Anti-apoptotic effects of valproic acid treatment on dopaminergic neuronal loss in a 6-hydroxydopamine model of Parkinson's disease in rats

Sıçanlarda 6-hidroksidopamin modeli ile oluşturulan Parkinson hastalığında valproik asit tedavisinin dopaminerjik nöronal kayıp üzerindeki anti-apoptotik etkileri

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Abstract

Aim: Parkinson's disease (PD) is characterized by the progressive loss of dopaminergic neurons resulting in deterioration of motor activity in patients. Currently, available therapies including Levodopa (L-DOPA) are more geared toward the treatment of symptoms. Therefore, developing effective neuroprotective therapies is needed. Valproic acid (VPA) has shown potent neuroprotective effects on dopamine (DA) neurons in various brain regions. The aim of this study is to investigate whether VPA attenuates the neuronal loss when co-treated with L-DOPA in a 6-hydroxydopamine (6-OHDA) induced PD model in rats.

Methods: Male Wistar Albino rats received intranigral injection of 6-OHDA unilaterally. Twelve days later rats received either saline, L-DOPA, VPA, or L-DOPA+ VPA for 9 days. To determine whether rats had dopaminergic neuronal loss apomorphine-induced rotation test was used. Immunohistochemical analyses were performed in the Substantia Nigra pars compacta (SNpc) by measuring the tyrosine hydroxylase (TH) positive neurons and the apoptotic neurons.

Results: 6-OHDA injection showed clinically impairment of the motor function with histologically significant damage to the dopaminergic neurons. VPA administration combined with L- DOPA protected neurons in SNpc by increasing the TH positive neurons and by decreasing the apoptotic neurons. L-DOPA given as a monotherapy, on the other hand, was ineffective on these parameters.

Conclusion: Our experiments demonstrated that VPA had a neuroprotective effect when used with L-DOPA in the PD rat model.

Keywords: apoptosis; Parkinson's disease; rat model; tyrosine hydroxylase; valproic acid; 6-hydroxydopamine

Öz

Amaç: Parkinson hastalığı (PH), hastalarda motor aktivitede bozulma ile sonuçlanan dopaminerjik nöronların ilerleyici kaybı ile karakterizedir. Levodopa (L-DOPA) dâhil olmak üzere mevcut tedaviler daha çok semptomların tedavisine yöneliktir. Bu nedenle, etkili nöroprotektif tedavilerin geliştirilmesine ihtiyaç vardır. Valproik asit (VPA), çeşitli beyin bölgelerindeki dopamin (DA) nöronları üzerinde güçlü nöroprotektif etkiler göstermiştir. Bu çalışmanın amacı, sıçanlarda 6-hidroksidopamin (6-OHDA) ile indüklenen PH modelinde VPA'nın L-DOPA ile birlikte tedavi edildiğinde nöronal kaybı azaltıp azaltmadığını araştırmaktır.

Yöntemler: Erkek Wistar Albino sıçanlarına tek taraflı olarak intranigral 6-OHDA enjeksiyonu yapıldı. On iki gün sonra sıçanlara, 9 gün boyunca salin, L-DOPA, VPA veya L-DOPA+ VPA verildi. Sıçanlarda dopaminerjik nöron kaybı olup olmadığını belirlemek için apomorfin kaynaklı rotasyon testi kullanıldı. Substantia Nigra pars compacta'da (SNpc) tirozin hidroksilaz (TH) pozitif nöronlar ve apoptotik nöronlar ölçümleri için immünohistokimyasal analizler yapıldı.

Bulgular: 6-OHDA enjeksiyonu, klinik motor fonksiyon bozukluğu ve dopaminerjik nöronlarda önemli hasar gösterdi. VPA tedavisi, TH pozitif nöronları artırarak ve apoptotik nöronları azaltarak SNpc'deki nöronları korumuştur. L-DOPA ise bu parametreler üzerinde etkisizdi.

Sonuç: Deneylerimiz, PH sıçan modelinde L-DOPA ile birlikte kullanıldığında VPA'nın nöroprotektif bir etkiye sahip olduğunu göstermiştir.

Anahtar Sözcükler: apoptoz; Parkinson hastalığı; sıçan modeli; tirozin hidroksilaz; valproik asit; 6-hidroksidopamin

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INTRODUCTION

Parkinson's Disease (PD) is a neurodegenerative disorder characterized by the progressive loss of DA neurons in SNpc. The prevalence is 0.3% in the industrialized population and 1% in patients over the age of 50. The available treatment of PD is generally symptomatic, therefore, understanding the mechanisms of the PD pathogenesis is crucial in the development of new therapies for PD (1).

Levodopa (L-DOPA) has been one of the major pharmacologic aids in the treatment of PD by supplying dopamine (DA) to the central nervous system. However, the symptomatic relief is not long-lasting due to increased oxidative stress, other adverse effects, and motor control fluctuations that occur in the long run (2). L-DOPA-induced dyskinesia has also been investigated extensively. Carbidopa or benserazide are combined with L-DOPA in order to inhibit peripheral decarboxylation of the drug. Decreasing the peripheral conversion of L-DOPA to dopamine, thus decreasing the peripheral side effects and increasing levodopa bioavailability in the central nervous system (CNS) (3).

