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β-carotene Has the Neuroprotective Effects in Parkinson's Disease by Regulating Mitochondrial Apoptotic Pathway Genes

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Abstract

Background: Parkinson's disease (PD) is one of the most common neurodegenerative diseases that cause disability. Finding treatment options that have no side effects can be very important.
Objectives: Therefore, in this study, the effect of β-carotene administration was investigated in the PD model of rats.
Methods: Induction of Parkinson's disease in rats was done by injection of 6-hydroxydopamine (6-OHDA) into the substantia nigra (SN). After induction rat behaviour was studied using an apomorphine-induced rotation test. The SN cells' viability was evaluated by MTT assay and apoptosis and necrosis were measured by flow cytometry. The expressions of *bax* and *bcl-2* genes were also studied using RT-PCR technique. Data analysis was done by GraphPad Prism V.8 software.
Results: The results showed a positive effect of β-carotene administration in PD rats, which led to improvement in apomorphine-induced rotation test, increased viability, and decreased apoptosis and necrosis of SN neurons. It also downregulated *bax* and overexpressed *bcl-2* gene expressions ($P < 0.01$).
Conclusions: β-carotene has therapeutic effects in PD conditions and its mechanism of action was attributed to regulating the expressions of genes involved in mitochondrial apoptosis. Therefore, its study in a clinical setting is recommended.

Keywords: Parkinson, β-carotene, Viability, Apoptosis

1. Background

Parkinson's disease (PD) is a disease of the central nervous system (CNS) that affects older people frequently (1). The death of dopaminergic cells in the substantia nigra (SN) is primarily responsible for the disease, and patients experience fatigue and boredom (2). The presence of Lewy bodies in the histopathological examination of the SN is a sign of Parkinson's disease (3). Symptoms include muscle stiffness, bradykinesia, akinesia, imbalance, and tremors at rest (4). This disease is treated with drugs, such as levodopa, amantadine, biperiden, and selegiline, which improve the patient's daily activities (5). However, these drugs do not completely block neurodegeneration and are associated with undesirable side effects such as movement fluctuations, dyskinesia, and neurological complications (6).

Mitochondrial-induced apoptosis has been reported to play a vital role in the pathogenesis of PD (7), which is associated with downregulation of anti-apoptotic *bcl-2* proteins (8) and overexpression of *bax* pro-apoptotic proteins (9). Under stressful conditions such as oxidative stress, cells undergo autophagy and this maintains cellular homeostasis and their survival (10). However, cellular apoptosis occurs when the rate of autophagy is excessive. As mentioned, one of the causes of dopaminergic cell death in SN is oxidative stress induced by increased free radicals and reactive oxygen species (ROS). Therefore, the administration of antioxidants in PD as a therapeutic strategy has been considered by researchers (11). β-carotene, a precursor for vitamin A, has antioxidant properties and protects against lipid peroxidation as a free radical scavenger, and can reduce oxidative stress (12). The role of vitamin A in preventing lipid peroxidation in the brain has been reported. However, there are little data on the effect of beta-carotene on PD (13).

2. Objectives

The current study was aimed to investigate the effect of β-carotene administration on PD model rats and evaluate the mechanism responsible for its therapeutic effects.

3. Methods

3.1. Materials

β-carotene was prepared from Sigma Aldrich (USA) corporation. 2.05 mg/kg body β-carotene was administrated to the rats (14) for 14 days.

