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The Protective Effect of Vitamin E on Locus Coeruleus in Early Model of Parkinson's Disease in Rat: Immunoreactivity Evidence

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ABSTRACT

Background: Free radical formation and oxidative stress might play an important role in the pathogenesis of Parkinson's disease (PD). *In vitro* data indicate that neuromelanin (NM) pigment is formed the excess cytosolic catecholamine that is not accumulated into synaptic vesicles via the vesicular monoamine transporter 2 (VMAT2). We designed this study to investigate the neuroprotective effects of vitamin E in the early model of PD. **Methods**: Male rats (n = 40) with unbiased rotational behavior were randomly divided into five groups: sham operated group (SH, n = 8), vehicle-treated SH group (SH + V, n = 8), vitamin E-treated SH group (SH + E, n = 8), vehicle-treated lesion group (L + V, n = 8) and vitamin E-treated lesion group (L + E, n = 8). Unilateral intrastriatal 6-hydroxydopamine (12.5 µl) lesioned rats were treated intramuscularly with α-tocopherol acid succinate (24 I.U/kg, intramuscular [i.m.]) 1 h before surgery and three times per week for 2 month post-surgery. To evaluate the vitamin E pretreatment efficacy, tyrosine hydroxylase (TH) immunoreactivity and immunostaining intensity (ISI) for monoamine transporter 2 were used. **Results**: TH immunohistochemical analyses showed a reduction of 20% in locus coeruleus (LC) cell number of vitamin E pretreated lesioned group but the cell number dropped to 60% in the lesioned group. The ISI of the cells was measured for VMAT2 in LC. Lesioned groups: 1) had the lowest VMAT2 ISI of all neurons; 2) There was an inverse relationship between VMAT2 ISI and NM pigment in the locus and 3) Neurons with the highest VMAT2 ISI also had high TH ISI. **Conclusion**: The data support the hypothesis that repeated i.m. administration of vitamin E exerts a protective effect on the LC neurons in the early model of PD. *Iran. Biomed. J. 12 (4): 217-222, 2008*

Keywords: Vitamin E, Parkinson's disease (PD), Neuromelanin (NM), Immunoreactivity

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INTRODUCTION

Free radical formation and oxidative stress might play an important role in the pathogenesis of Parkinson's disease (PD). The central nervous system shows an exceptionally high degree of vulnerability to reactive oxygen species (ROS) [1-3]. Aging is a major risk factor for neurodegenerative diseases including Alzheimer's disease (AD), PD, and amyotrophic lateral sclerosis. An unbalanced overproduction of ROS may give rise to oxidative stress which can induce neuronal damage, ultimately leading to neuronal death by apoptosis or necrosis. Numerous evidences indicate that oxidative stress is involved in the pathogenesis of AD and PD [4-8]. Several studies have shown that nutritional antioxidants (especially vitamin E and polyphenols) can block neuronal death *in vitro*, and may have therapeutic properties in animal models of neurodegenerative diseases including AD and PD [9-14]. Moreover, clinical data suggest that nutritional antioxidants might exert some protective effects against AD and PD [15]. Neuromelanin (NM) pigment synthetic byproduct of catecholamine

*Corresponding Author; Tel. (+98-21) 4464 4585; Fax: (+98-21) 6641 9072; E-mail: negar.omidi@gmail.com; **Abbreviations:** SH, sham operated; SH + V, vehicle-treated SH; L + V, V-treated lesion; SH + E, vitamin E-treated SH; L + V, vehicle-treated lesion; L + E, vitamin E-treated lesion; PD, Parkinson's disease; AD, Alzheimer's disease; ROS, reactive oxygen species; NM, neuromelanin; DA, dopamine; LC, locus coeruleus; 6-OHDA, 6-hydroxydopamin; TH-IR, tyrosine hydroxylase immunoreactivity; i.m., intramuscular; VMAT2, vesicular monoamine transporter 2; ISI, immunostaining intensity; DAergic, dopaminergic; ABC, avidin biotin complex

synthesis that NM accumulates with age in the substantia nigra dopamine (DA)-containing neurons, and in the locus coeruleus (LC) noradrenergic neurons [16, 17].