Although L-DOPA has been used for many years, due to its adverse effects other pharmacologic approaches are being investigated. Valproic acid (VPA) is being used in the treatment of epilepsy, migraine, schizophrenia, and bipolar disorders. It can cross the blood-brain barrier and acts by increasing GABA activity, blocking Ca^{+2} and Na^{+} channels, and decreasing NMDA-mediated excitation. It has also been demonstrated that VPA pretreatment protects dopaminergic neurons against the toxic effects of lipopolysaccharides (LPS) in cell cultures and suppresses the activation of microglia by LPS (4). VPA treatment prevents 1-methyl-4-phenylpyridinium (MPP+) induced death of dopaminergic neurons in an *in vitro* model of midbrain neuron-glia cultures. Importantly, Kidd and Schneider showed that prophylactic VPA treatment has protective effects in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD.

VPA has also been shown to inhibit histone deacetylases (HDACs) (5). Histone deacetylation is related to a more condensed chromatin state and transcriptional repression. The relaxed chromatin, also known as heterochromatin, represses gene transcription. Therefore, the balance between the activities of histone deacetylases and histone acetyltransferases is important in gene regulation. Histone deacetylases target various non-histone proteins such as transcription factors, cytoskeleton proteins, and cellular proteins (6). Histone deacetylase (HDAC) inhibitors are considered neuroprotective by increasing acetylation levels in the brain and by affecting many genes involved in cell cycle regulation, apoptosis, and DNA repair processes (7).

Ribosomal S6 kinase (RSK) has also been implicated as an antiapoptotic protein which can be very important in PD. It is a family of serine-threonine kinases which has important functions in cell proliferation, differentiation, and survival. RSKs are regulated by the Ras/mitogen-activated protein kinase pathway (MAPK) and have two main classes: the P90RSK (MAPK-activated protein kinase) and the P70RSK (s6 kinase) (8).

The main purpose of this study was to investigate the neuroprotective effects of VPA and discover whether VPA can be an adjunct treatment to L-DOPA. We measured Tyrosine hydroxylase activity and a total number of apoptotic neurons, using TUNEL assay, in the right SNpc of rats by 6-OHDA induced neurodegeneration PD model. Western blot (WB) analyses were, also, performed to quantify Phospho-S6 Ribosomal Protein (Ser235/236) & Phospho-p90RSK (Ser380) aiming to explain the molecular mechanism of neuroprotection.

MATERIALS AND METHODS

Animal Procedures

Adult male Wistar albino rats (250-300 g) were obtained from the Yeditepe University Experimental Research Center (YUDETAM). All experimental protocols were approved by the Ethical Committee of Yeditepe University Experimental Research Center (Approvel number: 334, date: 02.04.2013) and the use of animals was in compliance with the US National Institutes of Health Guide for Care and Use of Laboratory Animals. Animals were maintained in standard housing conditions with constant temperature, humidity, 12-hour light/dark cycles, and *ad-libitum* for food and water.

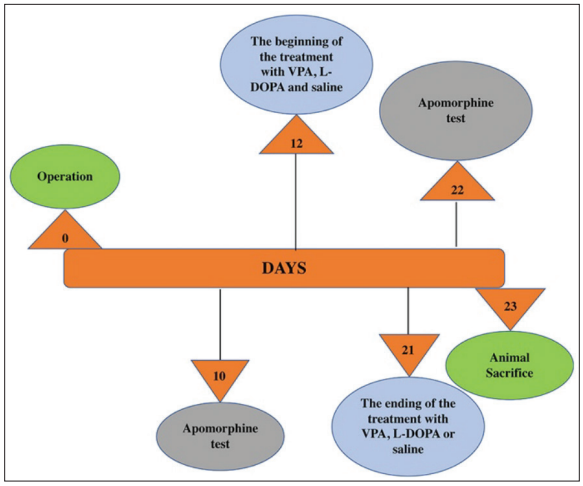

Figure 1: The time schedule of the experiments.

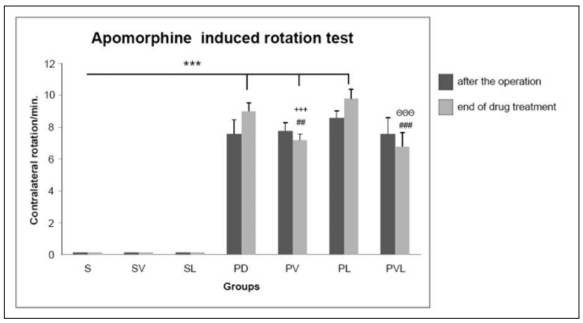

Figure 2. Apomorphine induced rotational behavior of the animals. Apomorphine induced rotation test was performed after the operation -dark colored bars- and at the end of drug treatment -light colored bars-. Sham operated (S), Sham operated and VPA treated (SV), Sham operated and L-DOPA treated (SL), 6-OHDA injected into the substantia nigra (PD), 6-OHDA injected into the substantia nigra and VPA treated (PV), injected into the substantia nigra 6-OHDA injected and L-DOPA treated (PL), injected into the substantia nigra 6-OHDA injected and VPA and L-DOPA treated (PVL) groups. Data are presented as contralateral rotations per minute. $^{***}p<0.001$ compared with S, SV and SL groups, $^{\#\#}p<0.01$. $^{\#\#\#}p<0.001$ compared with PD group, $^{\Theta\Theta\Theta}p<0.001$ compared with PL group. Data are expressed as mean ± standard error

42 rats were randomly divided into 7 experimental groups as follows: sham operated (S), sham operated and VPA treated (SV), sham operated and L-DOPA treated (SL), 6-OHDA injected into the substantia nigra (PD), 6-OHDA injected into the substantia nigra and VPA treated (PV), 6-OHDA injected into the substantia nigra and L-DOPA treated (PL), 6-OHDA injected into the substantia nigra and VPA and L-DOPA treated (PVL). Each group comprised of 6 animals (Table 1).

