Olive leaf extract reverses the behavioral disruption and oxidative stress induced by intrastriatal injection of 6-hydroxydopamine in rats

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Abstract
Introduction: Oxidative stress seems to play a critical role in the degeneration of dopaminergic neurons in Parkinson’s disease (PD). Antioxidant compounds can deactivate and scavenge free radical. Olive leaves are considered as a useful source of phenolic compounds. Therefore, this study was designed to investigate the effects of methanolic olive leaf extract (OLE) on neurobehavioral activity and antioxidant enzyme activity, malondialdehyde (MDA) and glutathione (GSH) levels in striatum of rats in an experimental model of PD.

Methods: The PD was induced in animals by intrastriatal injection of 6-hydroxydopamin unilaterally. Animals were pretreated with the OLE (50, 100 and 150 mg/kg body weight) for 7 weeks, and then behavioral activity (narrow beam and grip testes) and antioxidant parameters were evaluated.

Results: In our study behavioral testes showed improvement in motor coordination and balance behavior in rats pretreated with OLE. Furthermore the extract of olive leaf restored the activity of antioxidant enzymes (superoxide dismutase, glutathione peroxidase, glutathione reductase and catalase) and decreased MDA and increased GSH levels in the brain of rats.

Conclusion: Our results suggest that OLE shows a neuroprotective effect in animal models of Parkinson’s disease.

Original Article

Introduction
Oxidative stress is defined as an imbalance between the production and the decomposition of oxidants or a defect in antioxidants (Dias et al., 2013). Evidence from clinical and experimental studies indicate that oxidative stress and antioxidants may be directly or indirectly involved in the neural disorders such as Parkinson’s (PD) and Alzheimer’s (AD) diseases (Mosley et al., 2006; Uttara et al., 2009; Agostinho et al., 2010). Parkinson's disease is a progressive movement disorder which is the result of the loss of dopamine-producing brain cells in the substantia nigra (Betarbet et al., 2002; Emborg, 2004; Zaitone et al., 2013). Symptoms of PD include rigidity, tremors, bradykinesia (slowness of movement), postural instability (impaired balance and coordination) and gait disturbances (Dexter and Jenner, 2013; Peterson, 2014; Vervoort et al., 2015). 6-
hydroxydopamine (6-OHDA), hydroxylated analogue of dopamine neurotransmitter, is the most common neurotoxins used as a preclinical model of Parkinson’s disease. Intrastriatal injections of 6-OHDA able to induce depletion of the nigro-neostriatal dopamine (DA) system associated with oxidative stress and motor disturbances in the striatum (Blum et al., 2001; Schober, 2004; Reyhani-Rad et al., 2011; Galindo et al., 2014). Recently several reports suggested that the phenolic compounds in medicinal plants have neuroprotective activity against 6-OHDA-induced toxicity in PD models (Baluchnejadmojarad et al., 2010; Hritcu et al., 2011; Khuwaja et al., 2011; Li and Pu, 2011; Shrivastava et al., 2013). Olive tree (*Olea europaea, Oleaceae*) is an important medicinal plant widely used to treat neurodegenerative disease such as Huntington’s disease (Silva et al., 2006). Also another study reported that olive leaf extract (OLE), was evaluated towards the decrease of amyloid beta(Aβ) aggregation, which is inherent to AD.

Olive leaf extract is rich in caffeic acid, verbascoside and oleuropein (Lee-Huang et al., 2003; Lafka et al., 2013; Ahmed et al., 2014; Guinda et al., 2015). These chemical compound can be a powerful natural antioxidant towards reactive species (Carcel et al., 2010). Previous studies have shown that OLE widely used as an antioxidant, anti-inflammatory and anti-HIV agents (Lee-Huang et al., 2003). In this present study we investigated the neuroprotective activity of OLE against behavioral changes and oxidative stress indices induced by 6-OHDA toxicity in rats.

