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B Vitamins Supplement Potentiates Antiparkinsonian Effect of Flunarizine: the Behavioral and Biochemical Evidences From 6-Hydroxydopamine Animal Model

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Abstract

Introduction: Prominent data indicate that flunarizine (flu), a calcium channel blocker, has neuroprotective effect. However, several authors have reported that the chronic use of flu can produce drug-induced Parkinsonism. Previously, we showed that B vitamins supplement (B com) has antiparkinsonian effect. In the present study, we evaluated the effect of pretreatment with flu and a combination of flu and B com on the 6-hydroxydopamine (6-OHDA) - induced Parkinsonism.

Methods: 6-OHDA (4 µl, 4 µg/µl) was injected into right striatum by stereotaxic surgery. Different groups of rats received flu (5 or 10 mg/kg) or B com or a combination of them before the toxin to three weeks after that. The severity of Parkinsonism was assessed by conventional behavioral tests and also biochemical measurement of striatal dopamine level. Furthermore, malondialdehyde (MDA) concentration was measured in the serum and brain suspension.

Results: Pretreatments with flu or B com significantly attenuated apomorphine- induced rotations and improved rotarod performance, but they had little effect on the 6- OHDA-induced swinging behavior. The pretreatments also reduced the decreasing effect of 6-OHDA on the striatal dopamine level. These antiparkinsonian effects were potentiated when animals were pretreated with a combination of flu and B com. In addition, B com alone or in combination with flu reduced MDA concentration especially in the brain tissue. On the other hand, flu increased MDA concentration in the serum.

Conclusion: Our data show that co-administration of B com with flu potentiate largely the antiparkinsonian effect and may attenuate its adverse effects.

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Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease affecting 1 to 3% of the population over the age of 50. PD is characterized by the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc). Due to the controversy of the basic molecular pathogenesis of PD and the selective death of DA neurons, many studies suggest that mitochondrial oxidant stress is an important step in the events leading to the DA neuronal death (1-4). In spite of prominent advances, all current treatments are symptomatic and unable to halt or retard DA neuronal death. Therefore, current studies are being directed toward the identification of new drugs, materials or strategies for the protection of DA neurons against biological processes inducing or promoting DA neurodegeneration.

Calcium is a very important mediator of neuronal functions regulating neurotransmitter release, neuronal excitability and integration of electrical signals, sensory perception, synaptic plasticity, gene expression, metabolism and programmed cell death (5-8). Plasmalemmal voltage-dependent calcium channels (VDCC) are the main routes of calcium entry from extracellular fluid into the excitable cells. An imbalance between calcium influx and efflux leads to Ca^{2+} overload and apoptotic cell death. For instance, the L-type VDCC activator bay K 8644 augments and nimodipine, a calcium channel blocker, inhibits apoptosis and mitochondrial disruption (5). Also, Ca^{2+} overload involves in the progressive and delayed death of nerve cells occurring in cerebral injury and cerebrovascular diseases such as stroke and trauma (9). Recent studies suggest that calcium entry through calcium channels leads to elevated mitochondrial oxidant stress in DA neurons and controlling of the activity of these channels prevents the neuronal death in PD (7). In agreement with this report, studies have shown that the dihydropyridine calcium channel antagonists protect striatal DA terminals and their parent cell bodies against intrastriatal injection of the neurotoxin 6-OHDA (10-11). Besides, our previous data shows that pretreatment with nifedipine which is a well-known L-type calcium channel blocker reduces the behavioral symptoms of 6-OHDA- induced Parkinsonism (12).

In addition to L-type calcium channels, N-type calcium channels are highly expressed in the CNS and play an important role in the control of neurotransmitter release (8). Recent data shows that oxidative stress induces remarkable changes on the N-type calcium channels in SNc and the balance between the up-regulation and down-regulation of these channels have a potential role in the treatment of the PD symptoms (11). Furthermore, it has been reported that Na^{+} channels involve in Ca^{2+} overload and the increase in the intracellular concentration of sodium ions induces a rapid Ca^{2+} overload through the reverse operation of the Na^{+}/Ca^{2+} exchanger (13).