All animals were anesthetized by intramuscular (IM) injections of xylazine (10 mg/kg) and ketamine (80 mg/kg). After they were deeply anesthetized, rats were fixed in a stereotaxic frame in the flat skull position. The scalp was shaved, and a small central incision was made to expose the skull. Bregma and lambda points were located.

The coordinates were anterior/posterior from bregma (AP) = -4.8 mm, medial/lateral (ML) = -1.8 mm and dorsal/ventral (DV) = -8.2 mm. The coordinates were confirmed according to the Paxinos rat brain atlas (9). Freshly prepared 6-OHDA (8 µg/per rat in 2 µL saline with 0.1 percent ascorbic acid) was infused with a 5 µL, 26-gauge Hamilton syringe at a flow rate of 0.2 µL/min into the right SNpc. Sham operated animals were subjected to the same procedure but instead of 6-OHDA, 2 µL vehicle (0.9 percent saline containing 0.1 percent w/v ascorbic acid) was injected into the SNpc. Due to the restrictions of the ethics committee, we did not have a non-lesioned sham group. In the literature, generally, investigators did not observe significant differences between lesioned and non-lesioned groups (10). The needle was left in place for an additional 5 minutes before retraction. Then, the surgical area was sutured. All operations were performed under aseptic conditions.

Treatment of L-DOPA, benserazide, and VPA acid in rats

All rats were intraperitoneally (IP) injected either with valproic acid, or L-DOPA combined with benserazide hydrochloride or saline for 10 days after the surgical operation. The rats in SL and PL groups were treated with L-DOPA (10 mg/kg per day) combined with (2 mg/kg per day) to inhibit peripheral decarboxylation of L-DOPA. Rats in S and PD groups were IP injected with saline. In SV and PV groups the rats received IP injections of valproic acid (300 mg/kg per day). In the PVL group, the rats received intraperitoneal injections of valproic acid (300 mg/kg per day) and L-DOPA (10 mg/kg per day) combined with benserazide hydrochloride (2 mg/kg per day) 14. All of the drugs were dissolved in saline (11).

The sequence of operation, apomorphine test, and drug treatments are explained (Figure 1). The apomorphine test was carried out twice after the opera-

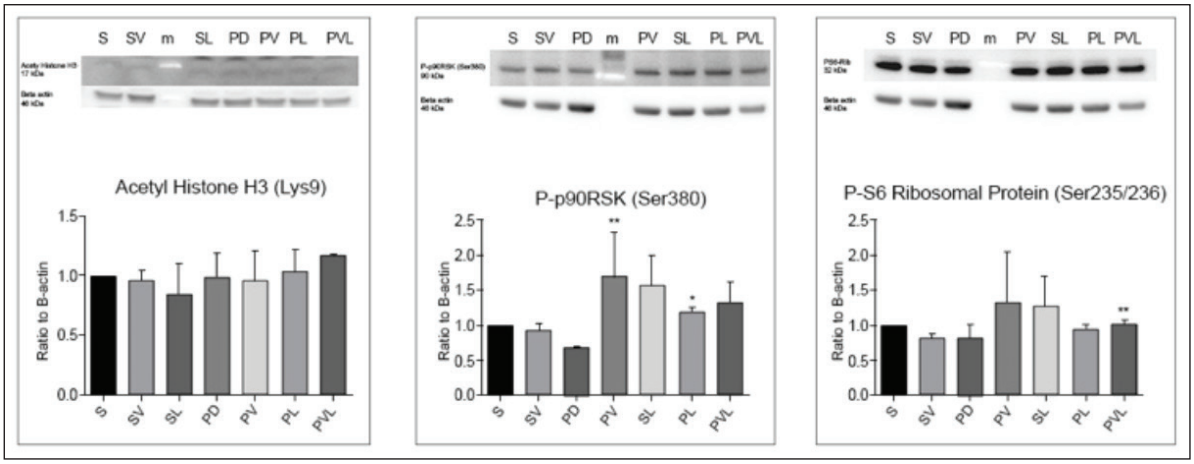

Figure 3. Western blot analysis for 17 kDA band of Acetyl-Histone H3 (Lys9), 42 kDA band of Phospho-p90RSK Ser380) and 90 kDA band of Phospho-S6 Ribosomal Protein (Ser235/236) in striatal tissue. β-actin (42 kDA) was used as an internal control. Sham operated (S), Sham operated and VPA treated (SV), Sham operated and L-DOPA treated (SL), 6-OHDA injected into the substantia nigra (PD), 6-OHDA injected into the substantia nigra and VPA treated (PV), 6-OHDA injected into the substantia nigra and L-DOPA treated (PL), 6-OHDA injected into the substantia nigra and VPA and L-DOPA

tion. Unilateral injection of 6-OHDA leads to asymmetric circling motor behavior after the administration of dopaminergic drugs such as apomorphine, due to physiologic imbalance between the lesioned and non-lesioned sites. This circling behavior gives reliable information on the degree of the lesion in SNpc (12).