**Materials and methods**

**Animals**

Forty-two adult male Wistar rats from the Pasteur Institute (Amol, Iran), weighing 200-250 g at the time of surgery, were used. Animals were housed five per cage, in a room with a 12:12 h light/dark cycle (lights on at 07.00 h) and controlled temperature (23±2°C). Animals had free access to food and water and were permitted to adapt to the laboratory conditions for at least 1 week. All procedures in this study were in agreement with the Guide of Care and were approved by the ethics committee on animal experimentation of the University of Mazandaran.

Adult male rats were randomly divided into 6 groups: control group, vehicle group (were gavaged by distilled water and received intrastriatal injection of ascorbic acid-saline), lesioned group (were gavaged by distilled water and received intrastriatal injection of 10μg 6-OHDA/2μl in 0.1% ascorbic acid-saline) and extract-pretreatment lesioned groups (were gavaged by 50, 100 and 150 mg/kg body weight OLE, daily for 7 weeks and received intrastriatal injection of 10 μg 6-OHDA/2μl in 0.1% ascorbic acid-saline).

**Surgery**

After 3 weeks of OLE treatment, all the animals in experimental and vehicle groups were anesthetized intraperitoneally with ketamine hydrochloride (60 mg/kg) and xylazine (5 mg/kg) and fixed in a stereotaxic frame. The stainless steel guide cannula (22-gauge) was implanted unilaterally in the right striatum of the basal ganglia coordinates: AP: +1 mm; L: +2.5 mm; D: +4.5 mm) according to Paxinos and Watson (1998). It was then fixed to the skull with acrylic dental cement. Thereafter, all the animals in the experimental groups were lesioned by injecting 10μg 6-OHDA/2μl in 0.1% ascorbic acid-saline into the right striatum, whereas the vehicle group received 2.0μl of the 0.1% ascorbic acid-saline (Shrivastava et al., 2013).

**Apopomorphine-induced circling behavior**

After 3 weeks of lesioning the rats were tested for drug-induced rotational behavior. Contralateral rotations of animals were recorded after giving 0.5mg/kg apomorphine (in ascorbic acid-saline) subcutaneously and their rotational scores were collected over a period of 60min intervals (Khuwaja et al., 2011).

**Grip test**

Grip strength was measured by recording the length of time the rat was able to hold onto a steel wire, 2mm in diameter and 35cm in length. Measures of grip strength were taken at seven days after injecting the 6-OHDA, for 3 consecutive weeks (Shear et al., 1998).

**Narrow beam test**

The animals were evaluated for narrow beam test at 3 weeks after the surgery. Briefly, the animals were allowed to walk on a stationary wooden narrow flat
beam (L105cm×W1cm) placed at a height of 80cm from the floor. The total time taken to cross the beam was recorded. The maximum time allowed for the task was 2min (Allbutt and Henderson, 2007).

**Brain sampling**
The rats were anesthetized with ketamine, brains were quickly removed and then striatum areas were collected. The striatum samples (150 to 200 mg of right striatum tissue) were homogenized in 1ml of buffer (0.32 mol/l sucrose, 1 mmol/l EDTA and 10 nmol/l Tris–HCl, pH 7.4) in a Teflon glass homogenizer. The homogenate was centrifuged at 13,600g for 30min, and the supernatant was collected and used for the measurement of antioxidant parameters.

**Determination of catalase (CAT) activity**
Catalase activity was assayed following the method of Genet et al. (2002). Briefly, the reaction mixture consisted of 50 mM sodium phosphate buffer pH 7.0, 10mM hydrogen peroxide and 20μl of the brain tissue supernatant. The absorbance of the supernatant was then measured spectrophotometrically at 240 nm for 5min at 25°C against a blank containing all the reagents except the homogenated tissue. The enzyme activity is expressed as μmole of H₂O₂ consumed/min/mg protein.

**Determination of glutathione peroxidase (GPx) activity**
Glutathione peroxidase activity was assayed following the method of Sharma and Gupta (2002). Briefly, the reaction mixture consisted of 1ml of 0.4 M phosphate buffer (pH 7.0) containing 0.4mM EDTA, 1ml of 5 mM NaN₃, 1ml of 4 mM glutathione (GSH) and 200 μl of the brain tissue supernatant was preincubated at 37°C for 5min. The enzyme activity is expressed as the amount of enzyme required to oxidize 1nmol GSH/min.

