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# Docosahexaenoic acid provides protective mechanism in bilaterally MPTP-lesioned rat model of Parkinson's disease

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**Abstract:** Docosahexaenoic acid (DHA), a major polyunsaturated fatty acid (PUFA) in the phospholipid fraction of the brain, is essential for normal cellular function. Neurodegenerative disorders such as Parkinson's disease (PD) often exhibit significant declines in PUFAs. The aim of this study was to observe the effects of DHA supplementation in an experimental rat model of PD created with '1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine' (MPTP). Adult male Wistar rats were divided into four groups: (1) Control; (2) DHA-treated; (3) MPTP-induced; and (4) MPTP-induced + DHA-treated. Motor activity was investigated using the 'vertical pole' and 'vertical wire' tests. The dopaminergic lesion was determined by immunohistochemical analysis for tyrosine hydroxylase (TH)-immunopositive cells in substantia nigra (SN). Immunoreactivities of Bcl-2, Akt and phosphorylated-Akt (p-Akt) in SN were evaluated by immunohistochemistry. MPTP-induced animals exhibited decreased locomotor activity, motor coordination and loss of equilibrium. Diminished Parkinsonism symptoms and decreased dopaminergic neuron death were detected in the MPTP-induced + DHA-treated group compared to the MPTP-induced group. Moderate decreases in Akt staining were found in the MPTP-induced and MPTP-induced + DHA-treated groups compared to controls. p-Akt immunoreactivity decreased dramatically in the MPTP-induced group compared to the control; however, it was increased in the MPTP-induced + DHA--treated group compared to the MPTP-induced group. The staining intensity for Bcl-2 decreased prominently in the MPTP-induced group compared to the control, while it was stronger in the MPTP-induced + DHA-treated group compared to the MPTP-induced group. In conclusion, DHA significantly protects dopaminergic neurons against cell death in an experimental PD model. Akt/p-Akt and Bcl-2 pathways are related to this protective effect of DHA in experimental PD. *(Folia Histochemica et Cytobiologica 2012, Vol. 50, No. 2, 228–238)*

**Key words:** Parkinson's disease, MPTP, docosahexaenoic acid, dopaminergic neuron survival, Akt/p-Akt, Bcl-2, rat

## Introduction

Docosahexaenoic acid (DHA) is the major polyunsaturated fatty acid (PUFA) in the phospholipid fractions of the brain and is required for normal cellular function [1, 2]. The maintenance of adequate concentrations of this PUFA is essential for cognition, learning and memory [2–4]. However, with aging, membrane PUFA concentrations decline, leading to cognitive impairment. It is evident that there is a significant decline in DHA and other PUFAs in neurodegenerative disorders such as Parkinsonism and Alzheimer's disease [2]. Supplementation of these PUFAs may help delay the onset of such diseases or may reduce the insult to brain functions [2, 5].

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Parkinson's disease (PD) is triggered by certain predisposing factors including environmental factors, free radicals, oxidative stress, mitochondrial dysfunction, excitotoxicity, calcium cytotoxicity, trophic factor deficiency, inflammatory processes, genetic factors and/or undefined insults, resulting in a progressive loss of dopaminergic neurons in the nigrostriatal pathway [6–8]. The loss of dopaminergic afferents to the striatum and putamen results in extra-pyramidal motor dysfunction, including tremor, rigidity and bradykinesia [6, 7]. Although other neurotransmitter systems are affected in this condition, dopamine (DA) depletion is the major neurochemical alteration [9]. The exact cause of this neuronal loss is still unknown, but recent human post mortem studies have suggested that, in PD, nigral dopaminergic neurons die by apoptosis [10] as do dopaminergic neurons in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated rodents [10, 11], an *in vivo* model of PD.

MPTP has been used in animal models to investigate the process of neurodegeneration with the aim of developing antioxidant neuroprotective drugs. MPTP is a potent neurotoxin that selectively destroys the nigrostriatal dopaminergic neurons in humans, sub-human primates, and lower animals [12–14]. MPTP is a highly lipophilic compound and can cross the blood brain barrier [15]. MPTP itself is not toxic. Inside the brain, the pro-toxin MPTP is rapidly metabolized into the toxic cation 1-methyl-4-phenylpyridinium ($MPP^+$) the active neurotoxin, by monoamine oxidase B (MAO-B) in glial cells. Furthermore, $MPP^+$ is selectively accumulated in dopaminergic neurons where it interferes with the mitochondrial metabolism via inhibiting the electron transport chain, decreasing mitochondrial membrane potential, and inducing disturbances in $Ca^{2+}$ homeostasis, which could eventually lead to cell death and the build-up of free radicals, toxic molecules that contribute further to neuronal cell destruction [16–19].

