicative of a possible antioxidant effect, also decreasing lipid peroxidation levels (Lucia *et al.,* 2008).

In this study we observed the neuroprotective effect of vit-C and our results are in agreement with neuroprotective actions of vit-C reported in previous studies that vit-C play a important role to induced radical free content changes during epileptic seizure in adult rats (Xavier *et al.,* 2007).

In summary, the results showed that PTZ induced seizure and apoptotic neurodegeneration in adult rats while the pretreatment of vit-C decreased PTZ-induced apoptotic neurodegeneration rat brains. Finally we suggest, that vit-C a readily available and safe agent, could be used for the treatment and prevention of the seizure and apoptotic neurodegeneration in adults rat brain.

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