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Synergistic Antiparkinsonian Effect of Flunarizine, Glibenclamide and B Vitamins in a Rate 6-Hydroxydopamine Model; The Role of Malondialdehyde

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Abstract

Background: The current study evaluated the effects of a combination of flunarizine (flu) a calcium channel blocker, glibenclamide (Glib), a KATP channels blocker and B vitamins (B com) on the behavioral symptoms of 6-hydroxydopamine (6-OHDA)-induced model of Parkinson disease to evaluate the synergistic antiparkinsonian effects of the drugs and supplements. Also the level of malondialdehyde (MDA) was measured in blood and brain suspensions to find probable neuroprotective mechanism of these materials.
Methods: 6-OHDA was injected into striatum of rats by stereotaxic surgery. Pretreatment with flu, Glib and B com was started before the surgery and continued to three weeks after the surgery. Development and severity of Parkinson disease were evaluated by the conventional behavioral tests. MDA values were measured spectrophotometrically, using thiobarbituric acid test and the MDA standard curve.
Results: Pretreatment with a combination of flu, Glib and B com ameliorated the behavioral symptoms of Parkinson disease. The effect of the combination was significantly more potent than those of flu, Glib or B com, solely. Pretreatment with the combination or using only Glib or B com separately, reduced the level of MDA in blood and brain, significantly. However, the effect of the combination was significantly more potent than those of Glib or B com, solely.
Conclusions: Since the severity of the behavioral symptoms in the 6-OHDA-induced model of Parkinson disease reflects the degree of the lesion in substantia nigra (SN) dopaminergic neurons, it is suggested that using the combination had neuroprotective effects. The obtained data suggest a synergistic neuroprotective and antiparkinsonian effect for flu, Glib and B com. At least, a part of this effect was mediated through inhibition of oxidative stress.

Keywords: 6-Hydroxydopamine, Flunarizine, Glibenclamide; B Vitamins, Behavioral Symptoms, Malondialdehyde

1. Background

Parkinson disease (PD) is a chronic and progressive neurodegenerative disorder affecting approximately 1.5 million individuals in the US alone and with the extension of life expectancy this number is expected to rise remarkably within the next decades.

The pathological hallmark of PD is the loss of the nigrostriatal dopaminergic (DA) neurons.

The cell bodies of these neurons are in the compact part of substantia nigra pars compacta (SNpc), and they project primarily to the putamen. At the onset of symptoms, putamen dopamine is depleted by 80%, and 60% of SNpc dopaminergic neurons are already lost (1-4).

Current PD medications treat symptoms; none of them halt or retard DA neuron degeneration. Therefore, the current research aimed to prevent DA neurons degeneration.

Flunarizine (flu) is a wide spectrum calcium channel blocker which blocks the T-, L- and N-type calcium channels (5, 6), and also Na^+ channels and prevents the overloading of the cell with Ca^{2+} under pathological and ischemic conditions (7). Neuroprotective effect of this drug is wildly investigated and it is shown that flu has cytoprotectant actions in neuronal cultures (5, 8), chromaffin cell cultures (9), hippocampal slices (10) and in animal models of stroke (11).

ATP-sensitive potassium (KATP) channels are suggested as a potential pharmacotherapeutic target for neuroprotection in some neurodegenerative diseases, including PD (12, 13). They are considered to play an important role in maintaining the membrane potential and mitochondrial matrix volume during ATP decline (12, 14). Studies showed that DA neurons in the SNpc have a high density of KATP channels (15). Glibenclamide (Glib) is a second generation sulfonylurea; it exhibits an inhibitory effect on surface and mitochondrial KATP channels (16). Several authors reported that Glib provides neuroprotective effects (17-21).

Also, evidence shows that B vitamins (B com) provide neuroprotective effects, probably through prevention of mitochondrial dysfunction and inhibition of oxidative stress (22). Previously, it was shown that B vitamins can reduce severity of behavioral symptoms in 6-hydroxydopamine (6-OHDA)-induced model of Parkinson disease (23, 24).

