Short-Term Treatment with Silymarin Improved 6-OHDA-Induced Catalepsy and Motor Imbalance in Hemi-Parkinsonian Rats

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Introduction
Parkinson’s disease (PD) is a progressive neurodegenerative disorder of the central nervous system which affects more than 4 million people over age 60 worldwide.1 The disorder affects different areas of the brain, specially a region called the substantia nigra pars compacta (SNc) that controls balance and movement.2 Often the first manifestation of PD is tremor of a limb, particularly when the body is at rest. Typically, the tremor begins on one side of the body, usually in one hand. Other characteristic symptoms include rigidity or stiffness of the limbs and trunk, slow movement (bradykinesia) or the inability to move (akinnesia), and impaired balance and coordination (postural instability).3 These features occur when dopaminergic neurons in the SNc die or become impaired.2,3 Normally, dopaminergic cells produce dopamine, which transmits signals within the brain to produce smooth physical movements. When these dopamine-producing neurons die or become impaired, communication between the brain and muscles weakens, and eventually, the brain is unable to control muscle movement. The primary cause of PD is still unknown although aging appears to be a major risk factor. Indeed, mitochondrial impairment and elevated oxidative stress have been linked to the PD pathogenesis.4 It is well agreed that chronic neuro-inflammation has part in the pathogenesis of the disease.5,6 Milk thistle (Silybum marianum) extract, silymarin (SM), is a polyphenolic flavonoid which has become so popular among herbalists throughout the world to maintaining liver health. SM is commonly used to treat liver diseases and has antioxidative, anti-apoptotic, anti-inflammatory and neuroprotective effects.7,9,10 SM effect against oxidative stress is due to the scavenging inflammatory,9,10 and increase in reduced glutathione.14 Furthermore, previous studies showed protective effects of SM in several experimental models of neuronal injury, especially in focal cerebral ischemia and cerebral ischemia–reperfusion-induced brain injury in rats.13,14 Because of less information about its effect on PD, in this study we

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investigated the effect of SM on 6-OHDA-induced motor disturbance in hemi-parkinsonian rats.

Materials and Methods

Chemicals
All chemicals were purchased from Sigma Chemical Co. (USA). Solutions were made freshly on the days of experiment by dissolving drugs in physiological saline (0.9% NaCl) except for SM which was dissolved in 50% polyethylene glycol (PEG). The drugs were injected intraperitoneally (i.p) except for 6-OHDA which was injected into SNc.

Animals
Male Wistar rats (240±20 g) were used in this study. The animals were given food and water at liberty and were housed in standard polypropylene cages, four per cage at an ambient temperature of 25±2 °C under a 12-h light/12-h dark cycle. Animals were habituated to the testing conditions including being transferred to the experimental environment, handled, weighed, and restrained on the test platform for 10 min; 2 days before the behavioral investigations were conducted. The present study was carried out in accordance with the ethical guidelines for the Care and Use of Laboratory Animals of Tabriz University of Medical Sciences, Tabriz, Iran (National Institutes of Health Publication No. 85-23, revised 1985).

Experimental protocol
In the beginning of study only the rats that showed normal immobilization in bare test were subjected to further experimentation. The healthy animals were allocated randomly into 8 groups each consisting of eight rats by using simple random sampling method. Rats in group 1 (control group) received no injection and were left untreated for the entire period of the experiment as intact animals. Rats in group 2 (sham-operated) were injected with saline into SNc and were left untreated for the entire period of the experiment. Rats in group 3 (Lesioned (L) group) received only administration of 6-OHDA into SNc. Rats in group 4 (L + vehicle) were received intra-nigral administration of 6-OHDA in the same way as group 3 and then, after recovery period, treated with PEG for 5 consecutive days (days 22 to day 26). Rats in groups 5, 6 and 7 (L + SM) were received intra-SNc administration of 6-OHDA in the same way as group 3 and then, after recovery period, all animals that showed parkinsonian features were treated with i.p injection of SM 100, 200, 300 mg/kg, once daily for 5 consecutive days. Rats in group 8 (SM treated healthy animals) were treated with i.p injection of SM 200mg/kg once daily (9 a.m.) for 5 days.

