Neuroprotective Effect of Silymarin in 6-Hydroxydopamine Hemi-parkinsonian Rat: Involvement of Estrogen Receptors and Oxidative Stress

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Running title: Silymarin protects against Parkinson’s disease

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Abstract
This study examined neuroprotective effect of silymarin (SM) in a model of Parkinson’s disease (PD). Unilateral intrastriatal 6-hydroxydopamine (6-OHDA)-lesioned rats were pretreated i.p. with SM (100 and 200 mg/kg) 1 hour before neurotoxin injection. Fulvestrant was used to evaluate the involvement of estrogen receptors. Net apomorphine-induced rotations and number of Nissl-stained neurons of substantia nigra pars compacta (SNC) were counted in addition to measurement of oxidative stress markers. SM administration only at a dose of 200 mg/kg attenuated the rotational behavior in 6-OHDA-lesioned rats and protected the neurons of SNC against its toxicity and fulvestrant partially attenuated this beneficial effect of SM. In addition, pretreatment with SM at a dose of 200 mg/kg significantly decreased the 6-OHDA-induced thiobarbituric acid reactive substances (TBARS) formation. SM exhibits a dose-dependent neuroprotective effect against 6-OHDA toxicity, partly through attenuating oxidative stress and via an estrogenic pathway.

Key words: Silymarin, 6-hydroxydopamine, Parkinson’s disease, Estrogen receptor, Oxidative stress
**Introduction**

Parkinson's disease (PD) is a neuropathological disorder involving the degeneration of dopaminergic neurons in the substantia nigra with debilitating motor disturbances [24,34]. Oxidative stress, low glutathione levels, DNA damage and iron deposition has been reported as the main causes of dopaminergic neurons degeneration in PD [11,15,25]. Oxidative stress not only destroys the dopaminergic neurons, but it also compromises mitochondrial oxidative phosphorylation, leading to decreased energy output and eventually to secondary cell death [6]. Although great advances have been made in development of agents to treat PD, none yet address the underlying problem associated with it, i.e. the progressive loss of dopaminergic neurons [3,19,36]. Among natural products, silymarin (SM), classified within the group of flavonolignans and isolated from milk thistle plant *Silybum marianum* [21], routinely used to treat liver disorders [14], could prevent lipid peroxidation [8,33], promote regenerative processes, stabilize cell membranes, and possibly through binding to an estradiol binding site, may stimulate the synthesis of ribosomal RNAs [4,29,30]. SM can also increase antioxidative enzyme levels [31]. Neuroprotective effect of SM on oxidative stress in rat brain has already been reported [18]. Furthermore, SM through inhibiting glial cell monoamine oxidase (MAO) or scavenging peroxide products may be useful to attenuate oxidative neuronal damage associated with various neurodegenerative diseases [16]. SM could also lower damage to dopaminergic neurons through inhibiting lipopolysaccharide-induced activation of microglia and reducing production of inflammatory mediators [35]. SM due to its estrogenic activity [26] prevents bone loss in ovariectomized rats and shows mild proliferative effects in uterus [10]. Silymarin is capable of binding to cytosolic estrogen receptors and this binding is exclusive to the estrogen receptor β (ERβ). Treatment of ovariectomized rats with SM or estradiol may allow differentiation of biological effects mediated by the ERα or ERβ [26]. In addition, *in vitro* and *in vivo* studies have described estrogen’s protective effects against amyloid β peptide (Aβ)-induced toxicity [12] and mitochondria toxins such as *N*-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine (MPTP) [7]. One of the
important points regarding SM is that it is a safe herbal product, since no health hazards or side effects are known in conjunction with the proper administration of designed therapeutic dosages [17]. This study was conducted to investigate for the first time the possible neuroprotective effect of SM administration in an early model of Parkinson’s disease in rat and to evaluate the related involvement of estrogenic pathway and oxidative stress.