Flunarizine (flu) is a wide spectrum calcium channel blocker which blocks the T-, L-, and N-type calcium channels (14) as well as sodium channels and it prevents the Ca^{2+} overload under pathological and ischemic conditions (15). The neuroprotective effect of this drug is wildly investigated and it has been shown that flu has cytoprotectant actions in neuronal cultures (14, 16), chromaffin cell cultures (17), hippocampal slices (18), and in experimental models of stroke (19). However, several studies have shown that the chronic use of flu produces extrapyramidal side effects and movement disorders (20-22). Moreover, some studies have shown that flu reduces the viability of dopamine-rich human neuroblastoma cells in vitro (23).

Our previous data show that supplement of B vitamins reduces severity of behavioral symptoms in 6-OHDA-induced Parkinsonism (24-26). In the present study, we did

experiments to find that: can B vitamins supplement potentiate the neuroprotective and the antiparkinsonian effect of flu? We hypothesized that B com potentiates antiparkinsonian effects of flu and can reduce its effective dose and therefore its adverse effects. To evaluate this hypothesize, we assessed the antiparkinsonian effect of flu alone and in combination with B com in the 6-OHDA- induced Parkinsonism. Also, to define the antiparkinsonian mechanism of flu and B com, MDA concentration which is a biomarker of lipid peroxidation and oxidative stress was measured both in blood and in midbrain portion of the brain.

Methods

Animals and experimental groups

Male Wistar rats (Razi Institute, Karaj, Iran), in weighing range of 250–300 g were housed in large cages (38 × 59 × 20 cm) at a temperature-controlled room by a regulated light/dark cycle. All procedures carried out in this study were according to the guidelines of animal experiments of Research Council at Qazvin University of Medical Sciences and all animals had free access to tap water and standard food throughout the experiments.

Rats were divided into six experimental groups of: control (con, n=9) which did not receive any pretreatment; vehicle (veh, n=10) which received ethanol as the solvent of flu; B com (n=9) which received a combination of all B vitamins 5-folds of that in normal MEM (minimum essential medium); low F (n=9) and high F (n=10) which received flu at doses of 5 and 10 mg/kg, respectively; B com + F (n=10) which received both flu (5 mg/kg) and B vitamins. Additional B vitamins were dissolved in drinking water as described in previous researches (Haghdoost-Yazdi et al., 2012, Haghdoost-Yazdi, H. et al., 2014, Sophiabadi, M. et al. 2013). Ethanol and flu were administrated once per day intraperitoneally. In addition, the data of another group of rats (n=8), considered as healthy rats, were also used to analyze the data obtained from rotarod test. This group consisted of intact rats which did not receive 6-OHDA or any other intervention. All of B vitamins, flu, 6-OHDA and apomorphine were purchased from SIGMA-ALDRICH Company.

Experimental design

Except the healthy group, other groups of rats received 6-OHDA. All pretreatments were performed a few hours before 6-OHDA injection and continued to three weeks after that (figure.1). In this regard, we assured that animals received a full period of treatment to prevent 6- OHDA – induced neurodegeneration (27-28). Apomorphine- induced rotational test and elevated body swing test (EBST) were performed at three separated steps: within the second, fourth and eighth weeks post-surgery. Rotarod test was performed in the sixth week post-surgery. Blood sampling and serum extraction were performed after rotarod test. After the last behavioral tests, animals were decapitated and suspension from the brain tissue was prepared.