Dietary fish oils rich in DHA may offer a protective role against neuron death seen in PD. However, the underlying mechanisms are not well understood. Supporting a role for DHA in neuroprotection, a study has shown that DHA promotes neuronal survival by facilitating membrane translocation/activation of Akt, a downstream effector in the phosphoinositide 3-kinase (PI3K) pathway, and *in vivo* reduction of DHA by dietary depletion increased hippocampal neuronal susceptibility to apoptosis [20]. It has been shown that pharmacological inhibition of PI3K blocks the cell survival effect of DHA, and enrichment of cells with DHA partially rescues the phosphorylation and activity of the protein kinase Akt in the absence of serum [20].

Because there are multiple downstream effectors of Akt, it is not completely clear how the survival signal is transduced. One possibility is that the PI3K/Akt signaling pathway might act through modulation of Bcl-2 expression, and it has been shown that expression of Bcl-2 is induced by $\alpha$-synuclein [21]. Supporting this possibility, several studies have demonstrated that activated Akt stimulated changes in Bcl-2 and Bax expression and showed anti-apoptotic effects in many different cell types, including hippocampal neurons and PC12 cells [22, 23]. Acting via the Akt signaling pathway, DHA might increase Bcl-2 expression in neuronal cell membranes, thereby protecting against apoptosis.

Currently, drug therapy cannot completely cure PD. However Youdim et al. demonstrated that chronic treatment with a low dose of rasagiline (N-propargyl-1-(R)-aminoindan) which is a highly potent irreversible MAO-B inhibitor, increased DA in the rat striatum [24]. Furthermore it has been demonstrated that Parkinsonian subjects respond to low doses of rasagiline, (0.5–2 mg daily) in controlled monotherapy and adjunct L-Dopa therapy, as shown in early and late PD studies [25–27]. Nonetheless, efforts are still being made to investigate new drugs with both antiparkinsonian and neuroprotective effects. In this context, the main objective of the present study was to investigate some of the signaling proteins involved in the mechanisms of DHA neuroprotection observed in experimental PD.

## Material and methods

**Animals.** Adult male Wistar rats (12 months old, weighing 375–425 g) were obtained from Akdeniz University Animal Research Unit. The animals were housed in stainless steel cages (4–5 per cage) in an air-conditioned room (22°C with a 12 hour light:12 hour dark cycle) with food and water available *ad libitum*. All experimental protocols conducted on rats were performed in accordance with the standards established by the Institutional Animal Care and Use Committee at Akdeniz University Medical School.

**Experimental design.** Rats were randomly divided into four experimental groups as follows: (1) control; (2) DHA-treated; (3) MPTP-induced; (4) MPTP-induced + DHA-treated. DHA (Sigma–Aldrich, St. Louis, MO, USA) was dissolved in corn oil at a concentration of 0.046 M and was given to the treatment groups for 30 days (36 mg/kg/day) by gavage [28–33]. In order to eliminate the effects of daily gavage and vehicle, the control and MPTP-induced rats received a similar volume of corn oil alone.

**Surgery.** On the 23rd day of gavage treatment, MPTP-induced and MPTP-induced + DHA-treated animals were anesthetized with 400 mg/kg chloral hydrate intraperitoneally (i.p.).

MPTP (100 $\mu$g in 1 $\mu$l saline) was infused bilaterally into the medial forebrain bundle (MFB) using a Hamilton micro-syringe at a rate of 0.33$\mu$l/min [34], according to the following coordinates adapted from the Pellegrino [35]: anteroposterior (AP) — 2.2 mm from the bregma; mediolateral (ML) ± 1.5 mm from midline; and dorsoventral (DV) — 8.0 mm from the skull. After surgery, the animals were allowed to recover from anesthesia in a temperature-controlled chamber and then placed in individual cages. All four animal groups continued on their normal diets for an additional week after surgery.

**Tests of motor activity**. Seven days after the creation of the experimental PD model, motor activity of the rats was investigated using the 'vertical pole' and 'vertical wire' tests. The results of these tests confirmed that the created model of PD was reliable [36, 37].

For the vertical pole test, the animal was placed face up on a cloth-tape-covered pole (3.0 cm diameter, 150 cm length), which was held in a horizontal position, then the pole was gradually lifted to a vertical position and the time a rat stayed on the pole was recorded for a maximum of 120 s. In this test, an animal with deficits in motor coordination and balance will fall off the pole [36].

We also analyzed the rat catalepsy state on vertical wire netting (size 56.5 × 23.5 cm; mesh 1 × 1 cm; wire diameter 2 mm). The rats were placed with all paws on the wire net and the time taken for at least one paw to be actively displaced from the bar (descent latency) was determined [37].