3.2. Animals

4 adult male rats were purchased from Pasteur Institute Tehran-Iran. The rats were kept under the standard condition (12 h of light/dark cycle, 25 $\pm$ 2°C temperature, and 50% $\pm$10% RH). All animals were fed with the same proportions of corn, wheat, barley, and pellets under the same nutritional conditions, and free access to water was available to all. After one week, the rats were randomly divided into four groups as follows:

(1) Control group
(2) Control group receiving 2.05 mg/kg β-carotene
(3) PD group
(4) PD group receiving 2.05 β-carotene

3.3. Induction of Parkinson's Disease

3% sodium pentobarbital (45 mg/kg i.p.) was used to anesthetize rats. Then, unilateral lesions of the left medial forebrain bundle were performed, followed by stereotaxic injection of 6 hydroxydopamine (6-OHDA). For the preparation of 6-OHDA, it was first dissolved in sterile 0.01% ascorbate saline (4 μg/μL) and was injected unilaterally (0.5 μL/min) at the coordinates described by the atlas of Paxinos and Watson (1986). To confirm the induction of the PD, we used the Apomorphine-induced rotation test, and the results confirmed the PD induction in the rat model (15).

3.4. Apomorphine-induced Rotation

If injection of 6-hydroxy dopamine causes extensive neuronal damage in the midbrain, two to four weeks after surgery, animals show successive rotations toward the injection site in response to Apomorphine injection. The number of these rotations per time unit is a measure of the severity of neuronal damage in the midbrain. To perform the test, after 14 days of β-carotene administration, the rats were first placed in a transparent plastic cylinder (28 cm $\times$ 38 cm), and after 15 min 0.5 mg/kg body weight Apomorphine hydrochloride was injected into rats. After 60 seconds, the number of rotations to the injection site or vice versa was recorded at ten-minute intervals for one hour. Finally, the number of rotations toward the injured side was subtracted from the opposite side, which indicated the number of net rotations to the opposite side. Further rotation indicated the severity of the lesion and the loss of dopaminergic cells (16).

3.5. Preparation of Substantia Nigra Cells

After treating PD and control rats with β-carotene for 14 days, the rats were transferred to the operating room and anesthetized by intraperitoneal injection of ketamine and xylazine (5 mL ketamine and 3 mL xylazine). Then, the striatum tissue was rapidly separated from other brain tissues and placed in liquid nitrogen. After homogenizing the tissue in saline buffer solution with pH 7.4, the sample was centrifuged at 20,000 g for 20 minutes and stored for molecular analysis at -20°C.

3.6. MTT Assay

Tetrazolium (Sigma, USA), which forms insoluble purple crystals of Formazan was used in the MTT assay. First, 20 mg of tissue was lysed in a PBS buffer by sonicator or homogenizer and then centrifuged at 12000 rpm for 15 minutes at 4°C. Then, the supernatant was centrifuged at 10,000 g. Next, 50 μL of MTT solution was added to the tubes to reach a final concentration of 2 mg/mL. The tubes were incubated for 2 h at 37°C. After 120 minutes, 500 μ dimethyl sulfoxide was added and shaken well. The solution was poured on 96 wells plate, and after 1 h the absorption was read at 560 nm by spectrophotometry (17).

3.7. Flow Cytometry

During apoptosis, phosphatidylserine binds to the surface of the cell membrane and is detected by annexin-V. Therefore, in the present study, the Propidium iodide (PI) staining method was used to evaluate apoptosis or necrosis (18). Striatum cells were washed in N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid (HEPES) buffer and diluted to 106 c/mL. Then 5 μL of FITC-labeled annexin-V (Sigma), 10 μL of PI and CD3, CD4, and CD8 cell surface antigen antibodies were mixed in 100 μL of the cell suspension. After 15 minutes, 400 μL of HEPES buffer was added to the suspension to block the binding. Finally, a tube containing stained cells and antibodies was placed in a FACS caliber flow cytometer.

3.8. Gene Expression Analysis

3.8.1. RNA Extraction and cDNA Synthesis

RNA Extraction Kit (Denazist, Iran) was used to extract RNA from cerebellar tissue. The manufacturer's instructions were applied for RNA extraction. Agarose gel and nanodrop were used to determine the quality and quantity of extracted RNA, respectively.

cDNA synthesis was performed using the cDNA Synthesis Kit (Easy cDNA Synthesis Kit, DenaZist, Iran) based on the manufacturer's instructions. Quantitative measurement of DNA was performed with a nanodrop device.