Moreover, it has been reported that the levels of glutathione and vitamin E increased in the brain of patients with PD as a compensatory mechanism to deal with oxidative stress [18- 20]. Since vitamin E is an effective free radical scavenger in the brain [21, 22], its neuroprotective function is the issue of new therapeutic approaches in neurodegeneration diseases. In clinical trials, the vitamin E therapy might have retarded the progression of degenerative process in patients with PD [18, 23]. Dexter *et al*. [17] postulated that chronic vitamin E deficiency in rats produces a selective dopaminergic (DAergic) cell loss in substantia nigra. In addition, reactive oxygen radicals are involved in the toxicity of 6-hydroxydopamin (6-OHDA) that induce nigrostriatal lesions and is used as an experimental model of unilateral Parkinsonism [24]. In this respect, the pretreatment with tocopherol attenuates the toxic effects of 6-OHDA that causes in LC. Vitamin E may replace those protective enzymes that are deficient in nigral and LC neurons. In spite of these reports and the existence of controversies [25, 26], no direct evidence has revealed the protective actions of vitamin E on the LC neurons yet. Therefore, the present study is designed to evaluate the protective effects of vitamin E in restraining DAergic cell dysfunction and loss at the onset of their damage. For this purpose, tyrosine hydroxylase (TH) immunohistochemistry study was performed in 6-OHDA lesioned rats, since parenteral administration of acetylated forms of vitamin E can lead to a higher and longer plasma concentration [13, 27]. In the present study, we decided to use a repeated short-term intramuscular (i.m.) administration of a pharmacological dose of D-Q-a-tocopheryl acid succinate to examine neuroprotective effects of vitamin E on noradrenergic neurons.

MATERIALS AND METHODS

Adult male Sprague-Dawley rats (Pasteur Institute of Iran, Tehran and weighing 200 280 g at the start of the study) were housed three to four per cage in a temperature controlled colony room under light-dark cycle with food and water ad libitum. They were held in their cages for at least 10 days before being tested. All procedures of the study were according to the guidelines of Animal Experiments of Research Council at Tehran University of Medical Sciences (Tehran, Iran).

Surgical and pretreatment procedures. Rats that show no biased rotational behavior (net rotations less than 30/h) following i.p. injection of apomorphine hydrochloride (0.5 mg /kg), were selected for the present study. The animals (n= 40) were randomly divided into five groups: sham operated group (SH, n= 8), vehicle-treated SH group (SH + V, n = 8), vitamin E-treated SH group (SH + E, n = 8), vehicle-treated lesion group (L + V, n = 8) and vitamin E-treated lesion group (L + E, n = 8). Since no behavioral and differential effects were seen with the vitamin E or the vehicle in the sham groups, they were all considered as control group. Unilateral intrastriatal 6-OHDA injection was performed with 10-ql Hamilton syringe on anesthetized (ketamine 100 mg/kg and xylazine 5 mg/kg, i.p.) rats using stereotaxic apparatus (Stoelting, USA) at the coordinates: L-3 mm, AP +9.2 mm, V + 4.5 mm from the center of the interaural line, according to the atlas of (Paxinose and Watson [23]. At the end of the injection, the needle was left in place for an additional 5 min and then withdrawn at a rate of 1 mm/min. The L + V group received a single injection of 5 µl of 0.9% saline containing 2.5µg/µl of 6-OHDA (Sigma, USA) and 0.2% (w/v) ascorbic acid at a rate of 1 µl/min and in addition to i.m. administration of the vehicle, propylene glycol (Merck, USA). The SH group received an identical volume of ascorbate-saline solution. The L + E group, in addition to the neurotoxin, received a solution of D-a-tocopheryl acid succinate (24 I.U./kg, i.m, Bioglan, UK) in 0.8 ml/kg of propylene glycol 1 h before the intrastriatal injection of neurotoxin. Then, one day after and with 2-day intervals this administration continued for 8 weeks.