**Tissue collection.** At the end of the treatment period, rats were anesthetized with a combination of ketamine (80 mg/kg, i.p.) and xylazine (15 mg/kg, i.p.), perfused transcardially with heparinized saline and their brains were removed immediately. For immunohistochemical studies, brain tissues containing SN were fixed in 4% formaldehyde for 8 h and washed with tap water for approximately 6 h afterwards; brain tissues were dehydrated in ethanol and embedded in paraffin for immunohistochemical stainings. 5-$\mu$m thick sections were collected onto poly-l-lysine-coated slides (Sigma–Aldrich, St. Louis, MO, USA).

**Immunohistochemistry.** For tyrosine hydroxylase (TH), Akt, p-Akt and Bcl-2 immunohistochemistry, paraffin sections were deparaffinized in xylene and rehydrated in a graded series of ethanol. Sections were immersed in 3% hydrogen peroxide in methanol for 15 min to block endogenous peroxidase activity. Slides were then incubated with universal blocking reagent (BioGenex, San Ramon, CA, USA) for 10 min at room temperature. Afterwards, excess serum was drained and sections were incubated with primary antibodies mouse monoclonal anti-TH (1/100; Calbiochem, CA, USA; #657010), rabbit polyclonal Akt (1/50; Cell signalling; #9272), rabbit polyclonal p-Akt (1/50; Cell signaling; #9275) and rabbit polyclonal anti-Bcl-2 (1/600; Abcam plc., Cambridge, UK; ab7973) in a humidified chamber for 2 h at room temperature. For negative controls, the primary antibodies were replaced by normal rabbit IgG serum (Vector Labs., Burlingame, CA, USA) at the same concentration. After several washes in PBS, sections were incubated with biotinylated polyvalent secondary antibody (K0675; LSAB2 System-HRP; DakoCytomation, Glostrup, Denmark) for 30 min followed by LSAB streptavidin-peroxidase complex (Dako, Carpinteria, CA, USA) incubation for 30 min and were rinsed with PBS. Antibody complexes were visualized by incubation with diaminobenzidine (DAB) chromogen (BioGenex). Sections were counterstained with Mayer's hematoxylin (Dako), dehydrated, mounted and examined by an Axioplan microscope (Zeiss, Oberkochen, Germany). The images were taken using a 5MP Canon A95 camera integrated to the microscope.

**Evaluation of Tyrosine Hydroxylase (TH)-positive neurons.** TH-positive neuron numbers were assessed to confirm the accuracy of the MPTP-induced experimental PD model at the histological level. To assay changes in the number of dopaminergic neurons in the SN, the total numbers of TH-stained neurons were counted independently by two observers blinded to the type and source of the tissues under a light microscope (40 × magnification) in six slides from each of the groups. The average of counts was used (data not shown) [33].

**Measurement of densitometrical staining intensities.** The densitometrical staining intensities of the Akt, p-Akt and Bcl-2 in SN of animals were measured by values of immunostaining. Densitometric measurements were performed in six different regions of SN sections. Immunostaining intensities were presented as the mean of measured layer value minus mean of measured background value. Morphometric analysis was performed with a Zeiss Axioscop-2 Plus microscope at 20 × magnification coupled with Image System Analysis, Axiovision Ver. 4.7 (Carl Zeiss, Jena, Germany).

## Results

### *Motor activity*

According to the motor activity tests, a significant decrease in motor activity was found in the MPTP-induced groups compared to the control and DHA-treated groups. Moreover, the motor activity was significantly improved in the MPTP induced + DHA-treated group when compared to the MPTP-induced group, but did not reach control levels (data not shown).

### *Quantitative analysis of Tyrosine Hydroxylase (TH) Positive cell in the Substantia Nigra (SN)*

The compact, reticular and lateral parts of the rat SN were easily distinguished by TH immunohistochemistry. The immunoreactivity for TH was observed in

neuron bodies and processes. No immunoreactivity was observed in glial cells or the endothelium (Figure 1).

The TH-immunoreactive neuron numbers in the SN of the control, MPTP-induced, DHA-treated, and MPTP-induced + DHA-treated groups were counted by two observers. According to these counts, the dopaminergic neuron numbers in the MPTP-induced group were significantly lower compared to all the other groups (data not shown) which confirmed that the model of PD was achieved successfully. Moreover, the dopaminergic neuron numbers significantly increased in the MPTP-induced + DHA-treated group when compared to the MPTP-induced group (data not shown).

Dopaminergic neuron processes of the MPTP-induced group were found to be sparse and disorganized compared to the other groups. In the MPTP--induced + DHA-treated group, the neuron processes were more organized compared to the MPTP-induced group. No staining was observed in the negative sections (Figure 1).