Materials and Methods

Adult male Wistar rats (250-300 g; n = 80) (Pasteur’s Institute, Tehran) were housed in a temperature-controlled colony room under light/dark cycle with food and water available ad libitum. Procedures involving animals were conducted in conformity with NIH guidelines for the care and use of laboratory animals. Only rats not showing any biased rotational behavior (net rotations less than 30/hour) following intraperitoneal injection of apomorphine hydrochloride (0.5 mg/kg) were selected. The animals were randomly divided into seven groups: sham-operated group (SH), silymarin-treated sham-operated groups (SH+SM100 and SH+SM200) at doses of 100 and 200 mg/kg, lesion group (L), silymarin-treated lesion groups (L+SM100 and L+SM200) at doses of 100 and 200 mg/kg and the treated lesion group receiving estrogen receptor antagonist fulvestrant one hour before neurotoxin injection (L+SM200+Ful; 10 microgram/rat, ICV). Unilateral intrastriatal 6-OHDA injection (left side) was performed through a 5 µl Hamilton syringe on anesthetized rats (ketamine 100 mg/kg and xylazine 5 mg/kg, i.p.) using stereotaxic apparatus (Stoelting, USA) at the coordinates: L –3 mm, AP +9.2 mm, V + 4.5 mm from the center of the interaural line, according to the atlas of Paxinos and Watson [20]. The lesion group received a single injection of 5 µl of 0.9% cold saline containing 2.5 μg/µl of 6-hydroxydopamine-HCL (6-OHDA, Sigma) and 0.2% ascorbic acid (W/V). The SH groups received an identical volume of ascorbate-saline solution. The L+SM100 and L+SM200 groups received the neurotoxin in addition to intraperitoneal pretreatment with a single low and/or high dose of silymarin (100 and 200 mg/kg dissolved in propylene glycol; Sigma) 1 hour before surgery.

Behavioral testing
The animals were tested for rotational behavior by apomorphine hydrochloride (0.5 mg/kg, i.p.) one week before (baseline) and two weeks after the surgery. The rotations were measured according to a method as described previously [1]. Briefly, 1 min after apomorphine injection, full rotations were counted in a cylindrical container (a diameter of 33 cm and a height of 35 cm) at 10-min intervals for 60 min in a quiet isolated room. Net number of rotations was defined as the positive scores minus the negative scores.

**Histological study**

Half of the animals in each group was randomly used for histological assessment. Following behavioral experiment, the rats were deeply anesthetized with a high dose of ketamine (150 mg/kg) and perfused through the ascending aorta with 50 ml of 0.9% saline followed by 100-150 ml of fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) followed by 100 ml of 0.1 M PB containing 10% sucrose. Following perfusion, the brains were removed from the skull, the blocks of forebrain and brainstem were prepared and were cut at a thickness of 40 μm on a freezing microtome (Leica, Germany) and sections collected in PB (0.1 M). Every second section was Nissl-stained with 0.1% cresyl violet (Sigma, USA).

**Neuronal counting**

For each animal, mesencephalic sections (Interaural 2.9-4.2 mm) were examined by a method as described previously [1]. Briefly, Nissl-stained neurons of the SNC were counted (Light microscopy; X200). At least two sections representative of each of four Paxinos-Watson planes (4.2, 3.8, 3.2, 2.97; Interaural) were examined by scanning the entire extent on each side. Counting was done blind to the treatments received.

**Determination of midbrain MDA concentration**

The rats were anesthetized with ketamine (100 mg/kg), decapitated, brains were removed, anterior third part of left midbrain block was blotted dry, weighed, then made into 5% tissue homogenate in ice-cold 0.9% saline solution, centrifuged (1000×g, 4 °C, 10 min) to remove particulates, obtained supernatant was aliquotted, then stored at -80 °C. The MDA concentration in the supernatant was
measured as described before [22]. Briefly, trichloroacetic acid and TBARS reagent were added to supernatant, then mixed and incubated at 100 °C for 80 min. After cooling on ice, samples were centrifuged at 1000×g for 20 min and the absorbance of the supernatant was read at 532 nm. TBARS results were expressed as MDA equivalents using tetraethoxypropane as standard.

**Measurement of midbrain SOD activity**

SOD activity measurement was according to previous works [22]. Briefly, supernatant was incubated with xanthine and xanthine oxidase in potassium phosphate buffer (pH 7.8, 37 °C) for 40 min and NBT was added. Blue formazan was then monitored spectrophotometrically at 550 nm. The amount of protein that inhibited NBT reduction to 50% maximum was defined as 1 nitrite unit (NU) of SOD activity.

**Assay of midbrain NO concentration**

Supernatant NO content was assayed by the Griess method. Because NO is rapidly converted to the stable end products nitrate (NO3⁻) and nitrite (NO2⁻), the principle of the assay is the conversion of nitrate into nitrite by cadmium and followed by color development with Griess reagent (sulfanilamide and N-naphthyl ethylenediamine) in acidic medium. The total nitrite was measured by Griess reaction. The absorbance was determined at 540 nm with a spectrophotometer.

**Protein Assay**

The protein content of the supernatant was measured with Bradford method using bovine serum albumin ( Sigma Chemical, St. Louis, MO) as the standard [2].