TH and vesicular monoamine transporter 2 (VMAT2) immunohistochemistry. Rats were deeply anesthetized (Ketamine 150 mg/kg) and perfused through the ascending aorta with 200 ml of 0.9% saline followed by 500 ml fixative solution containing 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 over 30 min. Then, the brains were dissected immediately and the tissues were fixed in 10% neutral buffered formalin for at least one week. Prior to cutting, tissue blocks containing LC were placed in 20% sucrose in formalin for 1-2 weeks. Control sections were cut at 40%-µm thickness through the midbrain

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and rostral pons. A standard avidin biotin complex (ABC) immunohistochemical staining method was employed. In brief, the sections were washed in PBS, treated with 1% hydrogen peroxide in PBS and blocked in 5% normal goat serum in 0.3 Triton X-100 in PBS. Staining was performed by incubating sections with diluted primary antibodies overnight at room temperature. This was followed by incubating sections with 1.5 µg/ml biotinylated goat anti-rabbit IgG for 30 minutes at room temperature (Vector labs, Burlingame, CA), with an avidin/biotin/ peroxidase reagent (1:250 dilution ABC Elite, vector labs) for 1 hour at room temperature. Then, the sections were reacted in an acetate buffer (pH 6.0) containing 0.035% diamino benzidine tetrahydro-chloride, 2.5% nickel ammonium sulfate and 0.001% hydrogen peroxide for 5-10 minutes. All of the incubations were done on a shaker. A polyclonal antibody against TH (Protos Biotech Corp., NY) was used at 1:1,000 to 1:6000 concentrations. An affinity purified VMAT2 antibody was made against 13 amino acids in the rat C-terminus of the protein (Alpha Diagnostics, Inc, San Antonio, TX, USA.). An antibody made against the same sequence is also commercially available (AB1598P; Chemicon, Temecula, CA, USA) and we used it previously to immunostain VMAT2-containing neurons in the rodent [3]. The sections were immunostained with concentrations of 1:500 to 1:1000. When either primary antibody was omitted, the catecholamine-ergic somata did not immunostain. Densitometry methods were used to assess the immunostaining intensity (ISI) for each antibody within the somata of LC noradrenergic neurons. A Leica DMRE microscope equipped with a cohu video camera and stereo Investigator software (Micro Bright Field, Inc.) was employed for densitometry measurements (40 X objectives) on a scale of 0-255 and the optical density of cells outlined within the LC was determined. The ISI of 10 neurons within each region was also determined. In SH groups without NM pigments, the ISI was determined by taking the average intensity over the entire somata of cells without an unstained nucleus visible in the plane of the section. In the cells from lesion, L + V and L + E brains that contained NM pigment, the ISI was measured in the portion of the somata that contained little or no NM pigment. The mean intensity was subtracted from the background of ISI of the tissue section to calculate the average ISI for the cell region. In this way, the cells with dark staining had high ISI values and the cells with immunostaining had low ISI values.

Statistical analysis. All data were expressed as mean ± SEM. For each group, the values of TH-immunoreactive (TH-IR) or VMAT2 cell counts were compared by a paired students'*t*-test and the inter group differences were analyzed using one-way ANOVA.

RESULTS

TH and VMAT2- immunohistochemistry. Two months after striatal 6-OHDA-induced lesion, the number of neurons in the LC of L + V and lesion groups was decreased ($P<0.01$) (Fig .1C and 1G, compared with L + E group Fig. 1B and 1F). However, the rats in the L + E group had more intact neurons in the LC ($P<0.05$) compared with lesion and L + V groups and obviously less intact neurons in comparison with the SH group (Fig .1A and 1E). The levels of VMAT2 ISI were inversely related to the amount NM pigment in LC. In the brain of L + V group, the LC neurons contain high levels of NM pigment and faint amount of VMAT2 ISI (Fig .1C and 1G) whereas, neurons in the brain of L + E group exhibited 2.9-fold VMAT2 ISI higher than the L + V group and had low detectable NM pigment (Fig. 1B and 1F). The examined TH ISI for LC neurons was lower for lesion and L + V groups than SH and L+E groups. In SH group, there is little NM pigment and LC neurons were stained intensely with TH-IR whereas in lesion and L + V groups, LC neurons were stained faintly. Neurons of LC in lesion and L + V groups contained low levels whereas in L + E group contain high levels of TH-IR. Neurons in SH group also were stained intensely. In different groups, (DA cell contained different intensities of VMAT2 and TH-IR. In all brains examined, cells with the darkest ISI were found within SH groups and intermediate ISI was observed in L + E groups. Neurons in the lesion and L + V groups had the lowest ISI values. For cells in the lesion and L + V groups and SH and L + E groups, this relationship was quantified, VMAT2 ISI was lowest within the lesion and L + V groups and highest within the SH group with controlling the effects of the immunostaining protocol on intensity of the immunostaining reaction.