Surgical procedures
The animals were anesthetized by i.p injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). After the rats were deeply anaesthetized (loss of corneal and toe pad reflexes), they were fixed in a stereotaxic frame (Stoelting, Wood Lane, IL, USA) in the flat position and stereotactically injected with 6-OHDA into SNc through a 23 gauge sterile stainless steel guide cannula. The coordinates for this position were based on the rat brain atlas; anteroposterior from bregma (AP) = -5.0 mm, mediolateral from the midline (ML) = 2.1 mm and dorsoventral from the skull (DV) = -7.7 mm. Desipramine (25 mg/kg, i.p) was injected 30 min before intra-SNc injection of 6-OHDA to avoid degeneration of noradrenergic neurons. Then 6-OHDA (8 μg/ per rat in 2 μl saline with 0.2 % ascorbic acid) was infused by infusion pump at the flow rate of 0.2 μl/min into the right SNc. At the end of injection, guide cannula was kept for an additional 5 min and then was withdrawn slowly. All of these procedures were repeated in Sham-operated animals but they were received only 2 μl vehicle of 6-OHDA.

Cannula verification
For confirmation of placement of the cannula in the SNc, at the end of experiments all rats with guide cannula were euthanized by a high dose of ether. The brains with the injecting tube in situ were removed and placed in a formaldehyde (10%) solution. After 1 week, the tissues were embedded in paraffin. Then serial sections (3 μm) were cut with a microtome (Leitz, Germany), and the placement of the tip of the cannula in the SNc was microscopically controlled. Data from rats with an incorrect placement of the cannula were excluded from the analysis.

Behavioral analysis
All tests were carried out between 9.0 a.m. and 3.0 p.m. Animals were transferred to the experimental room at least 1 h before the test in order to let them acclimatize to the test environment. All experiments were carried out by an observer who was blind to the identity of treatments.

Catalepsy assay test
Catalepsy was assessed by using a standard wooden bar test in the days of 21, 22 and 26 after 6-OHDA injection (Figure 1). Anterior limbs of rat gently extended on 9 cm bar (0.9 cm in diameter) and the duration of retention of rats in this imposed posture was considered as the bar test elapsed time. The end point of catalepsy was designated to occur when both front paws were removed from the bar or if the animal moved its head in an exploratory manner. The cut-off time of the test was 600 sec. The animals were pre-trained to maintain their anterior limb on the bar for 2 consecutive days before main test. Catalepsy was assessed three weeks after neurotoxin injection and 1 and 5 days after intra-peritoneal (i.p) injection of SM in four consecutive times with one hour interval (time 5, 60, 120 & 180 minute).

Rotarod assay test
Assessment of motor coordination was done by using rotarod test. This test was performed on the day 21 after 6-OHDA injection, 1 and 5 days after i.p injection of SM (Figure 1) in four consecutive times, each lasting 720 s, with one hour inter-trial period. Rats were mounted on the rotarod (18 RPM) and the time latency to fall from the rod.
was automatically recorded. All rats were pre-trained for 2 days in order to reach a stable performance. Animals staying during 720 s were taken from the rotarod and their retention time on rotarod considered 720 s. Values were expressed as retention time on the rotarod in the four test trails (5, 60, 120 & 180 minute).

**Statistical analysis**
Statistical analysis of each data set was performed by use of SPSS software (version 16.0). Data were expressed as the mean±SEM, and were analyzed by one-way ANOVA in each experiment. In the case of significant variation (p<0.05), the values were compared by Tukey test.

**Results**

**Effect of intra-SNC injection of 6-OHDA on Catalepsy**
Rats were divided into three groups: normal, sham operated and 6-OHDA (8 μg/2μl/rat)-injected group. Drugs or vehicle were injected into the SNC through the implanted guide cannula. As it has been shown in Figure 2, 6-OHDA was able to induce significant (p<0.001) catalepsy in comparison with both normal and sham-operated rats.