Apomorphine induced rotation

Ten days after 6-OHDA or saline injection to the SNpc, all rats were evaluated for apomorphine induced rotations. All animals were subcutaneously (SC) injected with 0.5 mg/kg apomorphine and were immediately placed in a plastic cylinder of 20 cm diameter with a height of 28 cm. The rotational behavior was monitored for 30 minutes. All observations were made between 9 a.m. and 12 p.m. It has been shown that more than 5 contralateral rotations account for approximately 90 % dopamine loss in the striatum (13). As such only the rats showing pronounced rotational behavior (more than 5 contralateral turns/min) were selected for the study.

Tyrosine Hydroxylase immunohistochemistry processing

Tyrosine hydroxylase is the rate-limiting enzyme in the biosynthesis of dopamine; it catalyzes the conversion of L-tyrosine to L-DOPA, so it is a selective and specific indicator of dopaminergic neurons in SNpc in PD.

After the last measurement, animals were immobilized and anesthetized by an experienced animal technician and were decapitated in the guillotine and their brains were removed. The brains were rapidly frozen on dry ice and stored at -80°C until used. The striata of the brains were used for the biochemical analysis of oxidative stress and Western blot experiments.

The brains of the animals were embedded in a mounting medium (Tissue-Tek O.C.T. Compound, Sakura, Finetek USA,) and cut into 16 µm coronal sections on a cryostat (Leica) at -20°C. Coronal sections were taken through substantia nigra (-4.8 mm posterior to bregma) and placed on poly-l-lysine coated slides. The slides were dried on a slide warmed at 40°C to dehydrate and ensure effective tissue adhesion to the slides. Then, they were stored at -80°C until used.

Prior to the histological staining, the tissue slides were incubated for 20 minutes in the oven (37°C). Then, they were immersed in 10% neutral formaldehyde for 20 minutes and rinsed with 2x phosphate-buffered saline (PBS). Tissue sections were placed in 0.1% sodium citrate and 0.1% Triton X-100 for 4 minutes at 4°C. Endogenous peroxidase activity was halted by incubating the slides in 0.3% H_2O_2 solution for 30 minutes at 21°C. The slides were washed with 1x PBS. Then, they were blocked for nonspecific binding with the Vectastain Universal Quick kit (RTU Vectastain; Vector Laboratories, Burlingame, CA, USA).