### *Immunoreactivity of Akt and p-Akt in the Substantia Nigra (SN)*

Immunohistochemical analysis showed that Akt immunoreactivity intensity was strong in the control (995) and DHA-treated (1,041.93) groups, while it was found to be decreased in the MPTP-induced (876.94) and MPTP-induced + DHA-treated (882.63) groups (Figure 2). In addition, there was very weak p-Akt immunoreactivity in the MPTP-induced group (790.11). On the other hand, p-Akt revealed a moderate immunostaining in the MPTP-induced + DHA-treated group (1,129.74) while it exerted a strong immunoreactivity in the control (1,602.32) and DHA-treated (1,590.74) groups (Figure 3). No immunoreactivity was observed on the slides where primary antibodies were replaced with normal rabbit IgG (data not shown).

### *Immunoreactivity of Bcl-2 in the Substantia Nigra (SN)*

Bcl-2 protein was mainly localized to SN dopaminergic neuron bodies, and to a lesser extent to their processes. The staining intensity for Bcl-2 in the MPTP--induced group was weak (934.49) while it was moderate (1,045.82) in the MPTP-induced + DHA-treated group. A strong immunoreactivity was observed in the control (1,237.07) and DHA-treated (1,080.92) groups (Figure 4).

## Discussion

In the present study, we have demonstrated that DHA treatment can alleviate MPTP induced nigrostriatal dopaminergic neuron degeneration and motor impairments in adult rats. Moreover, our findings indicate that the beneficial effect of DHA treatment on experimental PD was associated with the induction of prosurvival molecules such as Akt and Bcl-2.

In our study, neurotoxin MPTP was infused bilaterally into the MFB. Historically, MFB lesion model is the most widely used [34, 38, 39]. Because the dopaminergic axons of the mesolimbocortical pathway also transverse the MFB, this meant that injecting MPTP at this site also lesioned the ventral tegmental cell bodies and their terminals in the forebrain. The dopaminergic neuron numbers in the MPTP-induced group were significantly lower compared to all other groups [33]. There was a ~70% decline in TH immunopositive neurons in SN by one week after injection of MPTP into MFB, in agreement with previous reports [40–43]. MPTP caused damage to nigral dopaminergic neurons as seen in PD [44, 45]. The dopaminergic neuron numbers significantly increased in the MPTP-induced + DHA-treated group compared to the MPTP-induced group [33]. This indicates a relationship between TH positive neuron number depletion and diminished Parkinsonism symptoms that were detected in the DHA supplemented MPTP group. Our TH immunohistochemistry results have shown that DHA may partially restore dopaminergic neuron numbers in this model of PD. Previous studies have suggested that a high n-3 PUFA diet prevents the MPTP-induced decrease of a TH-labeled nigral cell [46, 47].

In the present study, the DHA dose was selected according to previous studies [28–33]. Daily intake of DHA was used with different doses in different experimental studies. Low DHA concentrations were found to be effective as a therapeutic agent, since high doses were cytotoxic to both normal and pathologic cells [48].

Degeneration of nigrostriatal structures was associated with motor dysfunction while bilateral lesions in the nigrostriatal system produce akinesia, rigidity and catalepsy [49]. There was a high degree of correlation between dopaminergic neuron degeneration and motor impairment in MPTP-induced Parkinsonism model [49]. Measurement of motor activity in experimental Parkinsonism models depends on the performance of animals in well defined tasks. We have performed these tests to confirm that the created model of PD was reliable [33]. MPTP-administered rats displayed typical behavioral characteristics of PD in the vertical pole and catalepsy tests [33]. These findings were in agreement with results from previous studies which demonstrated the impairment of motor activity in MPTP-induced Parkinsonism models [50–54]. On the other hand, DHA administration re-