The present study evaluated the effect of a combination of Glib, flu and B com on the behavioral symptoms of

6-OHDA–induced model of Parkinson disease in rats. It was hypothesized that each of these materials provides neuroprotective effects by mechanism(s) which differ from the mechanisms of the other ones. Therefore, it was supposed that they have synergistic antiparkinsonian effect. To find probable neuroprotective mechanism of these materials, the study measured the concentration of Malondialdehyde (MDA), which is a biomarker of lipid peroxidation and oxidative stress both in the serum and in the midbrain portion of brain. The study focused on oxidative stress since 6-OHDA selectively destroys catecholamine neurons by production of reactive oxygen species (ROS). Also, many studies suggest that oxidative stress plays an important role in the events leading to degeneration of DA neurons in humans with PD (1-4).

2. Methods

2.1. Animals and Experimental Groups

The current study was an original/basic scientific research performed in the cellular and molecular research center of Qazvin University of Medical Sciences, Qazvin, Iran. All procedures carried out throughout this study were according to the guidelines of animal experiments of research council at Qazvin University of Medical Sciences. Adult male Wistar rats (Razi Institute, Karaj, Iran), weighing 250 - 300 g at the beginning of the study were housed in large cages (38 × 59 × 20 cm) at a temperature-controlled colony room under light/dark cycle with free access to tap water and standard food.

Animals were divided into six experimental groups as follows: control (con, n = 9) which did not receive any pretreatment; vehicle (veh, n = 10) which received ethanol as the solvent of flu and Glib; B com (n = 9) which received a combination of all of B vitamins five times higher than that in normal MEM (minimum essential medium); F (n = 9) which received flu 5 mg/kg; G (n = 9) which received Glib 5 mg/kg; and F+G+B com (n = 10) which received a combination of flu and Glib 5 mg/kg and also B com. Additional B vitamins were dissolved in drinking water as described before (24). Ethanol, Glib and flu were daily administrated, intraperitoneally. In addition, results of another group of rats (n = 8) referred to healthy rats, were also used to analyze data obtained from rotarod test. Healthy rats were intact rats which did not receive 6-OHDA or any other treatments. All of B vitamins, flu, Glib, 6-OHDA and apomorphine were purchased from SIGMA-ALDRICH Company (Germany).

2.2. Experimental Design

All animals (except healthy group) received 6-OHDA through stereotaxic surgery. All pretreatments were started a few hours before 6-OHDA injection and continued up to three weeks after the surgery (Figure 1). Apomorphine-induced rotational test and elevated body swing test (EBST) were performed during the second, fourth and eighth weeks post-surgery. Rotarod test was performed in the sixth week post-surgery. Blood sampling and serum MDA assay were performed after rotarod test. After performing the behavioral tests, animals were decapitated and MDA concentration in the brain tissues was measured.

2.3. Surgical Procedures

After anesthetization of rats with intraperitoneal injection of a solution containing ketamine (100 mg/kg) and xylazine (5 mg/kg), 4 μL of 6-OHDA (4 μg/μL) dissolved in isotonic NaCl solution containing 0.2 mg/mL of ascorbic acid was injected into the four sites at right striatum using stereotaxic apparatus (Stoelting, USA) through a 10-μL Hamilton syringe. Coordinates for injections were anterior/posterior (AP): 1.5 mm, left biased (L): -2.5 mm, dorsal/verbal (DV): -6 mm and AP: 0.8 mm, L: -3 mm, DV: -6 mm, and AP: 0.1 mm, L: -3.2 mm, DV: -6 mm and AP: -0.5 mm, L: -3.6 mm and DV: -6 mm. AP and L were measured from bregma and DV from the surface of skull according to the atlas of Paxinos and Watson (25).

2.4. Behavioral Testing

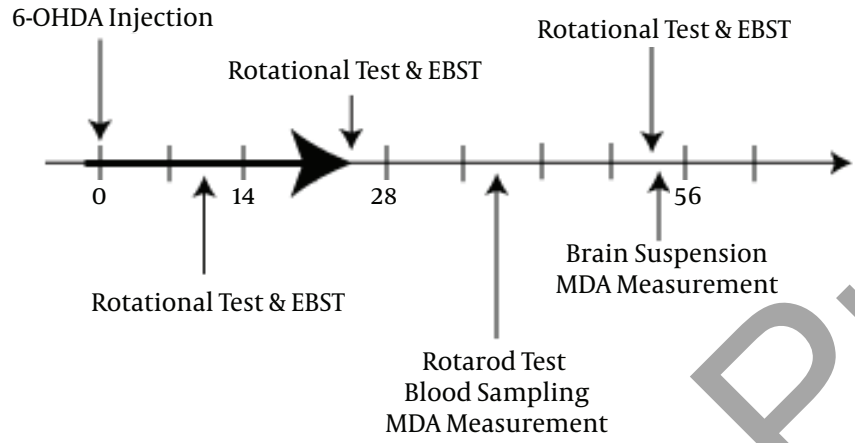
2.4.1. Apomorphine-Induced Rotational Test