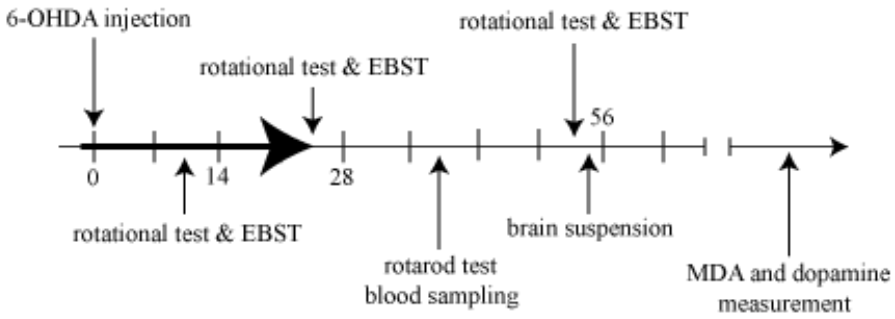

Figure 1. Time schedule used for animal experiments: Animals were tested by apomorphine-induced rotational test and elevated body swing test (EBST) at three different steps: in the second, fourth and eighth weeks after 6-OHDA injection. Rotational tests were performed at least one hour after the EBST. Rotarod rod test and blood sampling were performed in the sixth week after 6-OHDA. Preparation of the brain suspension was performed in the eighth week post-surgery. All pretreatments started a few hours before 6-OHDA injection and continued to three weeks after that (black arrow). Numbers show the days after 6-OHDA.

Surgical procedures

Rats were anesthetized with intraperitoneal injection of ketamine (100 mg/kg) and xylazine (5 mg/ kg). Then 4 µl of 6-OHDA (4 µg/µl) dissolved in isotonic sodium chloride solution including 0.2 mg/ml of ascorbic acid was unilaterally injected into 4 sites at right striatum using stereotaxic apparatus (Stoelting, USA) and through a 10-µl Hamilton syringe. Coordinates for injections were AP: 1.5, L: -2.5, DV: -6 and AP: 0.8, L: -3, DV: -6, and AP: 0.1, L: -3.2, DV: -6 and AP: -0.5, L: -3.6, DV: -6. AP and L were measured from bregma and DV from the surface of skull according to the atlas of Paxinos and Watson 2007 (29). After 6-OHDA injection, the needle was held in place for another 5 minutes and then it was taken away at a rate of 1 mm/min.

Behavioral testing

Apomorphine-induced rotational test

Apomorphine-induced rotational test was performed according to the method described previously by Fujita et al. in 1996 (30). Briefly, after a 5-min habituation time, apomorphine hydrochloride (0.5 mg/ kg, dissolved in saline) was injected intraperitoneally into animals. Then, the number of full rotations was counted for one hour in a cylindrical container (28 and 38 cm, in diameter and height, respectively). Contralateral and ipsilateral rotations (far away and toward the lesion side, respectively) were counted as positive and negative scores and the net number of rotations was defined by algebraic summation of the positive scores minus the negative ones.

Elevated body swing test (EBST)

The EBST was performed according to a slightly modified method described by Borlongan et al., in 1995 (31). Briefly, the animal was placed in a cylindrical container and allowed to habituate for several minutes to attain a position in which all four paws sit on the floor. After that, the animal's tail was held approximately at 2 cm from its base. Then, the animal was lifted up 2 cm above the surface and was held in the vertical axis with no deviation of more than 10^0 to either side. Whenever the animal moved its head out of the vertical axis to either side, a swing was recorded. The animal had to return to the vertical position before attempting another swing. Duration of the experiment for each rat was 1 min and simultaneously one person held the rat and another person

timed the test session and recorded the direction and the frequency of swings. All tests were blinded to the groups. Biased swing behavior was calculated as follows: L/ (L + R) (%) for left-biased swings and R/(R + L) (%) for right-biased swings (L = amount of left-biased swings, R = amount of right-biased swings).

Rotarod test

A rotating rod apparatus (M.T6800, Borj Sanat, Iran) was used to examine the motor performance of rats and measure their ability to improve motor skill with training. Rotarod test was performed at three consecutive days with two sessions in a day. Each session lasted 200 sec and the rotation of the rotating rod accelerated from 5 to 40 rpm over the first 120s of the trial and remained at maximum speed for the remaining 200 sec. Rats were scored for their latency (in seconds) to fall (height 30 cm) in each trial. A minimum rest of 30 min between trials was given to rats to avoid fatigue. Rotarod data are expressed as the area under the curve (AUC), which were calculated by the following formula:

AUC= time on the rod (s) × [time on the rod(s) × 0.44/2]

where 0.44 is the acceleration speed per sec.