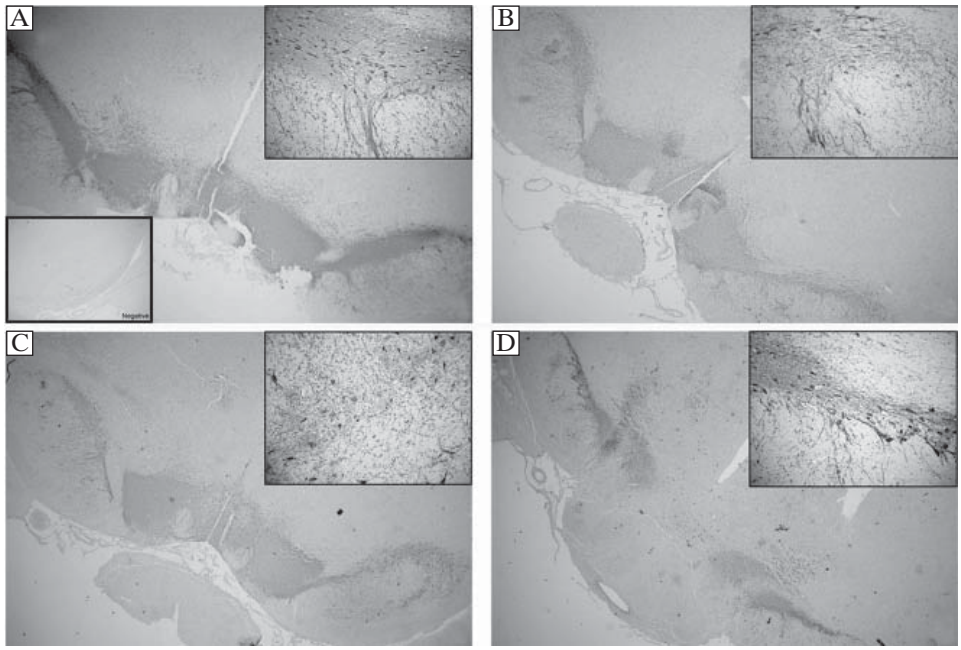


**Figure 1.** TH-immunoreactivity in the SN. (**A**) Control group; (**B**) DHA-treated group; (**C**) MPTP-induced group; (**D**) MPTP-induced + DHA-treated group. Notice the neuronal morphological alterations; the loss of dopaminergic neurons and the disorganized fibers in the MPTP-induced group. Scale bars = 400 $\mu$m

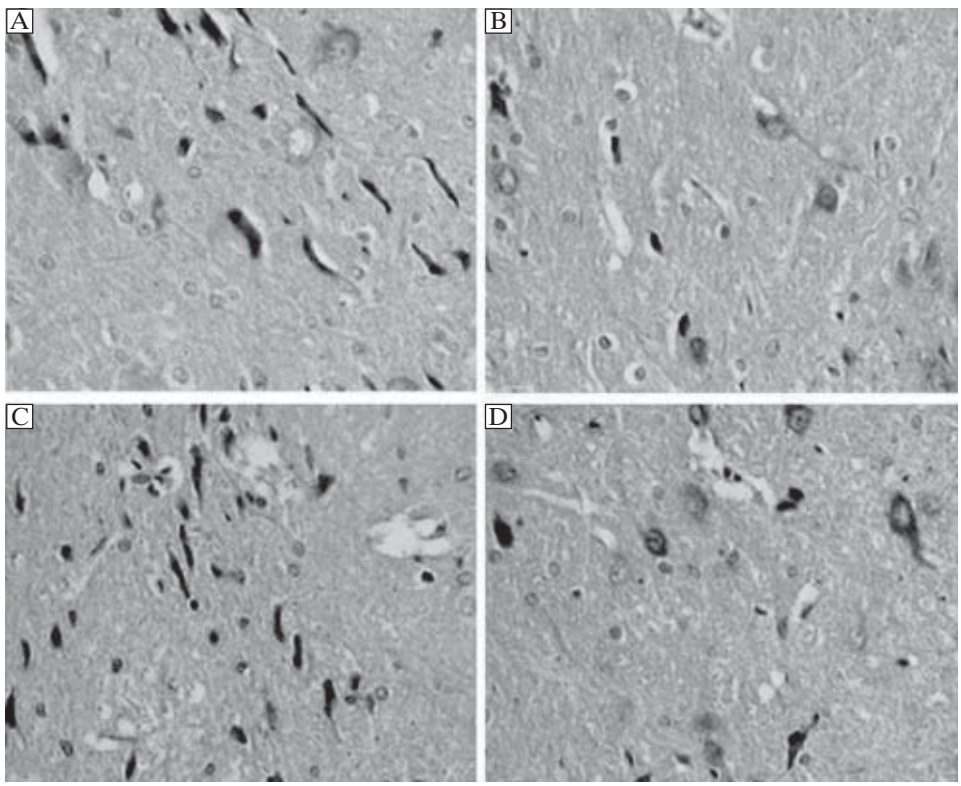


**Figure 2.** Akt immunoreactivity in the SN. (**A**) Control group; (**B**) DHA-treated group; (**C**) MPTP-induced group; (**D**) MPTP-induced + DHA-treated group. Akt immunostaining intensities were lower in the MPTP-induced and MPTP-induced + DHA-treated groups compared to the other two groups. Scale bars = 100 $\mu$m