**Statistical analysis**

All data are expressed as mean ± S.E.M. For rotational behavior, one-way ANOVA followed by Tukey post hoc test was used. For each group, the values of Nissl-stained cells for the injected and non-injected sides were compared using two-tailed student’s t-test for paired samples and the inter-group differences were analyzed using one-way ANOVA followed by Tukey’s post-hoc test. The latter test was also used for biochemical assays. In all analyses, the null hypothesis was rejected at a level of 0.05.
Results

Regarding apomorphine-induced rotations (Table 1), there were no significant differences among the groups at baseline (before surgery). Statistical analysis of the total net number of rotations made over a 60-min period 2 weeks after the surgery showed that apomorphine caused a very significant contralateral turning in the rats of L group (p<0.0005) and less significant rotations in L+SM200 and L+SM200+Ful groups (p<0.0005) in comparison with SH group. Moreover, groups L+SM200 and L+SM200+Ful showed a significant reduction of rotations (P<0.0005 and p<0.05 respectively) when compared to L group. Meanwhile, although L+SM200 group receiving fulvestrant as an estrogenic receptor antagonist exhibited a higher number of rotations as compared to L+SM200 group, but the existing difference was non-significant. In addition, number of rotations in SH+SM100 and SH+SM200 groups did not show any significant change relative to SH group.

The results of histochemical studies (Table 2 and Figure 1) showed that there is no significant differences for the number of Nissl-stained neurons on the right and left sides of SNC in SM-treated and untreated SH groups, a significant reduction was observed for L group (P<0.01), L+SM100 (p<0.005), and L+SM200+Ful (p<0.05) and no such significant difference was obtained for L+SM200 group. Meanwhile, number of Nissl-stained neurons on the left side of SNC showed a very significant reduction in L group as compared to SH group (p<0.005) and this difference was abolished to a large extent only in L+SM200 group. Furthermore, fulvestrant administration to the latter group caused a non-significant reduction in the number of neurons on the left side of SNC as compared to L+SM200 group.

Regarding midbrain oxidative stress markers (Figures 2-4), intrastriatal injection of 6-OHDA resulted in significant elevation of MDA and nitric oxide content (p<0.01) and significant reduction of SOD activity (p<0.05) and treatment of lesioned rats with SM at a dose of 200 mg/kg significantly attenuated the increased MDA content (p<0.05). However, level of SOD was slightly higher and level of NO was non-significantly lower in L+SM200 as compared to L group.
Meanwhile, fulvestrant administration to L+SM200 group slightly and non-significantly increased level of MDA and nitrite and decreased SOD activity.

**Discussion**

The unilateral damage of the nigrostriatal dopaminergic system through intrastriatal injection of 6-OHDA is followed by a reduction in the striatal dopamine level and an upregulation of dopaminergic postsynaptic receptors at the same side. These changes produce a prominent functional and motor asymmetry that can be evaluated by dopaminergic agonists like apomorphine [25]. These rotations are considered as reliable indicators of nigrostriatal dopamine depletion [27]. According to our previous study [23], an early model of PD could be induced by a lower dose of 6-OHDA (12.5 µg/rat) that is accompanied with a lower reduction in the number of SNC neurons and consequently a lower rotational behavior as observed in this study. In the present study, a significant attenuation of the apomorphine-induced rotational behavior was observed in SM200-treated 6-OHDA-lesioned group after two weeks and fulvestrant partially attenuated this beneficial effect, indicating part of SM effect is mediated through estrogenic pathway. The observed attenuation of rotational behavior in SM-treated lesioned group in this study could also be attributed to possible protective effect of SM against nigral neurodegeneration. In this respect, nigrostriatal neurons within SNC have been mainly preserved against neurodegenerative effects induced by the neurotoxin 6-OHDA. Since number of rotations in L+SM200 group was not restored to normal levels relative to L group in spite of a better condition for the number of Nissl-stained neurons in the former group, this indicates that capability of dopaminergic cells within SNC has still some dysfunctions.

Oxidative stress is considered as an important contributing factor in the progression of PD [13,19]. Antioxidant enzymes such as SOD as well as antioxidant molecules protect the cells against oxidative stress [32]. In the present study, the level of SOD was significantly decreased in the rat brain 2 weeks after 6-OHDA injection, which indicated that the antioxidant enzymes were depleted during the process of combating oxidative stress. Meanwhile, a marked increase in the MDA level
as a reliable marker of lipid peroxidation and level of nitrite was observed in the 6-OHDA lesioned rats. The level of these factors, especially MDA that showed a significant reduction, was reversed in the lesioned group by SM at a dose of 200 mg/kg. SM in this study may have attenuated neuronal damage and loss through counteracting oxidative stress, possibly via regulating antioxidant defense system as well as inhibition of free radical generation [18].