![Figure 2. The results of bar test in normal, sham-operated and 6-OHDA-lesioned (8 μg/2 μl/rat) rats. Each bar represents the mean±SEM of elapsed time (s); n=8 rats for each group; †p<0.001 as compared with normal and sham-operated groups.](https://example.com/image)

**Effect of short-term administration of SM on 6-OHDA induced catalepsy**
The effect of short-term treatment with SM (100, 200 and 300mg/kg, i.p) and its vehicle on catalepsy was investigated in 6-OHDA-lesioned rats for 5 consecutive days (Figure 3). In these groups catalepsy was assessed on day 21 after surgery for 4 repeated times (5, 60, 120 and 180 min). Rats that showed catalepsy were treated with SM and then catalepsy was assessed by bar test at 1 and 5 days after beginning of treatment. The results indicated that treatment with SM for 5 days (in all 3 doses) significantly (p<0.001) attenuated the severity of 6-OHDA induced catalepsy, but in the first day of treatment, only SM 300 mg/kg (p<0.05) decreased 6-OHDA-induced catalepsy (Figure 3A). No alteration was observed on bar test elapsed time in vehicle-treated rats (Figure 3B).

![Figure 3. The results of bar test in 6-OHDA (8 μg/2 μl/rat)-lesioned rats which were treated with silymarin (100, 200 and 300mg/kg, i.p for 15 days) (Figure 3A) and silymarin vehicle (Figure 3B). Each bar represents the mean±SEM of catalepsy time (s); n=8 rats for each group; *p<0.05; ***p<0.001 as compared with 6-OHDA Lesioned group; †p<0.001 when compared with normal rats. (SM=Silymarin); (V=Vehicle of Silymarin); (D = Day).](https://example.com/image)
**Effect of intra-SNC injection of 6-OHDA on motorbalance**

The effect of intra-SNC injection of 6-OHDA on motor coordination was evaluated by rotarod test. The time duration to fall from rotating rod was evaluated in three groups of rats: normal, sham operated and 6-OHDA (8 μg/2μl/rat)-lesioned rats. Drugs and vehicle were injected into the SNC through the implanted guide cannula. As it has been shown in Figure 4, 6-OHDA was able to induce significant (p<0.001) motor imbalance in comparison with both normal and sham-operated.

![Figure 4](Image 49x778 to 545x789)

**Effect of SM on 6-OHDA induced motor incoordination**

The effect of SM (100, 200 and 300mg/kg, i.p) and its vehicle on 6-OHDA-induced motor incoordination was assessed by rotarod. In these groups motor balance was tested 3 weeks after surgery for 4 repeated times (5, 60, 120 and 180 min). Rats that showed motor imbalance were treated with SM and then motor-balance was assessed 1 and 5 days after beginning of treatment. The results indicated that SM (100, 200 and 300mg/kg) after 5 days significantly (p<0.05, 0.01 and 0.001) improved motor balance in 6-OHDA-lesioned rats in a dose and time dependent manner. However, SM (300mg/kg) increased (p<0.001) motor balance in 6-OHDA-lesioned rats in the first day of treatment (Figure 5A). In vehicle treated rats, there was not any alteration on rotarod elapsed time (Figure 5B).