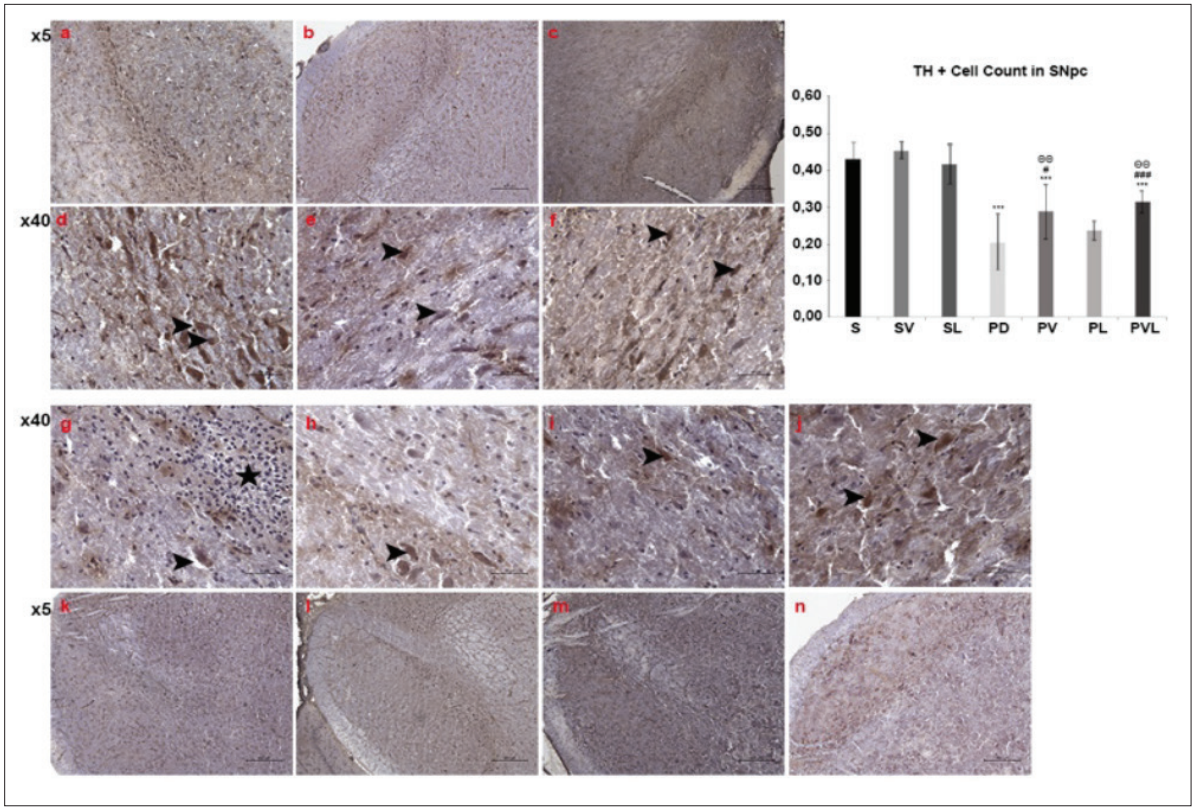

Figure 4. Photomicrographs demonstrate Tyrosine Hydroxylase immunoreactivity in right SNpc. Dopaminergic neurons are demonstrated with arrowhead and lymphocyte infiltration is demonstrated with asterisk. The location of the substantia nigra is evident in x5 magnifications (scale bar: 400 µm). Dopaminergic neurons are marked at x40 magnifications (scale bar: 50 µm). Sham operated (a-d), Sham operated and VPA treated (b-e), Sham operated and L-DOPA treated (c-f), 6-OHDA injected into the substantia nigra (g-k), 6-OHDA injected into the substantia nigra and VPA treated (h-l), 6-OHDA injected into the substantia nigra and L-DOPA treated (i-m), 6-OHDA injected into the substantia nigra and VPA and L-DOPA treated (j-n) groups It is apparent that dopaminergic neurons are clearly seen in the Sham operated, Sham operated and VPA treated, and Sham operated and L-DOPA treated groups, while neurons are decreased in the 6-OHDA injected group.

Tissue samples were incubated in 1:1000 dilution of the primary antibody (code n. AB152, Sigma-Aldrich, Merck, Darmstadt, Germany) overnight at 4°C in a humidified chamber. Then, the slides were rinsed with 1x PBS. Biotinylated secondary antibody (anti-rabbit/mouse/goat IgG) was applied to the sections for 30 minutes in humidified chambers, at room temperature. Streptavidin peroxidase preformed complex solution was applied to the sections for 30 minutes at room temperature in humidified chambers. The slides were washed with 1x PBS. Finally, chromogen diaminobenzidine tetrahydrochloride (TA 125 TD; Thermo Fisher Scientific, Leicestershire, UK) was used for the detection. They were counterstained with Mayer's hematoxylin and dehydrated in alcohol, cleared in xylene and cover slipped in Entellan mounting medium.

In Situ Cell Death Detection [Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)] Processing

TUNEL assay is a reliable method to measure neuron apoptosis death. In this technique, terminal deoxynucleotidyl transferase (TdT) enzyme adds fluoresceine labeled nucleotides to the free 3'-OH DNA ends which are, then, visualized with an anti-fluorescein antibody labeled with Peroxidase (POD) and substrate reaction.

The sections were dried at 37°C for 20 minutes and then were fixed in 4% paraformaldehyde (pH 7.4) solution for 20 minutes at room temperature. Then, the slides were washed twice with 1x PBS (pH 7.4) and were permeabilized in permeabilization buffer (0.1% tri-sodium citrate dihydrate, 0.1% triton X-100 in distilled water) for 4 minutes and washed with PBS. This