Blood sampling

Blood samples were collected from the caudal vain using a scalp vein while the animals were restricted within a restrainer. Samples then were allowed to clot and sera were separated by centrifugation at 5000 rpm (Eppendorf 5415D) for 5 minutes and stored at -80ºC until MDA measurement.

Dopamine measurement

Animals were decapitated under diethyl ether anesthesia and the brain was removed immediately and then the striatum was isolated. Dopamine concentrations were measured using the Dopamine Research ELISA™ Kit (BA E-5300; Nordhorn, Germany), according to the manufacturer's instructions. Briefly, striatal tissues were homogenized in hydrogen chloride (0.01 N; 1 mL for 50 mg of tissue) with 1 mM EDTA and 4 mM sodium metabisulfite. Under these conditions, dopamine is charged positively, and its solubility reaches the optimized level. Afterwards, the homogenate was centrifuged at 15000×g for 15 minutes (4ºC), and the supernatant was collected for the measurements. The measurements were performed in 20 µL of standard and diluted samples using a microplate reader at 450 nm (reference wavelength, 620 to 650 nm). The concentration of dopamine in the samples was calculated according to 6 standards from 0 to 90 ng. The ELISA kit provides a very sensitive approach (lower limit, 0.7 ng/mL) for the measurement of dopamine.

MDA measurement

To measure MDA concentration in midbrain, it was isolated, washed with normal saline, and sonicated in cooled KCl solution (1.5%) to give a suspension. MDA concentration was measured spectrophotometrically by the method described by Albro et al., in 1986 (32) using thiobarbituric acid (TBA) and MDA standard curve. 1, 1, 3, 3-tetramethoxypropane was used as standard. MDA reacts with TBA to produce a pink colored solution that has maximum absorbance at 532 nm. The results are expressed as µmol/L (for blood samples) and µmol/g (for brain suspension).

Statistical analysis

Data were initially analyzed by Kolmogorov-Smirnov test to find the normality of the data. Since data did not have normal distribution, the analysis was performed by Kruskal–Wallis nonparametric ANOVA followed by a two-tailed

Mann–Whitney U test. Data are expressed as the mean ± SD and a *P* value ≤ 0.05 was considered statistically significant.

Results

Pomorphine- induced rotational behavior

Prominent contralateral (away from lesion side) rotations were observed in all experimental groups indicating that none of the pretreatments could prevent 6-OHDA-induced neurotoxicity and Parkinsonism. However, as it has been displayed in left plots in figure 2, some of pretreatments had significant effects. The most significant effect was observed in B com + F group. In this group and in all rotational tests, the number of net contralateral rotations was significantly less than that in con and veh groups ($P<0.05$ and $P<0.01$). Such effects but with less potency were observed in high F group too ($P<0.05$). Treatment with a low dose of flu also reduced the number of rotations, however, this effect was statistically significant only in the third rotational test ($P<0.05$). Nourishment of rats with B com also reduced the severity of rotational behavior ($P<0.05$).