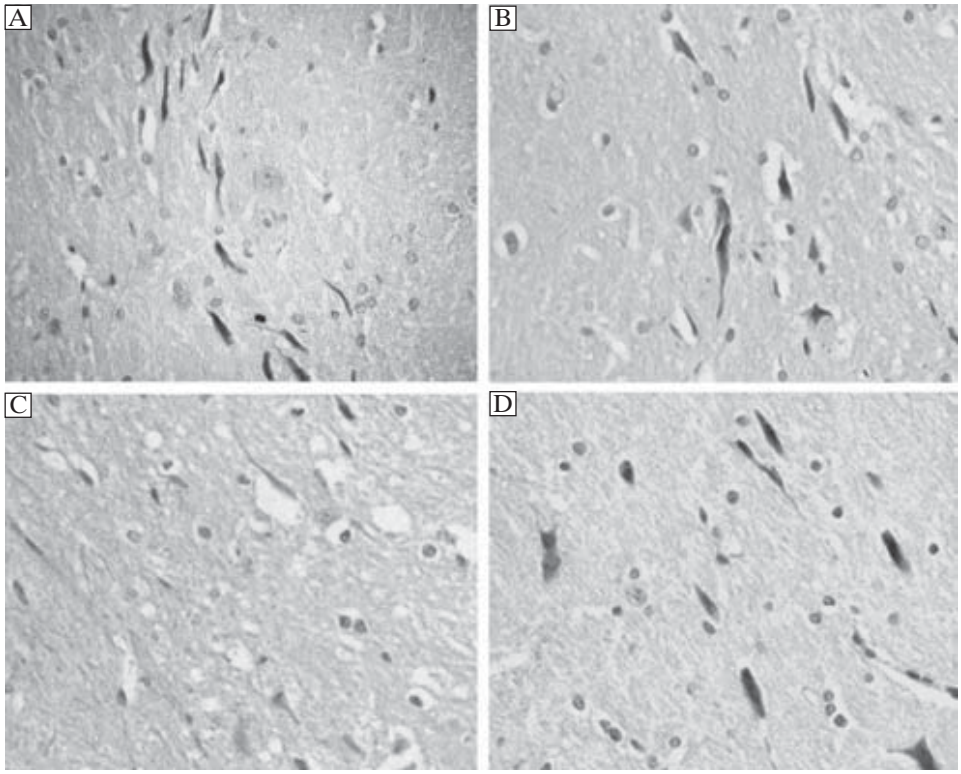


**Figure 3.** p-Akt immunoreactivity in the SN. (**A**) Control group; (**B**) DHA-treated group; (**C**) MPTP-induced group; (**D**) MPTP-induced + DHA-treated group. A significant decrease in p-Akt immunostaining intensity in the MPTP-induced group is seen. However it was increased in the MPTP-induced + DHA-treated group compared to the MPTP-induced group. Scale bars = 100 $\mu$m

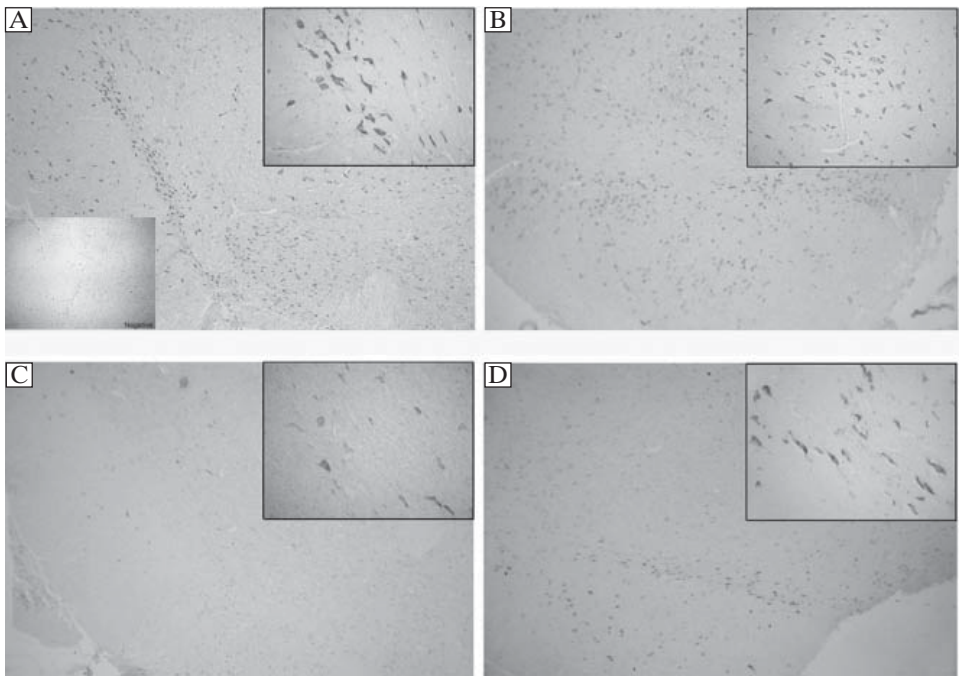


**Figure 4.** Bcl-2 immunoreactivity in the SN. (**A**) Control group; (**B**) DHA-treated group; (**C**) MPTP-induced group; (**D**) MPTP-induced + DHA-group. The dopaminergic neurons showed weak immunostaining with Bcl-2 in the MPTP-induced group (insets), while it was stronger in the MPTP-induced + DHA-treated group compared to the MPTP-induced group. Please note the absence of Bcl-2 immunostaining in the negative control slide (smaller inset A). Scale bars = 200 $\mu$m

duced these symptoms in the MPTP-induced + DHA--treated group [33]. It has been recently demonstrated that short-term administration of DHA reduced by about 40% the extent of levodopa-induced dyskinesias in a non-human primate model of Parkinsonism [55]. A previous study concluded that animals that received DHA for 24 days exhibited significant reductions (47%) in the number of d-amphetamine-induced rotations, compared to those in control rats [47].