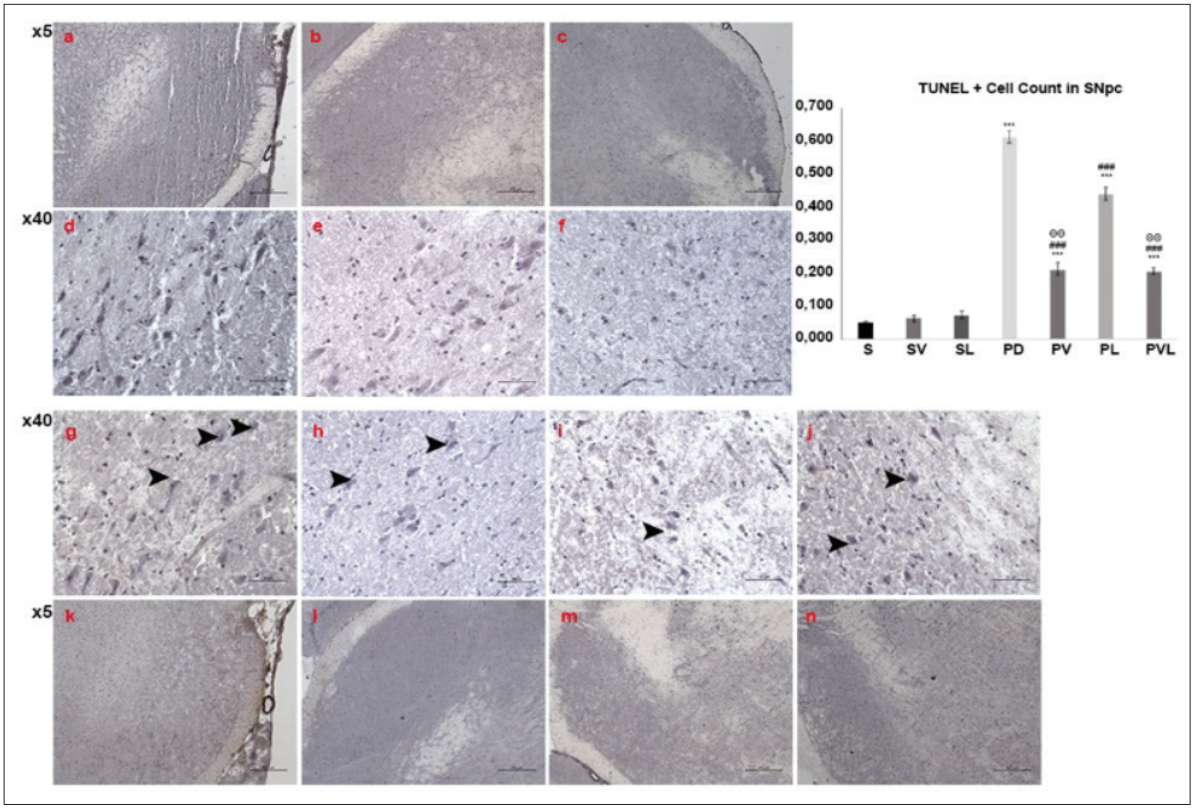

Figure 5. Photomicrographs demonstrate TUNEL staining in right SNpc. The arrowheads indicate nuclear fragmentation in dopaminergic neurons (TUNEL positive neurons). The location of the substantia nigra is evident in x5 magnifications (scale bar: 400 μm). Apoptotic dopaminergic neurons are marked at x40 magnifications (scale bar: 50 μm). Dopaminergic neurons are marked at x40 magnifications (scale bar: 50 μm). Sham operated (a-d), Sham operated and VPA treated (b-e), Sham operated and L-DOPA treated (c-f), 6-OHDA injected into the substantia nigra (g-k), 6-OHDA injected into the substantia nigra and VPA treated (h-l), 6-OHDA injected into the substantia nigra and L-DOPA treated (i-m), 6-OHDA injected into the substantia nigra and VPA and L-DOPA treated (j-n) groups It is apparent that apoptotic dopaminergic neurons are clearly seen in the 6-OHDA injected and 6-OHDA injected and L-DOPA treated groups, while apoptotic dopaminergic neurons are decreased in the 6-OHDA injected and VPA treated and 6-OHDA injected and VPA and L-DOPA treated groups.

step was repeated twice at 4°C. Slides were blocked with Vectastain Universal Quick kit (RTU Vectastain; Vector Laboratories, Burlingame, CA, USA). The sections were incubated in a TUNEL reaction mixture (45 μl label solution was mixed with 5 μL enzyme solution for one section) in dark, 37°C humidified chambers for 60 minutes. Then, the slides were washed with PBS three times for 5 minutes each. Converter-POD was added to the sections and incubated in dark, 37°C humidified chambers for 30 minutes. Then, the slides were thrice washed with PBS at room temperature. The reaction was visualized by using DAB (1000 μL DAB substrate buffer was mixed with 50 μL DAB chromogen) solution as a chromogen. The sections were counterstained with Mayer's hematoxylin and rinsed in tap water. Sections were dehydrated in increasing alcohol concentrations, cleared in xylene, and coverslipped in Entellan mounting medium (13).

Analysis of Tyrosine Hydroxylase Activity and Apoptotic Neurons

Tyrosine hydroxylase (TH) positive neurons and TUNEL positive neurons were counted in SNpc. Each section was examined via a stereological workstation equipped with a CCD digital camera (Optronics Microfire 1600x1200P, Goleta, CA, USA), computer-assisted motorized stage (Bioprecision, Howtrone, NY, USA), microcator (Heidenhein, Traunreut, Germany), image card (ATI FireGL Advance Micro Device, Camberly, UK) and light microscope (Leica DM 4000B, Wetzlar, Germany). Both right (lesioned side) and left (control side) SNpc of the rat brains were traced and

Table 1: The table shows the sequence of operation, apomorphine test and drug treatments

Group Name	Number of Animal	Treatments
Sham operated (S)	6	0.9 per cent saline containing 0.1 per cent w/v ascorbic acid
Sham operated and VPA treated (SV)	6	Valproic acid (300 mg/kg) IP
Sham operated and L-DOPA treated (SL)	6	Levodopa (10 mg/kg) combined with benserazide hydrochloride (2 mg/kg) I.P.
6-OHDA injected into the substantia nigra (PD)	6	6-hydroxydopamine (8 µg/per rat in 2 µl saline with 0.1 per cent ascorbic acid)
6-OHDA injected into the substantia nigra and VPA treated (PV)	6	Valproic acid (300 mg/kg) IP
6-OHDA injected into the substantia nigra and L-DOPA treated (PL)	6	Levodopa (10 mg/kg) combined with benserazide hydrochloride (2 mg/kg) IP
6-OHDA injected into the substantia nigra and VPA and L-DOPA treated (PVL)	6	Valproic acid (300 mg/kg) and Levedopa (10 mg/kg) combined with benserazide hydrochloride (2 mg/kg) IP

IP: Intraperitoneal, L-DOPA: Levodopa, VPA: Valproic acid, 6-OHDA: 6-hydroxydopamine

neurons were counted with a meander scan. Dopaminergic neurons were clearly distinguished depending on their shape and size.

Tyrosine hydroxylase and apoptotic index were calculated as TH positive neurons/total neurons and apoptotic neurons/total neurons respectively in the total substantia nigra. When analyzing apoptotic cells, shrinkage in the cytoplasm of neurons, fragmentation in their nuclei, and alteration of compact chromatin in the nucleus were examined. Apoptotic neurons have pyknotic nuclei (14).