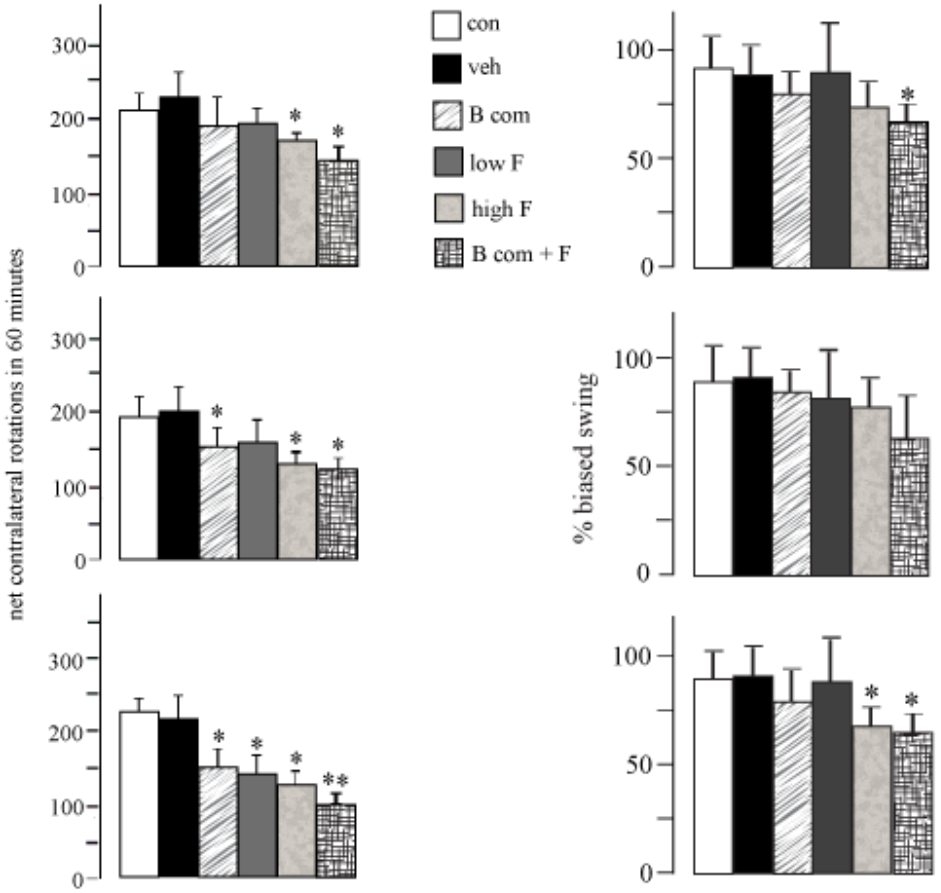

Figure 2. Left and right plots display respectively the number of apomorphine- induced net contralateral rotations and the findings of the EBST in different experimental groups at second (upper plot), fourth (middle plot) and eighth (lower plot) weeks after 6- OHDA. Note in right plots, 50% means that the number of left swings was equal to the number of right swings. Less than 50% means that most of swings were toward left (contralateral to lesion side) and more than 50% means that most of swings were toward right (ipsilateral to lesion side) side. Values are means ± S.E. of 12 animals.

*: P<0.05 and **: P<0.01 compared to veh group, Kruskall–Wallis nonparametric test followed by Mann–Whitney U test.

Swinging behavior

Right plots in figure 2 display findings of elevated body swing tests. Number of swings varied from 1 to 8 swings and almost all 6-OHDA lesion rats showed net ipsilateral (toward lesion side) swings. In B com + F group, a number of net ipsilateral swings were significantly less than that in con and veh groups (P<0.05). Also, the number of ipsilateral swings in high F group was significantly reduced in the 8th week after the surgery (P<0.05).

Rotarod test

Figure 3 shows the rotarod performance of different experimental groups. In healthy rats, the performance in each session was better than that in the previous session and rats reached maximum performance in sessions 4 to 6. All groups of 6-OHDA treated rats showed some degree of motor learning but significant differences were observed between 6-OHDA treated rats and healthy rats. None of the 6-OHDA treated group of rats reached maximum performance even in the last session. Also, the learning pattern was different and the performance did not improve in successive sessions. For example, in veh group, the stepping time on rotarod in session 5 (R5) was less than that in session 3 (R3) and 4 (R4). However, a significant difference was observed between 6-OHDA- treated groups. First of all, B com + F group of rats showed much better learning and AUC in R5 and R6 was significantly higher than that in veh group (P<0.01). In these sessions, although AUC in B com + F group was less than that in healthy group, but the difference was not statistically significant. In addition, the leaning pattern in B com + F group was similar to healthy group and it was prominently different with that in veh group. In contrast, the learning pattern in low F, B com and high F groups was more similar to that in veh group. However, AUC in R5 and R6 in high F and B com groups was significantly higher than that in veh group (P<0.05 and P<0.01, respectively).