**Discussion**

We have recently reported that pre-treatment with SM amends the 6-OHDA-induced motor impairments by inhibition of inflammatory pathways and oxidative stress. In this study we tried to investigate the effect of SM on 6-OHDA-induced catalepsy and motor imbalance which were assessed by bar test and rotarod respectively. Catalepsy which is commonly assessed by bar test is a valid marker of the nigrostriatal neurodegeneration. In rodents, this is a standard test for evaluation of 6-OHDA and neuroleptic drugs-induced akinesia. In the present study we observed that intra-SNC injection of 6-OHDA resulted in motor impairments (increased catalepsy and decreased motorbalance) in rats which were attenuated by short term treatment with SM. This is in accordance to findings of other researchers who reported that 6-OHDA (8-12μg) caused catalepsy and motor-impairment through induction of progressive neurodegeneration of SNC neurons. It is well established that 6-OHDA induced hemi-parkinsonian rats are a standard experimental model of PD and can be used in investigating of potential neuroprotective effect of drugs. 6-OHDA is a neurotoxin which structurally is similar to dopamine and norepinephrine. After its injection, 6-OHDA is up taken by dopaminergic neurons and then readily oxidized to hydrogen peroxide and paraquinone. It does not cross the blood–brain barrier. Thus, it is widely used to induce experimental models of PD by stereotoxic injection directly into the nigro-striatal region. It has been reported that 6-OHDA increases TNF-α levels in SNC, striatum and cerebrospinal fluid. Furthermore, 6-OHDA causes to neurodegeneration through inhibition of mitochondrial respiratory enzymes and induction of oxidative stress. It seems that also toxic effects of 6-OHDA are partly mediated through activation of microglia.

![Figure 5](Image 310x168 to 540x454)
Several useful effects have been reported for SM in different cultured neuronal cell lines and animal studies. However, less information is available about its effects on motor disturbances in experimental models of PD. In this study we observed that short-term (5 days) treatment with SM could improve 6-OHDA-induced catalepsy. An important finding of this study is that single high dose of SM (300mg/kg) in the first day could decrease catalepsy in bar test. This is in accordance with previous studies showing that pre-treatment with SM and its acute administration decreased catalepsy and rotational behavior in hemi-parkinsonian rats by protecting nigrostriatal neurons against neurodegenerative process. According to the results, intra-nigral injection of 6-OHDA impaired motor coordination so that rats fail to maintain their balances on rotarod and the duration of walking on the rotating drum significantly decreased in lesioned rats when compared with sham-operated group. On the other hand, our results showed a significant decrease in the 6-OHDA induced motor imbalance in lesioned rats which were treated by different doses of SM for five days. Furthermore, SM (300mg/kg) could improve motor-balance in the first day of treatment and increased retention time on rotarod. In rotarod, which is considered as a standard test for evaluation of motor-balance, performance is measured by the period of time that an animal stays up and walks on the rod and the latency to fall off the drum was recorded. In 6-OHDA-lesioned rats loss of dopaminergic neurons and reduction of striatal dopamine levels is an essential cause of motor incoordination. In fact, unilateral lesion of SNc obliges animal to change its weight irregularly for movement and balance; therefore this lead to motor disturbance and asymmetry. Because of high density of microglial in the SNc, the neurons of this area are over sensitized to inflammatory affront as a major exciter of neurodegenerative disease and activation of this microglia lead to release of pro-inflammatory cytokines, which have an important role in neurotoxicity. Previous studies revealed that SM down-regulates nuclear factor kappa B (NF-kB) activation in DA neurons, reduces brain myeloperoxidase activity and inhibits cerebral cyclooxygenase-2 (COX-2), and subsequently decreases release of pro-inflammatory cytokines. These properties may explain the observed motor improving effects of SM in hemi-parkinsonian rats. Additionally, SM decreases brain lipids peroxidation and Malondialdehyde as an index of the oxidative stress severity and increases the level of brain superoxide dismutase (SOD) and glutathione reductase (GR) as antioxidant enzymes in experimental models of PD. It can be postulated that reported neuroprotective effects of SM are mediated at least in part through its anti-oxidant activity.

Conclusion
The short-term administration of SM attenuated 6-OHDA induced catalepsy and motor imbalance. According to the obtained results, we suggest that SM may be used as a potential adjunctive therapy together with routinely used antiparkinsonian drugs. However, further clinical investigations should be carried out to prove it.

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Ethical Issues
Not applicable.

Conflict of Interest
The authors declare that they have no competing interest.

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