Statistical Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences statistical analysis program version 25.0 (SPSS Inc., Chicago, IL, USA). Descriptive analyses were presented using mean ± standard deviation for continuous data. Normality distribution assumption was tested by using Shapiro-Wilk's test. Homogeneity of variances assumption was tested using Levene's test. Since the variables were normally distributed and variances were homogenous, ANOVA was used to compare the group means. For the post-hoc analysis, Least Significant Difference (LSD) test was applied for multiple comparisons. A 5% type-I error level was used to infer a statistical significance. In our study, we performed post-hoc power analysis with software G*Power, (Version 3.0.10). Post-hoc power is the retrospective power of an observed effect, based on the sample size and parameters that provide estimates from a given data set.

RESULTS

VPA and VPA L-DOPA combination reduced Apomorphine induced rotation test

Apomorphine induced rotation test was performed in order to control the validity of the model. When the dopaminergic neurons die unilaterally in SNpc, the animals demonstrate a rotational behavior contralateral to the lesioned side. The number of the rotations prompts the severity of the model. Apomorphine-induced rotation test was performed after the operation (dark-colored bars) and at the end of drug treatment (light-colored bar) (Figure 2).

Sham operated groups did not show any rotational behavior. After the operation, all of the 6-OHDA lesioned animals showed similar pronounced rotational behavior compared to the sham operated control animals ($p<0.001$). After VPA treatment, in PV group the rotational behavior slightly decreased compared to the PD and PL groups ($^{**}p<0.01$ and $^{***}p<0.001$ respectively). Similarly, in PVL group rotational behavior significantly decreased compared with PD and PL groups ($^{**}p<0.01$ and $^{***}p<0.001$ respectively).

Acetyl Histone H3 (Lys9), Phospho-S6 Ribosomal Protein (Ser235/236) & Phospho-p90RSK Ser380)

Histone acetylation level was determined with Western blotting method to investigate whether valproic acid exerts its effects via modulating epigenetic mechanisms. For all of these experiments, the removed stria-

tal tissue of the animals was used and protein isolation was performed. The Acetyl Histone H3 (Lys9) levels in the PVL group were increased but not statistically significant (p=0,8892).

When Phospho-S6 Ribosomal Protein (Ser235/236) analyses were evaluated, we observed increased levels in the PL group compared to PD group and in the PV group compared to PD group ($^{*}p<0.05$ and $^{**}p<0.01$ respectively).

When Phospho-p90RSK (Ser380) analyses were examined, we observed an increase in the PVL group compared to PD group (p<0.01) treated (PVL) groups, m is marker. $^{**}p<0.01$ and $^{*}p<0.05$ vs PD. The data were all expressed as mean ± standard error of the relative intensity with respect to β-actin (Figure 3).

6-OHDA selectively induced dopaminergic degeneration, VPA and VPA L-DOPA treatment increased Tyrosine Hydroxylase in right SNpc/ Total SNpc Neurons

Coronal sections from all the groups were analyzed for tyrosine hydroxylase immunoreactive neurons. Immunohistochemistry for tyrosine hydroxylase gives reliable data on the total number of dopaminergic neurons. Photomicrographs demonstrate sections taken from the right SNpc stained with tyrosine hydroxylase (TH) (Figure 4).

The number of TH positive neurons was not different between S, SV, and SL groups. There was a pronounced loss of tyrosine hydroxylase positive neurons in 6-OHDA lesioned right SNpc in PD group as compared to the sham operated groups ($^{***}p<0.001$). VPA treatment significantly increased the number of tyrosine hydroxylase positive neurons in PV group as compared to PD group ($^{*}p<0.5$). Both VPA treatment and L-DOPA treatment significantly increased the number of tyrosine hydroxylase positive neurons in PVL group as compared to PD and PL groups ($^{***}p<0.001$ and $^{**}p<0.01$). However, the numbers of tyrosine hydroxylase positive neurons were still significantly lower in PV group as compared to S, SV and SL groups ($^{***}p<0.001$). The number of tyrosine hydroxylase positive neurons was not different between PD and PL groups (Figure 4).

6-OHDA selectively increased TUNEL positive neurons, VPA and VPA L-DOPA treatment decreased apoptotic neurons in right SNpc/ Total SNpc Neurons

Coronal sections from all the groups were analyzed for TUNEL positive neurons. TUNEL assay gives reliable data on the number of apoptotic neurons. Photomicrographs demonstrate sections taken from the right SNpc (Figure 5).

The number of TUNEL positive neurons was not different in SNpc of the animals between S, SV, and SL groups. 6-OHDA injected into the SNpc induced apoptosis in PD group as compared to all other groups ($^{***}p<0.001$). Valproic acid treatment significantly attenuated 6-OHDA induced apoptosis in PV and PVL groups as compared to PD group ($^{***}p<0.001$). However, the number of TUNEL positive neurons was still higher in PV group as compared to S and SV groups ($^{***}p<0.001$).

DISCUSSION AND CONCLUSION

In the present experiment, we were not able to observe any changes in histone 3 acetylation. We observed VPA and VPA combined with L-DOPA decreased the apomorphine induced rotations as compared to 6-OHDA lesioned and saline-treated animals. In addition to these results, we observed TH positive dopaminergic neurons and the apoptosis rate was decreased in the VPA treated animal.