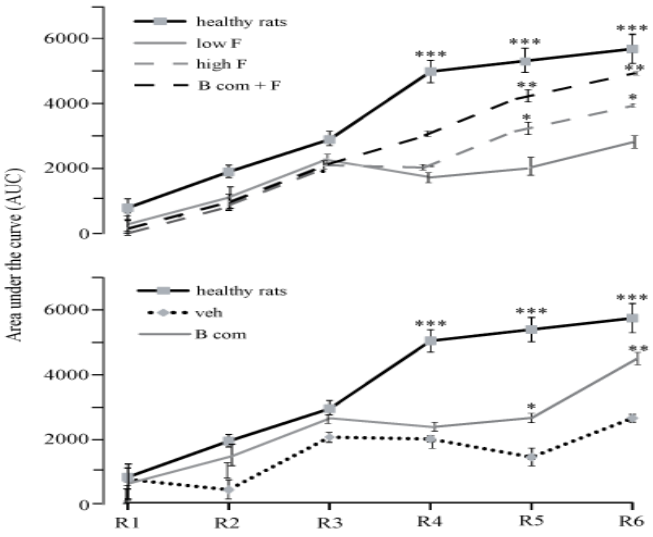

Figure 3. Diagrams display the motor performance of different groups of rats in rotarod test which was performed at three consecutive days, two sessions in each. Because con and veh groups of rats showed almost similar results, only data of veh group is shown here. Values are means ± S.E. of 12 animals.

*: P<0.05, **: P<0.01 ***: P< 0.001 compared to veh group, Kruskall–Wallis nonparametric test followed by Mann–Whitney U test. AUC: area under the curve. R1-R6: sessions of the test; R1: first session- R6: last session.

Dopamine assessments

The upper plot in Figure 4 quantifies the effect of 6-OHDA and the different pretreatments on striatal dopamine levels. 6- OHDA decreased remarkably right to left dopamine level. Pretreatment with B com, flu and a combination of them attenuated significantly. This decreasing effect indicates that they could protect striatal DA terminals against the 6-OHDA. Again, the effect of pretreatment combinations was more prominent.

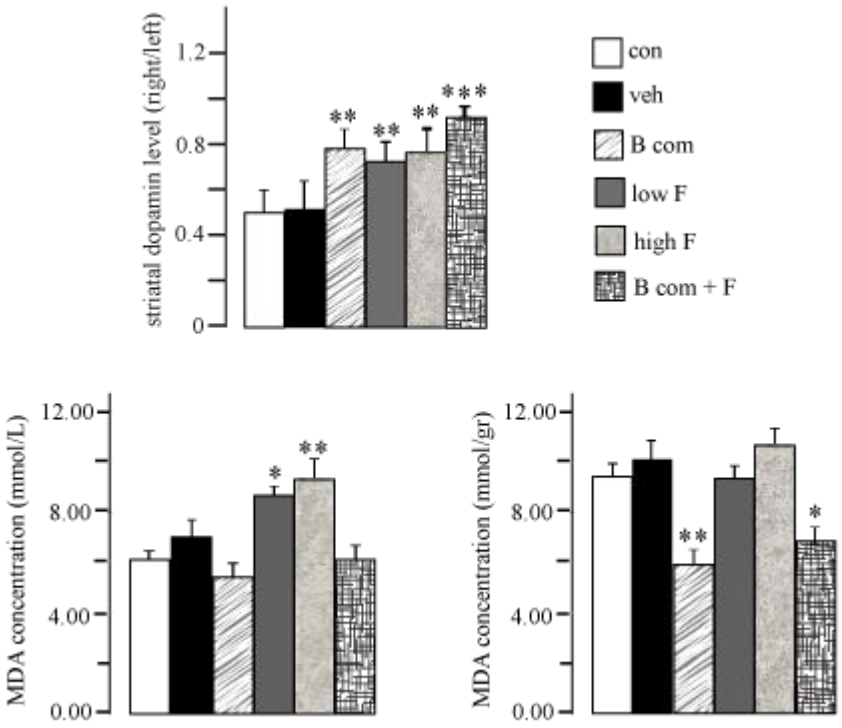

Figure 4. Upper plot displays striatal dopamine level and lower plots show malondialdehyde (MDA) concentrations in serum (left plot) and midbrain portion of the brain (right plot) in different experimental groups. Striatal dopamine levels in both hemispheres were measured using an immunosorbent assay kit and then calculated as a ratio of the injured side relative to intact side.