Apomorphine-induced rotational test was implemented according to Fujita et al., (26). Animals were first habituated for 5 minutes and then apomorphine hydrochloride (0.5 mg/ kg, IP, dissolved in saline) was injected. One minute later, the number of full rotations was counted for one hour in a cylindrical container (with diameter of 28 and height of 38 cm). Contralateral and ipsilateral rotations (away and toward the lesioned side, respectively) were counted as positive and negative scores and the net number of rotations defined as the positive scores minus the negative ones.

2.4.2. Elevated Body Swing Test

The elevated body swimming test (EBST) was performed according to the previously described method (27). Animal was allowed to habituate for 10 minutes in a cylindrical container, to attain a neutral position, defined as having all four paws on the floor. Then, the animal was held at a position two cm from the base of its tail. After that, the animal was lifted up 2 cm above the surface. The animal was held in the vertical axis, defined as no deviation of more than 100 to either side. Whenever the animal moved its head out of the vertical axis to either side a swing was

Figure 1. Time schedule for the experiments



Animals were tested by apomorphine-induced rotational test and elevated body swing test (EBST) three times: in the second, fourth and eighth weeks after 6-OHDA injection. Rotarod rod test, blood sampling and measuring the level of MDA were performed in the sixth week post-surgery. Preparation of the brain suspension and its MDA assay were performed in the eighth week post-surgery. All pretreatments were started before 6-OHDA injection and continued up to three weeks after that (black arrow). Numbers show the days after 6-OHDA injection.

recorded. For the next swing to be counted, the animal should have returned to the vertical position. Swings were counted for a period of one minute. One observer held the test session time and also determined and recorded the direction and the frequency of swings, while another observer held the rat. All tests were conducted blind 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).

2.4.3. Rotarod Test

The study used rotarod apparatus (M.T6800, Borj Sanat, Iran) to determine the motor performance and the ability of rats to improve motor skill with training. Rotarod test was performed at three consecutive days and two sessions a day. Each session lasted a maximum of 200 seconds, during which the rotating rod underwent a linear acceleration of 5 to 40 rpm over the first 120 seconds of the trial and remained at maximum speed for the remaining 200 seconds. Animals were scored for their latency (in seconds) to fall (height 30 cm) for each trial. Rats were given a minimum rest of 30 minutes between trials to avoid fatigue. Rotarod data were expressed as the area under the curve (AUC), which was computed according to the following formula:

$$AUC = \text{time on the rod (s)} \times [\text{time on the rod(s)} \times 0.44/2]$$

where 0.44 is the acceleration speed per second.

2.5. Blood Sampling and Preparing Brain Suspension

Venous blood samples were collected from the animal tails into a 2 mL microtube using a scalp vein; while the animals were restricted within a restrainer. Bloods were allowed to clot and sera were separated using centrifugation at 5000 rpm (Eppendorf 5415D) for 5 minutes and stored at -80°C until MDA measurement.

To prepare brain suspension, animals were decapitated under diethyl deep anesthesia and the brains were removed immediately. Then the midbrain portion of brain was isolated, washed with normal saline, and sonicated in cooled KCl solution (1.5%) to provide a suspension. Brain suspensions were stored at -80°C until MDA measurement.

2.6. MDA Measurement

MDA values were measured spectrophotometrically by the method described by Albro et al. (28), using thiobarbituric acid (TBA) test and MDA standard curve. 1,1,3,3-tetramethoxypropane was used as standard. MDA reacts with TBA to produce a pink colored solution with the maximum absorbance at 532 nm. The results were expressed as μM/L (for serum) and μM/g (for brain suspension).