Finally, one of the advantages of using phytochemicals like SM in these studies is their binding affinity for estrogen receptor β in CNS regions [26]. Neuroprotective effect of estrogens may be due to their attenuation of toxin-induced neurotoxicity in brain [9]. Estrogens may also exert their protective actions directly and synergistically with such antioxidants as glutathione [5, 28]. There is also evidence that estrogen prevents lipid peroxidation by sacrificing itself to oxidation [28].

Taken together, this study establishes for the first time the dose-dependent neuroprotective effect of SM administration in 6-OHDA hemi-parkinsonian rat, partly through attenuating oxidative stress and via an estrogenic pathway.

**Acknowledgements**

This work was supported in part by a grant from Research Council of Iran University of Medical Sciences (1385; Tehran, Iran).

**References**


Table 1: Net total apomorphine-induced rotations

<table>
<thead>
<tr>
<th></th>
<th>SH</th>
<th>SH+SM100</th>
<th>SH+SM200</th>
<th>L</th>
<th>L+SM100</th>
<th>L+SM200</th>
<th>L+SM200+Ful</th>
</tr>
</thead>
<tbody>
<tr>
<td>One week pre-surgery</td>
<td>-3.4 ± 4.1</td>
<td>-2.3 ± 2.5</td>
<td>0.2 ± 1.7</td>
<td>3.7 ± 4.7</td>
<td>-0.4 ± 0.9</td>
<td>-0.9 ± 2.7</td>
<td>-4.8 ± 2.3</td>
</tr>
<tr>
<td>Two weeks post-surgery</td>
<td>-2.7 ± 3.8</td>
<td>1.4 ± 2.6</td>
<td>-0.9 ± 3.2</td>
<td>243.2 ± 21.4*</td>
<td>207.6 ± 14.5*</td>
<td>131.4 ± 10.7**</td>
<td>181.3 ± 8.9††</td>
</tr>
</tbody>
</table>

Note that the positive values indicate turns contralateral to the side of lesion.

* P<0.005 (versus SH), † P<0.05, †† P<0.005 (versus L)

Table 2: Total number of Nissl-stained neurons on the left and right sides of SNC

<table>
<thead>
<tr>
<th></th>
<th>SH</th>
<th>SH+SM100</th>
<th>SH+SM200</th>
<th>L</th>
<th>L+SM100</th>
<th>L+SM200</th>
<th>L+SM200+Ful</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left</td>
<td>123.5 ± 9.1</td>
<td>134.6 ± 8.7</td>
<td>119.8 ± 7.3</td>
<td>68.6 ± 11.3***</td>
<td>76.2 ± 8.6****</td>
<td>101.3 ± 10.4</td>
<td>87.3 ± 10.7††</td>
</tr>
<tr>
<td>Right</td>
<td>131.4 ± 9.3</td>
<td>142.9 ± 9.4</td>
<td>128.6 ± 8.5</td>
<td>124.7 ± 9.7</td>
<td>136.8 ± 9.5</td>
<td>124.2 ± 10.9</td>
<td>125.3 ± 9.4</td>
</tr>
</tbody>
</table>

* P<0.05, † P<0.01, †† P<0.005 (in comparison with the right side)

† P<0.05, †† P<0.01, ††† P<0.005 (in comparison with SH group on the left side)
Fig. 1. Photomicrograph of coronal sections through the midbrain showing Nissl-stained neurons in sham-operated (SH), lesioned (L), silymarin-treated L (L+SM200), and SM-treated lesioned group receiving fulvestrant as an estrogenic receptor antagonist (L+SM200+Ful). A severe reduction in the number of neurons in SNC was observed in the lesioned group, but no such marked decrease was noted in the L+SM200 group in comparison with SH group. Scale bar = 250 μm (SNC and SNR = Substantia nigra pars compacta and pars reticulata respectively)
Fig. 2: MDA concentration in homogenate of midbrain from different groups
*p<0.05, **P<0.01 (in comparison with SH)
# P<0.05 (in comparison with L group)

Fig. 3: Superoxide dismutase (SOD) activity in homogenate of midbrain from different groups.
* P<0.05 (in comparison with SH)

Fig. 4: Nitric oxide (NO) content in homogenate of midbrain from different groups.
* p<0.01 (in comparison with SH)