Our results indicate that MPTP administration leads to loss of SN dopaminergic neurons. Previous studies have shown that MPTP caused oxidative stress and energy crisis [56, 57]. MPTP-induced mitochondrial defects may play a role in the development of apoptosis [58]. Although there is more than one pathway to induce apoptosis, the interaction between pro-apoptotic (Bad, Bax and Bak) and anti-apoptotic (Bcl-2 and Bcl-XL) members of the Bcl-2 family may determine the fate of cells by regulating the permeability of the mitochondrial membrane and controlling the release of cytochrome c (cyt c) from the mitochondria [59, 60]. Bcl-2 is a 26-kDa protein preferentially located at contact sites between the inner and outer mitochondrial membranes [61]. Phosphorylation inactivates Bcl-2, thus promoting apoptosis, possibly by releasing Bax from Bcl-2/Bax dimers [62–64]. The Bcl-2/Bax heterodimer is the active component for death protection [65, 66]. In response to apoptotic stimulation, Bax can be released from Bcl-2/Bax dimers and act as the channels for either ions or proteins [64, 67]. This proapoptotic protein Bax forms pores in the outer mitochondrial membrane which help in the release of cyt c [68]. Once released to the cytosol, cyt c could form the apoptosome together with apoptosis-activating factor-1 (Apaf-1) and procaspase-9, leading to the activation of caspase-9, and then activation of caspase-3 [69]. Alternatively, Bcl-2 and Bcl-XL are potent antioxidants in the mitochondria [70, 71].

Several studies have indicated that $MPP^+$ toxicity is associated with the translocation of cyt-c from the mitochondria to the cytosol and the activation of caspase-3 [45, 72–74]. Consistent with these observations, Bax null and Bcl-2 transgenic mice are both resistant to MPTP neurotoxicity [19, 75, 76]. Bcl-2 overexpression has been shown to prevent cell death [75–77], probably by inhibiting Bax translocation and insertion into mitochondrial membrane, or via a direct interaction with the channels [78]. Consistent with that result, MPTP lesion can also increase the level of phosphorylated Bcl-2 and decrease the interaction of Bcl-2 with Bax. Under MPTP intoxication, Bax is strongly upregulated in nigrostriatal dopaminergic neurons, whereas Bcl-2 levels are decreased [79]. In our study, Bcl-2 staining intensity was found to be decreased in the MPTP-induced group. This result was consistent with previous studies [58, 80–83]. In the present study, administration of DHA increased Bcl-2 levels in MPTP-induced + DHA-treated dopaminergic neurons compared to the MPTP-induced group. It has been demonstrated that DHA administration correlates with an increase in Bcl-2 levels in the brain [84, 85] and in retina tissues [84, 86]. In a cell study, German et al. [86] found that DHA treatment induced Bcl-2 expression in neurons. DHA protection in cells in culture and in *in vivo* models may involve neuroprotection D1 (NPD1) synthesis [87]. NPD1 is the first identified neuroprotective DHA-derived lipid mediator [88]. DHA plays an important role in the pathway leading to the formation of NPD1 [88]. DHA and NPD1 (10,17S-DHA) each showed enhanced expression of Bcl-XL, Bcl-2 and relative downregulation of Bax and Bik [84, 87] in human neural cells. Lukiw et al. [87] concluded that in human neural cells, DHA was used as a precursor of NPD1 biosynthesis. Notably, during oxidative stress in human retinal cells and ischemia/reperfusion in the brain, NPD1 elicits neuroprotection [88, 89]. Moreover, NPD1 inhibits IL-1 $\beta$-stimulated expression of cyclooxygenase-2 (COX-2) [84]. A further suggestion of the significance of NPD1 in Alzheimer's disease (AD) is the observation that hippocampal CA1 from AD patients shows a dramatic reduction in NPD1 [87]. It has been suggested that NPD1 may act at the level of signaling that regulates promoters of the genes encoding death repressors and effectors of the Bcl-2 family of proteins [90]. In contrast, translational or posttranslational events may also integrate a concerted response to counteract oxidative stress [90]. Mukherjee et al. [90] suggested that agents that stimulate NPD1 biosynthesis, NPD1 analogs, or dietary regimens may be useful as new preventive/therapeutic strategies for neurodegenerative diseases.