3.8.2. Primers

The primers for *bax* and *bcl-2* genes were designed using the three software Gene runner, Allele ID, Primer express software V.3.0 (Applied Biosystems, USA). In the current study, the *β-actin* gene was used as a reference gene (in our study we used the *β-actin* gene as internal control). The sequences of primers are given in Table 1.

Table 1. The Sequence of Designed Primers

Genes	Sequence [3'-5']
Rat-*bax*-F	AGGGTGGCTGGGAAGGC
Rat-*bax*-R	TGAGCGAGGCGGTGAGG
Rat-*bcl2*-F	ATCGCTCTGTGGATGACTGAGTAC
Rat-*bcl2*-R	AGAGACAGCCAGGAGAAATCAAAC
Rat-*β-actin*-F	CACCATTGGCAATGAGCGGTTC
Rat-*β-actin*-R	AGGTCTTTGCGGATGTCCACGT

3.8.3. Real Time PCR

Real-time PCR (ABI 7300) was done using a master mix and specific gene primers. The RT-PCR timing and temperature program started at 95°C for 30 seconds for cDNA denaturation. In the next step, 40 cycles of 95°C for 5 seconds and 60°C for 31 minutes were performed. In the next step, the temperature cycle of 95°C for 15 seconds, 60°C for 30 seconds, and 95°C for 15 seconds were used.

3.9. Statistical Analysis

The results were shown as mean ± SE. After confirming the normal distribution of data by the Kolmogorov-Smirnov test, we used a parametric test to evaluate the data. To compare the significant differences among the groups, the two-way analysis of variance test and Tukey Post hoc test were used. The level of probability for the significant differences among the groups was considered as $P < 0.05$.

4. Results

4.1. Apomorphine Induced Rotation Test

Induction of Parkinson's disease in rats resulted in a significant increase in the number of rotations in the apomorphine-induced rotation test in rats compared with healthy controls ($P < 0.0001$). Although β-carotene administration for 14 days did not have a significant effect on the number of rotations in healthy rats, a significant decrease in the number of rotations was observed in PD rats receiving β-carotene compared to PD rats ($P < 0.05$) (Figure 1).

4.2. SN Viability, Apoptosis and Necrosis

Viability of SN neurons was measured by MTT assay and apoptosis and necrosis by flow cytometry technique, and the results showed that induction of PD in rats led to a significant reduction in viability ($P < 0.001$), increased apoptosis, and necrosis ($P < 0.0001$) of SN neurons compared to controls. However, an increase in viability ($P < 0.01$) (Figure 2A) and a decrease in apoptosis ($P < 0.01$) (Figure 2B) and necrosis ($P < 0.001$) (Figure 2C) of SN neurons in PD rats receiving β-carotene were observed compared with PD rats. Therefore, β-carotene administration prevents SN cell death in PD conditions. The histograms of flow cytometry are demonstrated in Figure 2d.

4.3. bax and bcl-2 Gene Expressions

The overexpression of *bax* and downregulation of *bcl-2* genes were seen in SN neurons after induction of PD, indicating mitochondrial apoptotic pathway activation. However, when 2.05 mg/kg β-carotene was administrated for 14 days, *bax* gene expression was downregulated and *bcl-2* overexpressed compared with PD rats (Figure 3).

5. Discussion

The results of the study showed a reduction of rotations in the apomorphine-induced rotation test in the PD rats. Also, increased viability, decreased apoptosis and necrosis of SN neurons were observed in PD rats receiving 2.05 mg/kg β-carotene.