*: P<0.05, **: P<0.01 and ***: P<0.001 compared to veh group, Kruskall–Wallis nonparametric test followed by Mann–Whitney U test.

MDA analysis

MDA concentration was measured in the serum and midbrain portion of the brain of 6-OHDA-treated rats (Figure 4 lower plots). In the control group, MDA concentrations in serum and brain tissue were 6.16 ± 0.33 µmol/L and 9.34 ± 0.85 µmol/g, respectively. In veh group, although the concentrations were higher, but the differences were not significant. Serum MDA concentration in low F and high F groups was significantly higher than that in the veh group (P<0.05 and P<0.01, respectively). Besides, MDA concentration in midbrain of B com and B com + F groups was significantly less than that in the veh group (P<0.01 and P<0.05, respectively), while the difference between F groups and veh group was not significant.

Discussion

In the present study, we investigated the effect of pretreatment with flu, B com or a combination of them on the behavioral symptoms of 6-OHDA- induced Parkinsonism. We also measured the striatal dopamine level to assess effects of these pretreatments on the biochemical consequences of 6-OHDA neurotoxicity in the brain. Our findings show that flu, especially at a dose of 10 mg/kg and also B com significantly attenuate the severity of behavioral symptoms and reduce the decreasing effect of 6-OHDA on the striatal dopamine level. Pretreatment with a combination of flu and B com was more effective than either flu or B com alone indicating that supplement of B vitamins potentiates significantly the antiparkinsonian effect of flu. To evaluate the mechanism(s) of these effects, MDA concentrations in the serum and midbrain were measured. Our data show that B com alone or in combination with flu reduces MDA concentration especially in the brain tissue. On the other hand, pretreatment with flu increased MDA concentration in the serum, but not in the brain tissue.

Several studies have shown that there is a positive association between nigral cell death and the severity of behavioral symptoms in the 6-OHDA-induced Parkinsonism (33-36). The rotational test is the most conventional test in the evaluation of 6-OHDA-induced Parkinsonism (28, 33, 37). This test can distinguish between partial lesion and near complete lesion of the SN (36). Also, scores in rotarod test, inversely correlates with the DA neuronal death in SNc. Furthermore, several authors have confirmed that EBST is a valid behavioral test which can provide an accurate measure of motor functions mediated by dopamine (27, 31, 38). Based on these evidences, our data show that pretreatment with either flu or B vitamins or a combination of them have neuroprotective effect and reduce the neurotoxic effect of 6-OHDA on the SNc dopaminergic neurons. Previously we reported that pretreatment of rats with B vitamins supplement has antiparkinsonian effect (24, 26). Biochemical analysis showed that this effect of B vitamins is not mediated by lowering plasma Hcy (25, 39). The present study shows that B vitamins supplementation reduces MDA concentration in midbrain. MDA is a biomarker of lipid peroxidation and oxidative stress. It has been shown that mitochondrial dysfunction and oxidative stress are the main mechanisms of 6-OHDA- induced Parkinsonism. 6-OHDA metabolism generates a series of reactive oxygen species at physiologic pH, including hydrogen peroxide, para-quinone and superoxide and hydroxyl radicals. Therefore, at least in part, the antiparkinsonian effect of B com has been mediated by the suppression of 6-OHDA- induced oxidative stress. This finding is in line with several data indicating that B vitamins exert antioxidant effect (40, 41).

Several reports have shown that flu produces the neuroprotective effect. Flu has been shown to have cytoprotectant actions in neuronal cultures (14, 16), chromaffin cell cultures (17), hippocampal slices (18), and in experimental models of stroke (19). Flu also significantly reduces glutamate-induced neurotoxicity (42), augments functional recovery following sciatic nerve lesion in rats (43) and improves the survival of grafted dopaminergic neurons (44). Our data confirms these reports and shows that the chronic treatments (3 weeks) of rats with flu ameliorate the behavioral and biochemical consequences of 6-OHDA