Some studies demonstrate that the PI3K/Akt pathway is critical for neuronal survival [91, 92]. Akt, also known as protein kinase B (PKB), is a member of a larger class of serine/threonine kinases. Akt has an N-terminus pleckstrin homology domain that mediates the interaction of Akt with a plasma membrane phospholipid, phophatidylinositol 3,4,5-triphosphate (PIP3). Extensive studies have shown that recruitment of Akt to the plasma membrane, and its association with PIP3, is crucial for its activation [93, 94]. Several lines of evidence indicate that the Akt signaling pathway responds to oxidative stress [95] and exerts a neuroprotective function [96, 97]. Moreover, a large number of studies *in vitro* have illustrated that pharmacological compounds that protect cells against oxidative stress exert their neuroprotective effects through activation of the Akt pathway [98–102].

Activated Akt can modulate the expression of proteins influencing cell death, such as inhibitors of apoptosis Bcl-2 and Bcl-XL or inducers of apoptosis Bax, Bad [103, 104]. Akt, promotes cell survival by inhibiting the function of proapoptotic proteins [105]. Phosphorylation of the protease caspase-9 or forkhead transcription factors by Akt blocks the induction of apoptosis by these factors [106]. While Akt phosphorylation at both Ser473 and Thr308 provides maximum catalytic activity, phosphorylation at Thr308 (the site regulated by growth factors through PI3K signaling) is sufficient to activate the kinase and to maintain survival [107]. Dopaminergic neurons from PD patients have greatly reduced expression of phospho-Ser473 and –Thr308 Akt, but not of total Akt [101]. Consistent with this observation, phosphorylation of Akt at Thr308 showed a tendency to decrease in response to MPTP [108]. Our immunohistochemical analysis showed a decrease in p-Akt in the MPTP-induced group compared to the control group which is consistent with a previous study [38]. Furthermore, it has been demonstrated that active Akt protects nigral neurons from 6-hydroxydopamine (6-OHDA) intoxication [109].

Downstream from Akt are the prosurvival Bcl-2 and proapoptotic Bad proteins. Akt can modulate Bcl-2 family members. Akt can directly phosphorylate the protein Bad on its serine 136, thereby inhibiting its proapoptotic function [110]. Transcription factors such as nuclear factor kappa-B (NF-$\kappa$B) and cAMP response element-binding (CREB) are also regulated by Akt [111, 112]; NF-$\kappa$B induces expression of the Bcl-XL and Bcl-2, and brain-derived neurotrophic factor (BDNF) expression is up-regulated by CREB [23, 113, 114].

It remains of clinical relevance to find active drugs that selectively target the PI3K/Akt pathway for treatment of diseases showing deregulation of Akt signaling. The inhibitor of MAO-B, rasagiline, has been found to slow the functional decline in patients with an early, mild form of PD [26, 115]. In MPTP-treated mice, rasagiline was shown to protect against the toxin, a neuroprotective effect associated with activation of Akt [116, 117]. Our results show protection against MPTP toxicity by DHA involving the PI3K/Akt pathway. It has been shown that DHA activates positive regulators of cell survival by up-regulating Akt, extracellular-signal-regulated kinase (ERK) and/or Bcl-2 [85]. Besides, DHA also has a negative effect on damaging factor production such as inflammatory cytokines and free radicals [20, 86, 118, 119]. The mechanism of activation of Akt is still controversial, even though it has been widely investigated. It is clear that Serine (Ser) and Threonine (Thr) phosphorylation of Akt is required for its activation. A previous study demonstrated that DHA induced phosphorylation of Ser residues of Akt [20]. DHA promotes neuronal survival by translocation/activation and phosphorylation of Akt [20, 120].

Although the modulatory effects of DHA on cell-survival/cell-damaging molecules have been demonstrated in cell studies, involvement of the inactivation of damaging mechanisms and/or the activation of survival mechanisms in DHA-mediated PD neuroprotection was less known. Therefore, the present investigation was designed to evaluate the effect of pretreatment with DHA on experimental PD via stereotactic MPTP-intoxicated animals and to attempt to determine whether the cell-survival mechanisms contribute to DHA's effects. p-Akt and Bcl-2 are survival factors that can block apoptotic cell death.

In conclusion, the present study indicates that DHA pretreatment provides protection of dopaminergic neurons against MPTP-induced cell death by activating Akt/p-Akt pathway and increasing Bcl-2 level in rats. Increase of Bcl-2 may be due to Akt/p-Akt pathway.

The highest priority in PD research is to develop a neuroprotective therapy to prevent, stop, or even reverse, neurodegeneration. It may be beneficial to investigate the neuronal protective effect of several foods rich in DHA against PD. Additional studies are in progress to examine the details of protective effects of DHA for developing new therapeutic strategies.

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