One of the most important symptoms of PD is locomotor disorders, which are associated with the death of dopamine-producing nerves in the substantia nigra (19). Results of the present study showed that unilateral injection of 6-hydroxydopamine into the substantia nigra of the rat brain causes immediate and complete destruction of dopaminergic neurons, which is consistent with other studies in this field (20, 21). In the current study, β-carotene

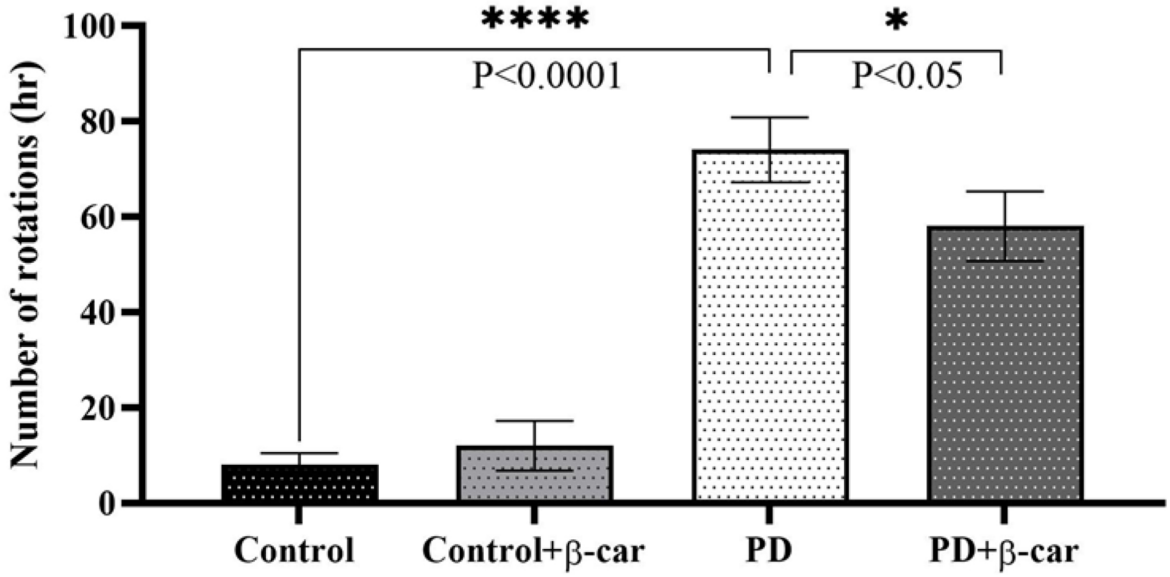

Figure 1. The Effects of β-carotene administration on the mean number of rotations in apomorphine-induced rotation test (n = 6). 2.05 mg/kg β-carotene was administrated for 14 days and one day after last dosing, the test was done.

appears to improve Parkinson's symptoms. Protein damage and lipid peroxidation induced by oxidative stress is involved in PD pathogenesis (22).

The results of studies have shown that oxidative stress plays a significant role in the pathogenesis of PD, and it seems that β-carotene has reduced oxidative stress (23, 24)

Although the results of a meta-analysis showed that β-carotene and vitamin C administrations did not have a positive therapeutic effect on PD (25), the present study showed that β-carotene has a positive therapeutic effect on PD conditions and leads to the prevention of SN dopaminergic cell death. This discrepancy can be attributed to the low number of studies used in meta-analysis (8 studies). Other studies have shown neuroprotective effects of β-carotene after traumatic brain injury (26) and ethanol-induced neurotoxicity (27). The antioxidant activity of this compound appears to be responsible for neuroprotective activity (28).

The *bcl-2* gene family plays an important role in the innate pathway of mitochondrial apoptosis and their expression determines the fate of the cells (29). In the present study, the expression of *bax* and *bcl-2* genes was studied by RT-PCR. *bax* is a protein that has pro-apoptotic activity, while *bcl-2* activity has a cell survival protective role and its anti-apoptotic activity in neurons and the protective effect of its activity on neurons have been shown (30). Its mechanism has been attributed to the reduction of oxidative stress and the increase of oxidative stress tolerance by increasing the content of antioxidant compounds such as GSH (31). *bax* protein interacts with *bcl-2* and inhibits the anti-apoptotic activity of *bcl-2*. In the present study, it was shown that induction of PD in rats leads to overexpression of *bax* and downregulation of *bcl-2* expressions, indicating an increase in apoptotic activity in neurons and the disappearance of dopaminergic neurons in the substantia nigra. However, administration of β-carotene decreased *bax* and overexpressed *bcl-2*, indicating their neuroprotective effect.