induced neurotoxicity. In contrast, several lines of data have shown that the chronic use of flu produces drug-induced Parkinsonism (DIP) (20-23). Flu and cinnarizine (another calcium channel blocker with less potency) represent one of the most common causes of DIP in many countries where they are prescribed (21). These drugs are widely used in Europe and South America for the treatment of migraine, vertigo, and cerebrovascular disorders. Probably, D2 receptor blockade by flu and cinnarizine is the major reason for the development of Parkinsonism (20). Flu exhibits an extraordinary capacity to accumulate in cell membranes, where it can reach mmol concentrations (45, 46). This can lead to the accumulation of flu in brain tissues following its repeated administration to patients, thus blocking dopamine release from striatal neurons and/ or blockade of striatal dopamine receptors (20, 47). Our data shows that the chronic administration of flu increases MDA concentration in serum and therefore can induce oxidative stress which may contribute to DIP. However, pretreatment with flu in combination with B com did not increase MDA concentration indicating that B com supplementation overrides flu- induced oxidative stress and therefore may reduce the adverse effects of flu.

Several explanations can describe the disagreement in neuroprotective effect of flu. First of all, different dosing of flu in experiments may be involved. While it has been shown that flu at a low dose has cytoprotective effect, at high concentrations, it exhibits cytotoxic effects (17, 48). Elimadi et al., in 1998 reported that flu at low concentrations inhibited the mitochondrial permeability transition (MPT) whereas at high concentrations, induces MPT (49). The MPT is induced by the opening of a non-specific pore between the mitochondrial inner and outer membranes and causes them become permeable to small solutes. MPT is usually accompanied by mitochondrial matrix swelling, destruction of the transmembrane potential, increase in the basal mitochondrial oxygen consumption, and the release of pro-apoptotic proteins such as cytochrome c (50). In the present study, the group of rats which received a combination of low dose of flu and B com showed less intensity concerning behavioral symptoms and more striatal dopamine level in comparison to the group of rats which received a high dose of flu. This finding clearly shows that the supplement of B vitamins can reduce the effective dose of flu and therefore its adverse effects. Another explanation is the difference in experimental protocols. Evidence shows that flu is more effective as a neuroprotective drug in pretreatment than in posttreatment protocols (51, 53).

What is the mechanism(s) of neuroprotective effect of flu? Our data showed that flu does not suppress 6-OHDA- induced MDA overproduction. This means that the neuroprotective effect of flu probably is not mediated by the suppression of oxidative stress. On the other hand, flu blocks Na^+ and Ca^{2+} channels and in this way prevents the overloading of the cell with calcium ions under pathological and ischemic conditions (13). It has been shown that Ca^{2+} overload is an important factor in the progressive and delayed death of nerve cells occurring in the cerebral injury and cerebrovascular diseases. Ca^{2+} overload produces functional disorders in the mitochondria and activates various calcium- dependent enzymatic reactions (5, 9). Also, Annoura et al., in 2002 reported that the activation of Na^+ channels is involved in the pathway of Ca^{2+} overload and the accumulation of intracellular Na^+ ions leads to a rapid Ca^{2+} overload by the reverse operation of the Na^+/Ca^{2+} exchanger (13). Another

explanation was reported by Qu et al., in 2014 (11). They showed that 6-OHDA modulates electrophysiological properties of SNc dopaminergic neurons through the activation of VGCCs which amplifies 6-OHDA-induced oxidative stress by increasing calcium entry from extracellular matrix. They also reported that N-type calcium channels in SNc change under oxidative stress and the balance between the up-regulation and down-regulation of these channels could be a potential therapy for PD symptoms.

Conclusion

Our data showed that flunarizine has antiparkinsonian and neuroprotective effect against 6-OHDA- induced neurotoxicity. Neuroprotective effect of flu is not mediated by suppression of oxidative stress and might be mediated through the inhibition of Ca^{2+} overload or modulation of the effect of 6-OHDA on the VGCC. We also showed that B vitamins supplement remarkably reduces oxidative stress and when administrated in combination with flu, it largely potentiates its neuroprotective effect. B vitamins supplement can also reduce the effective dose of flu. This data confirms this hypothesis that administration of B vitamins with calcium channels blockers can remarkably decrease their adverse effects.

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