2.7. Statistical Analysis

All data were presented as the mean $\pm$ SE. Data on behavioral tests and MDA measurements were first analyzed by Kolmogorov-Smirnov test to assess the normality of the data. Since distribution was not normal, data were analyzed by Kruskall–Wallis nonparametric ANOVA followed

by a two-tailed Mann–Whitney U test. A P value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Rotational Behavior

All experimental groups showed asymmetrical (contralateral to lesioned side) rotations in response to apomorphine, indicating that none of the pretreatments could prevent the development of 6-OHDA-induced model of Parkinson disease. However, as displayed in Figure 2, pretreatments significantly reduced severity of asymmetrical rotations. The most significant effect was observed in F + G + B com group. In this group and in all rotational tests, the number of net contralateral rotations was significantly less than those of veh or con groups ($P < 0.05$ and $P < 0.01$, respectively). Severity of rotational behavior in F + G + B group in the 2nd and 3rd post-surgery tests was significantly less than those of F, G or B com groups. In these tests, severity of rotational behavior in B com, F and G groups was also significantly less than that of the veh group ($P < 0.05$).

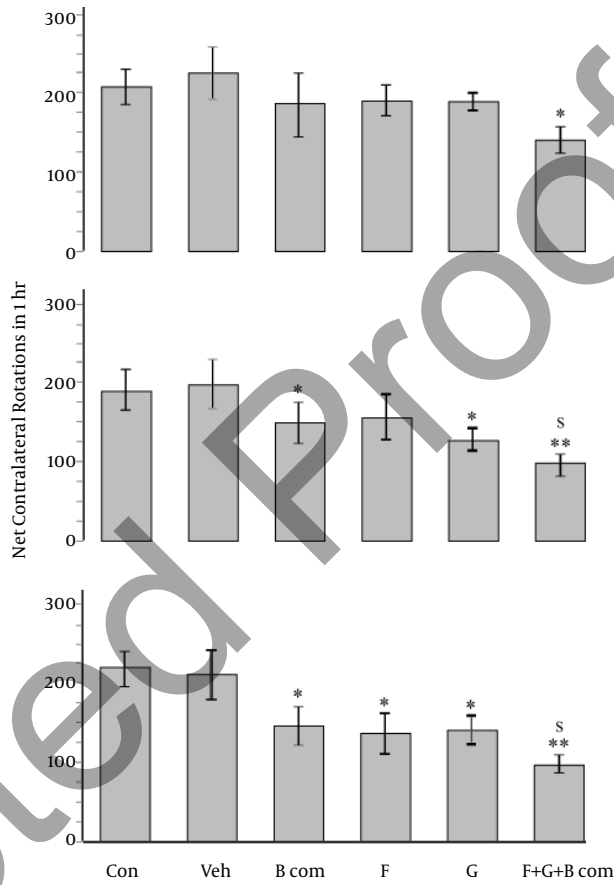


Figure 2. Apomorphine-induced net contralateral rotations of different experimental groups in the second (upper plot), fourth (middle plot) and eighth (lower plot) weeks post-surgery; values are means ± S.E of animals in each group. *, $P < 0.05$ and **, $P < 0.01$ compared to veh group; s, $P < 0.05$ compared to B com and F groups; Kruskall-Wallis nonparametric test followed by Mann-Whitney U test.

3.2. Swinging Behavior

Figure 3 displays findings of EBST. Number of swings varied from 1 to 8 and almost all of the 6-OHDA lesioned rats showed net ipsilateral (to lesioned side) swings. However, in B com and F + G + B com groups, the number of biased swings to ipsilateral side was significantly less than that of the veh group. In the third post-surgery test, the number of biased swings in F + G + B com group was also significantly less than that of the F group. In this test, the number of biased swings in the G group was also significantly less than that of the veh group.

3.3. Rotarod Test

Figure 4 illustrates capability of motor learning of different experimental groups in the rotarod test. In healthy rats, learning was complete and the performance in each session was better than that of the previous session and rats reached the maximum performance in sessions 4 to 6. All groups of 6-OHDA treated rats also showed some degree of motor learning, but there were not complete and significant differences between 6-OHDA treated rats and healthy ones. No one of the 6-OHDA treated group of rats reached the (normally expected) maximum performance, even in the last session. Also, learning pattern was different and the performance did not necessarily improve enough in successive sessions. However, some of the pretreatments had significant effects and improved learning of 6-OHDA lesioned rats. The best improvement was observed in F + G + B com and G groups. In these groups although stepping time on rotarod did not reach the maximum, learning pattern was very similar to that of the healthy rats. Stepping time in these groups in sessions 4 - 6 was significantly higher than that of the veh group ($P < 0.01$ and $P < 0.001$, respectively). Also, in these sessions stepping time in F + G + B com and G groups was significantly higher than that of F or B com groups ($P < 0.01$). Pretreatments with B vitamins increased stepping time in sessions 5 and 6, but pretreatment with flu had no effect.