When high concentration of VMAT2 antibody was employed (1:500), the neurons in L + V group and in L + E group exhibited low and high ISI values, respectively (Fig. 1). On the other hand, when low VMAT2 concentration was employed (1:1000), neurons in the L + V group exhibited no immune-

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reactivity at all and were only identified by their NM pigment content.

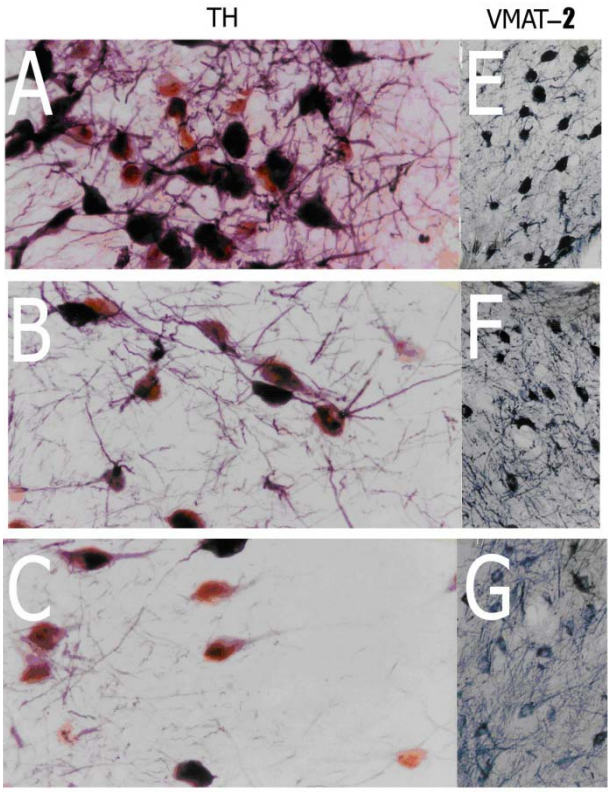

Fig. 1. Photomicrographs of typical frontal sections through the LC showing TH-immunohistochemically stained of LC (left side) and VMAT2 immunoreactivity stained neurons (right side), in SH (**A** and **E**), in L + V (**B** and **F**) and L + E **(C** and **G)** groups. There is an inverse relationship between VMAT2 immunoreactivity and neuromelanin pigment content. In L+V group neurons stain a little darkly for VMAT2, and a lot of NM pigment is visible within immunostained cells. LC neurons in L + E group stain very darkly with VMAT2 but contain some NM pigment granules golden brown) as in Figure 1 (Marker = 70 μm). A severe reduction in the number of neurons is observed in the L + V group, but no such marked decrease is noted in the L + E group in comparison with the SH group. Furthermore, in the L + E group there exist a greater number of TH-IR and VMAT2 neurons in LC than the L + V group compared to the SH group (Scale bar = 30 μm). LC, locus coeruleus; TH-IR, tyrosine hydroxylase-immunoreactive; VMAT2, vesicular monoamine transporter 2 and NM, neuromelanin pigment.

DISCUSSION

There are strong evidences that oxidative stress participates in the etiology of PD [4]. We designed this study to investigate the neuroprotective effect of vitamin E in the early model of PD. In this study, TH immunohistochemical analyses showed a reduction of 20% in LC cell number of vitamin E pretreated lesioned group but the cell number dropped to 60% in the lesioned group. The ISI of the cells was measured for VMAT2 in LC neurons. Lesioned group had the lowest VMAT2 ISI of all neurons and there was an inverse relationship between VMAT2 ISI and NM pigment in the locus and also neurons with the highest VMAT2 ISI had high TH ISI. Consistent with our findings, Hirisch *et al*. [28] also demonstrated that in midbrain and Pons DA neurons are an inverse relationship between NM pigment content and VMAT2 immunoreactivity.