Parkinson's disease is one of the most debilitating neurologic diseases. VPA is a well-tolerated, commercially available, and cheap medication. It has a high bioavailability rate in both humans and rodents. In addition to its inhibitory effects on sodium channels, calcium channels, and GABA transaminase, VPA is a histone deacetylase inhibitor as well. Recent studies indicate the neuroprotective effects of histone deacetylase inhibitors in various neurodegenerative disorders (15,16). Valproic acid decreases neuronal death through increasing histone 3 acetylation which increases the transcription of many anti-apoptotic proteins (17). Kidd and Snyder used 400 mg/kg of VPA and decapitated the animals right after the experiment.

They observed that VPA administration promoted histone hyperacetylation in the brain (5). On the other hand, Lei et al. observed improvement in locomotor function after spinal cord injury in rodents after administering a smaller dose of 300 mg/kg VPA twice daily and, also, decapitated the animals right after the experiment (17). Based on their finding, we decided to administer the same amount and dosing of VPA. However, we were not able to observe any changes in histone 3 acetylation in the present experiment. This can be due to the timing of the animal decapitation.

PD pathogenesis involves dopaminergic neuronal death. Although, VPA has been shown to decrease L-DOPA dyskinesia it has been blamed as a cause of PD by researchers (18). However, in a comprehensive review, Mahmoud et al. could not establish a relationship with either the dosage of VPA or its plasma level (19). Nonetheless, due to the use of neuroleptics in the elderly safe conclusions regarding VPA parkinsonism could not be extracted.

6-OHDA leads to the degeneration of dopaminergic neurons (20). 6-OHDA targets dopamine neurons via its specific affinity to DA transporters on dopaminergic neurons and its use in rats closely mimics PD. Once in the dopamine neurons, it increases the amount of reactive oxygen species while decreasing the activities of the antioxidant systems, leading to dopaminergic neuronal death. It also interferes with the mitochondrial respiratory chain which further increases the reactive oxygen species (15). Because 6-OHDA cannot cross the blood-brain barrier, it should be injected into SNpc, the medial forebrain bundle, or the striatum. In this study, 6-OHDA was injected into the right SNpc to induce rapid degeneration of the neurons, while the left non-lesioned side served as an internal control. The advantage of 6-OHDA in PD model over other methods is that, the validity of the method can be assessed while the animals are alive by the SC administration of apomorphine or other dopaminergic stimulants (12,16). In the present experiment, VPA and VPA combined with L-DOPA decreased the apomorphine induced rotations as compared to 6-OHDA lesioned and saline treated animals. These results might indicate that VPA diminishes the turning behavior by inhibiting neuronal loss.

In the present study, our models showed a clinical picture resembling subacute PD (ranging from days to weeks after the emergence of the symptoms), thus simulating more realistic conditions. Our results suggest that the given dose of VPA and the time window used, provided protection from toxic insults, while L- DOPA solely did not change the number of live dopaminergic neurons.

In the present experiment, TH expression and TUNEL assay were performed to morphologically examine tissues and define the number of live neurons. Inflammatory agents are known to be released from activated astrocytes and microglia (21). In this study, we observed once more the anti-apoptotic effects of VPA, since the apoptosis rate was decreased in the VPA treated animals. The previous study conducted in our laboratory has shown the antioxidant potential of VPA by antagonizing all the effects caused by 6-OHDA on Malondialdehyde (MDA), Superoxide Dismutase (SOD), Glutathione (GSH), and Glutathione S-transferase (GST) in the rat (22).

To understand the molecular mechanisms of VPA in detail, the phosphorylation level of p90RSK and ribosomal S6 protein was investigated. p90RSK is implicated in cell survival and proliferation processes by activating cell survival substrates that include transcription factor c-Fos, the cAMP response element-binding protein (CREB) (23). It is regulated under the MAPK pathway. p90RSK also halts apoptotic mechanisms by inhibiting proapoptotic protein BAD. It has been demonstrated that, while ribosomal S6 kinase phosphorylates ribosomal S6 protein at all sites, p90RSK phosphorylates ribosomal S6 protein at Ser 235/236 (8). Therefore, in the current study, an antibody specific to these phosphorylation sites was selected. L-DOPA combined with VPA showed a statistically significant increase of the S6 Ribosomal protein (Ser235/236) in PD animals. Given L-DOPA or VPA individually also caused a similar effect but it was not statistically significant (p=0,889). So both drugs seem effective in assisting neuronal survival. The combination of L-DOPA and VPA caused similar results on the Phospho-p90RSK (Ser380) levels. Further research is needed to explore the molecular mechanisms of VPA in attenuating PD.

In conclusion, we have demonstrated that VPA protected neurons by increasing TH positive dopaminergic neurons and decreasing apoptotic neurons in a rat model of PD induced by 6-OHDA. This might suggest that VPA can be an adjunct to L-DOPA in the treatment of PD since L-DOPA improves the motor symptoms while further loss of dopaminergic neurons might be inhibited by VPA.

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Conflict-of-interest and financial disclosure

The author declares that she has no conflict of interest to disclose. The author also declares that she did not receive any financial support for the study.

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