3.4. MDA Analysis

MDA concentrations were measured in the serum and brain suspension of rats. In the control group, MDA concentrations in blood and brain tissue were 6.16 ± 0.33 μM/L and 9.34 ± 0.85 μM/g, respectively. In vehicle group, these concentrations were insignificantly higher than those of

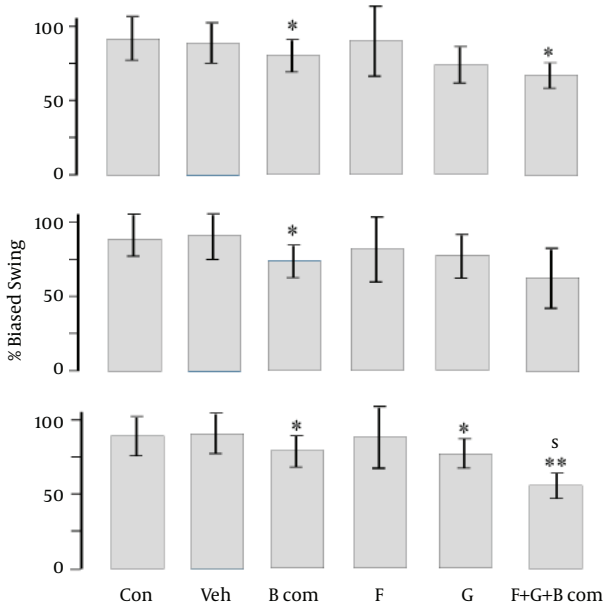


Figure 3. Plots display results of the EBST in the second (upper plot), fourth (middle plot) and eighth (lower plot) weeks post-surgery; 50% means that the number of the left swings was equal to the number of right swings. Less than 50% means that most of the swings were toward left (contralateral to lesion side) and more than 50% means that most of the swings were toward right (ipsilateral to lesion side) side. *, $P < 0.05$ and **, $P < 0.01$ compared to veh group; s, $P < 0.05$ compared to F group; Kruskall-Wallis nonparametric test followed by Mann-Whitney U test.

the control group (Figure 5). In F + G + B com group, MDA concentrations both in serum and brain tissue were significantly less than those of the veh and F groups ($P < 0.01$ and $P < 0.001$, respectively). Also, MDA concentrations in F + G + B com group in serum and brain were significantly less than those of the B com and G groups ($P < 0.05$ and $P < 0.01$, respectively). Pretreatment with B com or Glib also significantly decreased the level of MDA in brain tissue, but not in serum ($P < 0.05$ and $P < 0.01$, respectively). On the other hand, pretreatment with flu increased serum concentration of MDA ($P < 0.05$).

4. Discussion

The current study provided evidence that a combination of flu, Glib and B com has antiparkinsonian effect in the 6-OHDA animal model. This effect was significantly more potent than that of antiparkinsonian effects induced by flu or Glib or B com alone and indicating that flu, Glib and B com had synergistic antiparkinsonian effect. The study also measured MDA in the serum and midbrain portion of the brain. MDA is a biomarker of lipid peroxidation and oxidative stress. Pretreatment with Glib or B com or a combination of flu, Glib and B com reduced MDA concentration in brain. Again, using the combination was more effective than Glib or B com alone. The combination significantly reduced serum MDA level. On the other hand, using flu significantly increased serum MDA level.

A large body of evidence shows a positive relationship between nigral cell death and the severity of behavioral symptoms in the 6-OHDA-induced model of Parkinson disease. Rotational test is the most valid test in the evaluation of 6-OHDA-induced model of Parkinson disease (27, 29-31) and can discriminate partial and nearly complete lesions of the SN [31], and the time spent on the rotating rod in rotarod test, and inversely correlates with the cell loss in the SN. Also, many studies confirmed that EBST is a valuable behavioral test accurately measuring disorders of dopamine-mediated motor function (27, 32, 33). Based on the evidence, it is suggested that pretreatment with flu, Glib, B com or a combination of them had neuroprotective effect and reduced neurotoxic effect of 6-OHDA on the SN dopaminergic neurons. Since the antiparkinsonian effect of the combination was more than those of flu, Glib or B com alone, it is suggested that these pretreatments have synergistic neuroprotective effects.