In fact, some of the neurons make disproportionately more amine than can be stored in vesicles. Although the substantia nigra DA neurons that reside in the ventral portion of the nucleus (SNv) neurons possess relatively low levels of TH, they must also have proportionately lower levels of VMAT2 such that there is still a pool of DA that does not get stored in synaptic vesicles, which can be oxidized to ultimately from NM pigment [24, 29, 30]. The aminergic cell death in PD may depend upon several factors. Cells with relatively high levels of NM, like the LC and SNv neurons, may reflect neurons with relatively large pools of cytosolic amine that exist in addition to those in synaptic vesicles. This pool can be used for dendro-dendritic communication in the SN [31], a similar amine pool in the LC may also exist [32]. It can be hypothesized that when a sizeable non-vesicular pool of amine exists in the cytoplasm for a prolonged period of time, ROS are formed along with toxic DA-adducts. The ROS and adducts are stored in lysosomal structures which constitute the NM pigment granule [33]. It may be that after the neuron has accumulated an excessive amount of NM pigment, additional ROS and toxic DA-adducts can no longer be stored in the form of NM pigment, and once the toxins are in the cytoplasmic compartment the neuron becomes poisoned [34].

PD is known to be a chronic and progressive neurodegenerative disease caused by a selective DAergic of DAergic neurons in the brain. A large body of experimental evidence indicates that the

Table 1. Average total number of TH-immunostaining neurons on the LC.

	SH (n = 8)	L + V (n = 8)	L + E (n = 8)
LC	66.01 ± 4.25*	25 ± 3.98**	50.14 ± 3.30*
Reduction (%) (compared to SH)		60	20

$P<0.05$, *$P<0.05$ (L + E versus L + V) and **$P<0.01$ (compared with the SH group). LC, locus coeruleus; SH, sham operated group; L + V, vehicle-treated lesion group; L + E, vitamin E-treated lesion group.

factors involved in the pathogenesis of this disease are several occurring inside and outside the DAergic neuron [13]. Within few days following injection of 6-OHDA, TH-positive terminals in targeted region begin to undergo atrophy and degeneration. However, at least 1 month is needed for the complete degeneration of cell bodies in the LC [25- 27].

Some possible mechanisms may lead to the disappearance of LC neurons following striatal injury: one of the possibilities is the loss of striatal trophic support provided in retrograde fashion to LC DAergic neurons. The other possibility is chemical damage of the DAergic terminals in the striatum may cause the degeneration [13]. The important factor that contributes to aminergic cell death in PD relates to mitochondrial function and mitochondrial dysfunction has been implicated as an important trigger for PD like pathogenesis because exposure to the environmental mitochondrial toxin leads to PD-like pathology [35, 36].

Since vitamin E is an effective free radical scavenger in the brain, its neuroprotective function is the issue of new therapeutic approaches in neurodegenerative diseases [37]. In clinical trials, the vitamin E therapy might have retarded the progression of degenerative process in patients with some cortical diseases. Roghani and Behzadi [13] indicated that repeated i.m. administration of vitamin E exerts a rapid protective effect on the nigrostriatal DAergic neurons in the early model of PD. Antioxidant acts via Phosphatidylinositol-3 Kinase pathway to protect rat cortical neurons against cell death induced by NM and hydrogen peroxide. An unbalanced over production of ROS may give rise to oxidative stress which can induce neuronal damage, ultimately leading to neuronal death by apoptosis. Our data showed that dietary vitamin E may have a neuroprotective effect attenuating the risk of PD and is well match with the results of Roghani and Behzadi [13] and Etminan *et al*. [14]. More LC TH - IR neurons in vitamin E-pretreated animals as compared with 6-OHDA-lesioned group may be due to the preservation on neuronal resistance and probably to the recovery of atrophying neurons. Based on our result, we believe vitamin E slows functional decline in early model of PD. Therefore, people with relative PD, that have a greater chance of developing the disease, may decrease neurodegeneration by taking this supplement.

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