One of the limitations of the current study was experiments have been done on animal models, and caution should be taken in extending the results of current research to humans. Also, clinical studies should be performed in this field.

5.1. Conclusions

In general, β-carotene has neuroprotective effects in PD model rats. These protective effects were attributed to the upregulation of *bcl-2* and downregulation of *bax* expressions. However, this is a preliminary study of the neuroprotective effect of β-carotene and identification of the mechanisms of its neuroprotective effects requires further studies.

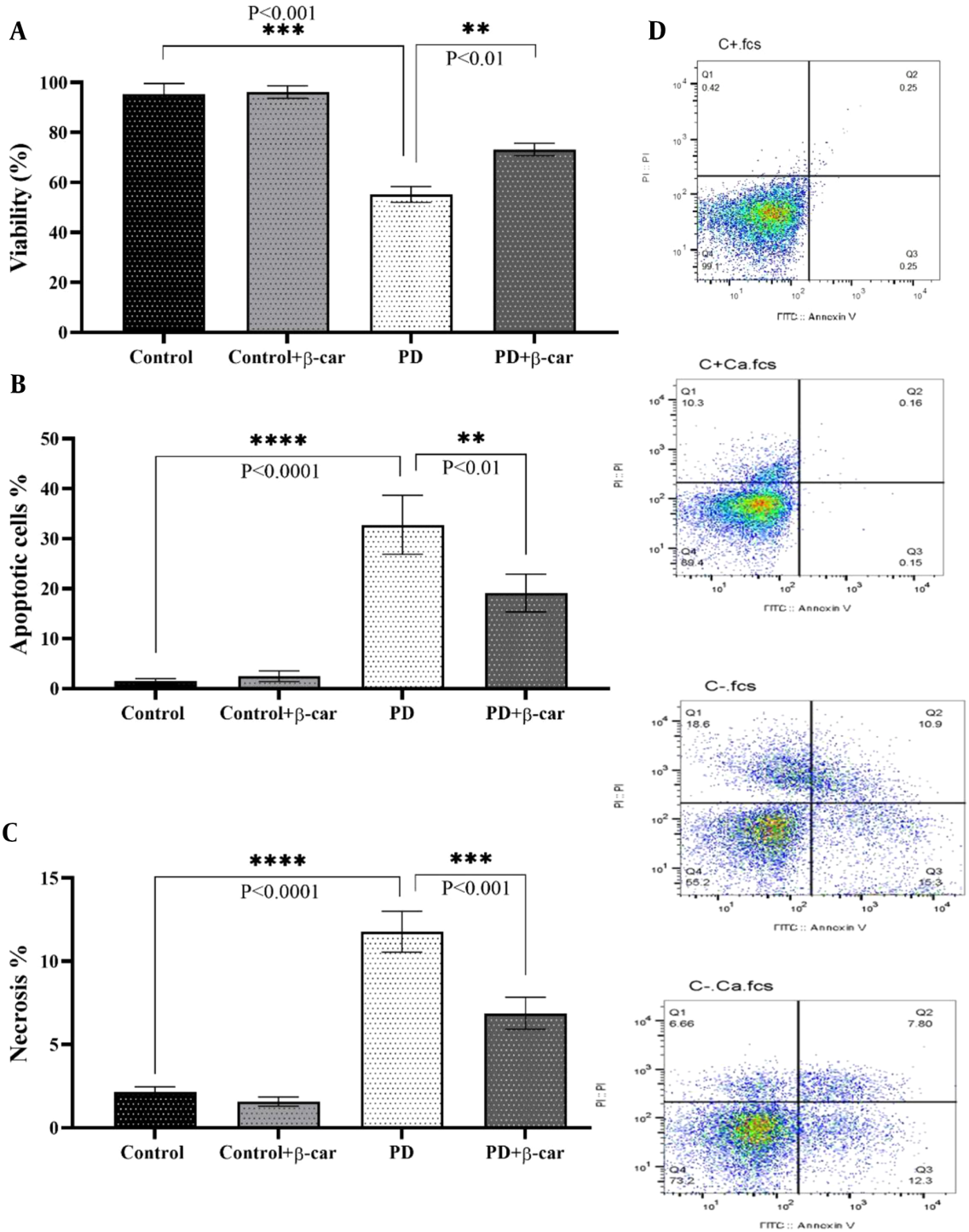

Figure 2. The effect of β-carotene on viability (A) and apoptosis (B) and necrosis (C) in brain cells of female rats. (D) The histograms of flow cytometry results (n = 4).

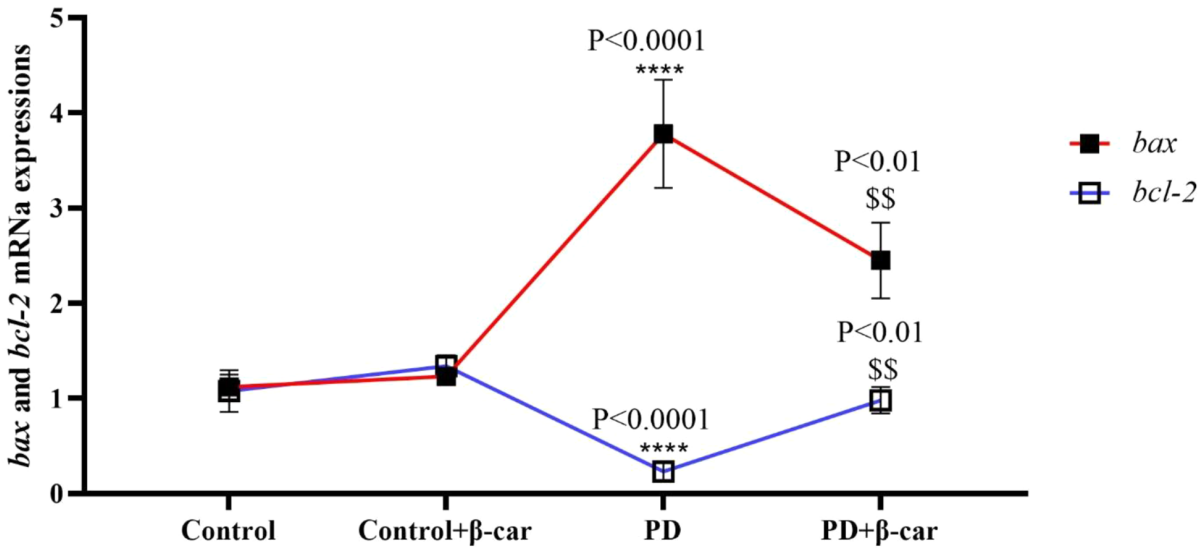

Figure 3. The expressions of *bax* and *bcl-2* genes in substantia nigra neurons of healthy and PD rats receiving β-carotene (n = 4). After last dosing of β-carotene, the rats were killed and SN neurons were prepared and the gene expression levels were measured by RT-PCR. ****vs. control, $$ vs. PD.

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Footnotes

Authors' Contribution: Behdokht Jamali and Mehrdad Hashemin: Conceptualization, methodology, software, validation, formal analysis. Maliheh Entezari: Supervision, project administration. Nahid Babaie and Mansour heidari: Review & editing, visualization, formal analysis.

Conflict of Interests: One of the authors of this article is a member of the committee board of the Journal of Human Genetics and Genomics.

Ethical Approval: The study was approved by Ethics Committee of Tehran Medical Science, Islamic Azad University (protocol code IR.IAU.PS.REC.13.99.208 and date of 2020/11/10).

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