Previously, authors reported that B com has neuroprotective effect against 6-OHDA-induced neurotoxicity (24). Also, biochemical analysis showed that neuroprotective effect of B com was not mediated by lowering plasma homocysteine. The present study showed that using B com reduces the level of MDA in the brain rats treated with 6-OHDA. Therefore, antiparkinsonian and neuroprotective effects of B com were probably mediated by reduction of oxidative stress. It was in agreement with several data showing that B vitamins had antioxidant effects (22-34).

Several reports addressed the neuroprotective effect of flu and Glib. Flu has cytoprotectant actions in neuronal cultures (5, 8), chromaffin cell cultures (9), hippocampal slices (10) and in animal models of stroke (11). Flu also significantly reduces glutamate-induced neurotoxicity (35), enhances functional recovery following sciatic nerve crush lesion in rats (36) and improves the survival of grafted dopaminergic neurons (37). The current study data clearly show that neuroprotective effect of flu is not mediated by inhibition or reduction of 6-OHDA-induced oxidative stress. Flu blocks Na^+ and Ca^{2+} channels and prevents the overloading of the cell with Ca^{2+} under pathological and ischemic conditions (6). The overload of Ca^{2+} plays an important role in the progressive death of nerve cells occurring in the cerebral injury and cerebrovascular diseases such as stroke and trauma (38, 39). Also, it was recently demonstrated that the accumulation of intracellular sodium ions causes a rapid Ca^{2+} overload through the reverse operation of the Na^+/Ca^{2+} exchange mecha-

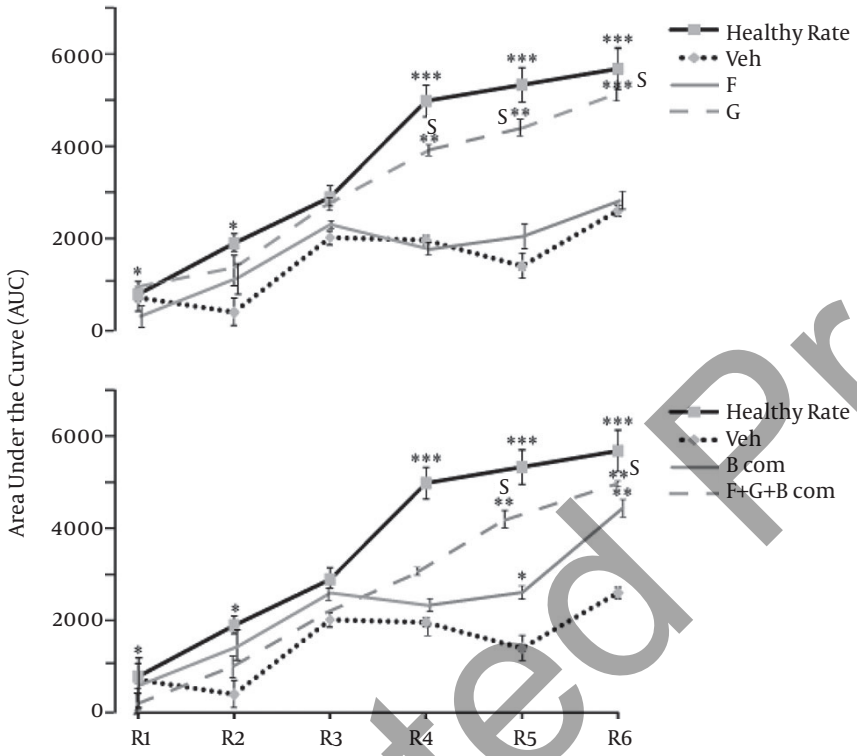


Figure 4. Motor performance of different groups of rats in rotarod test was examined in three consecutive days; two sessions a day. Since con and veh groups of rats showed almost similar results, only data of veh group are shown. *, P < 0.05; **, P<0.01; ***, P < 0.001 compared to veh group; s, P < 0.05 compared to F and B com groups; Kruskall-Wallis nonparametric test followed by Mann-Whitney U test; AUC, area under the curve; R1 - R6, sessions of the test; R1, first session and R6, last session.

nism (6). Another explanation was reported by Qu et al.. They showed that 6-OHDA modulates electrophysiological properties of SNpc dopaminergic neurons through activation of voltage gated calcium channels (VGCCs). Activation of these channels amplifies 6-OHDA-induced oxidative stress by increasing Ca^{2+} entry from extracellular matrix and accelerates cell death (40).

Regarding Glib, Lee et al. showed that Glib attenuates the cytotoxicity of MPP^+ in PC12 cells by suppressing changes in the mitochondrial membrane permeability (18). Kim et al. reported that centrally administration of sulfonylureas exerts a neuroprotective effect against kainic acid (KA)-induced hippocampal CA3 neuronal death (17). Also, studies on the midbrain DA neurons indicate that blockade of KATP channels protects these neurons against neurodegenerative agents (19, 20). Several mechanisms are suggested for neuroprotective effect of Glib. It is reported that Glib prevents the activation of endothelial caspase-3 through inhibition of the sulfonylurea receptors (SUR) 1-regulated NC (Ca-ATP) channels. Caspase-3 activation is described as a major cause of apoptotic processes (41). Glib also suppresses the inflammatory response, including the expression of proinflammatory cytokines IL-10 and TNF-α (42). The current study showed that Glib reduces oxidative stress and that antiparkinsonian effect of Glib may be mediated by its suppressing effect on the oxidative stress. Glib might also have neuroprotective effect by direct blocking of KATP channels. It is reported that 6-OHDA induces mitochondrial impairment and ATP deficiency through inhibition of mitochondrial complexes I and IV (31, 43, 44), which can cause neurodegeneration by persistent activation of KATP channels.

In conclusion, the obtained data showed that combination of flu, Glib and B com has antiparkinsonian effect in 6-OHDA animal model. This effect was significantly more potent than antiparkinsonian effect of flu, Glib or B com solely, suggesting a synergistic antiparkinsonian effect for them. MDA measurement indicated that at least a part of this effect was mediated through inhibition of oxidative stress by B com and Glib. Flu, through the blockade of Ca^{2+} and Na^+ channels, inhibited Ca^{2+} overload and its induced neurodegeneration. This mechanism probably potentiated neuroprotective effect of B com and Glib. Also, besides the inhibition of oxidative stress, Glib might in-

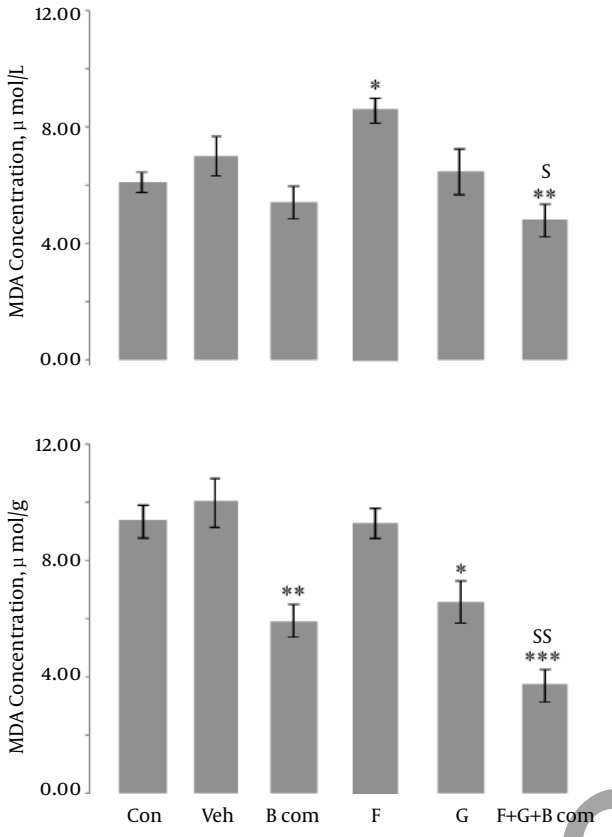


Figure 5. Malondialdehyde concentrations in blood (upper panel) and tissue suspensions prepared from midbrain portion of the brain (lower panel) of rats. *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$ compared to veh group; s, $P < 0.05$ and ss, $P < 0.01$ compared to G group; Kruskall-Wallis nonparametric test followed by Mann-Whitney U test.

hibit 6-OHDA-induced neurodegeneration by preventing the persistent activation of K ATP channels.

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Footnote

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