**Determination of superoxide dismutase (SOD) activity**
Superoxide dismutase activity was assayed following the method of Genet with some modification (Genet et al., 2002). Briefly, the reaction mixture consisted of 50mM sodium phosphate buffer, 0.1mM EDTA, 0.48mM pyrogallol and 20μl the brain tissue supernatant. The absorbance of the supernatant was then measured spectrophotometrically at 240nm for 5min at 25°C against a blank containing all the reagents except the homogenated tissue. The enzyme activity is expressed as the amount of enzyme that causes half maximal inhibition of pyrogallol autoxidation.

**Determination of glutathione reductase (GR)**
Glutathione reductase activity was assayed following the method of Romero et al. (2000). The reaction mixture consisted of 0.1M potassium phosphate buffer, pH 7.0, 2.5mM glutathione disulfide and 125mM NADPH. The absorbance of the supernatant was then measured spectrophotometrically at 340nm for 5min at 25°C against a blank containing all the reagents except the homogenated tissue. The enzyme activity is expressed as 1μmol of NADPH oxidized/min/mg protein.

**Estimation of lipid peroxidation**
Lipid peroxidation as a measure of thiobarbituric acid reactive substances (TBARS) formation was assayed following the method of Esterbauer and Cheeseman (1990). Tissue homogenates containing 1mg protein was mixed with trichloroacetic acid (1 ml, 20%) and thiobarbituric acid (2 ml, 0.67%). After incubation for 1h at 100°C and cooling, the precipitate was removed by centrifugation. The absorbance of the supernatant was then measured spectrophotometrically at 532nm using a blank containing all the reagents except tissue homogenates.

**Determination of reduced glutathione**
The concentration of glutathione was assayed following the method of Fukuzawa and Tokumura (1976). Supernatant (200 μg/l) was added to 1.1ml of 0.25 M sodium phosphate buffer (pH 7.4) followed by the addition of 130 μg/l DTNB [5,5′-dithio-bis (2-nitrobenzoic acid)] to form TNB (5-thio-2-nitrobenzoic acid) 0.04%. Finally, the mixture was brought to a final volume of 1.5 ml with distilled water and absorbance was read in a spectrophotometer at 412nm and results were expressed as μg GSH/μg protein.

**Estimation of protein concentration**
Protein was measured using the dye binding method of Bradford (1976) and bovine serum albumin was used as a standard.
Statistical analysis
All data are presented as mean ± SEM. Statistical analysis of the data was done using one-way analysis of variance followed by Tukey's post-hoc test. Results were considered statistically significant when $P<0.05$.

Results

Effect of OLE pretreatment on apomorphine-induced circling behavior in rats
To determine the extent of lesion in rats, we measured apomorphine induced contralateral rotations (Table 1). The 6-OHDA induced lesion rats showed a significant increase ($P<0.001$) in rotations compared to control group. Also the extract-pretreatment lesioned groups showed a significant decrease in the rotations compared to lesion group ($P<0.001$). In additions there were not any significant differences between the control, vehicle and OLE pretreated lesion (100 and 150 mg/kg) groups.

Effect of OLE pretreatment on narrow beam test
The crossing time on narrow beam was increased significantly in lesion group as compared to control group ($P<0.001$). The OLE pretreated lesion group has significantly improved the crossing time ability as compared to the lesion group ($P<0.001$, Fig. 1).

Effect of OLE pretreatment on grip strength test
The grip strength was found to be significantly decreased in the lesion group as compared to the control group ($P<0.001$). Whereas OLE pretreatment significantly improved the grip strength in the OLE pretreated lesion group (100 and 150 mg/kg) as compared to the lesion group ($P<0.001$, Fig. 2).

Effect of OLE pretreatment on the contents of malondialdehyde (MDA) and GSH
According to the results in Table 2, 6-OHDA increased lipid peroxidation level of striatum homogenates compared to the control group ($P<0.001$). OLE pretreatment showed significant decrease in MDA level in striatum as compared to the lesion group ($P<0.001$). Also these results showed that glutathione levels decreased in striatum homogenates ($P<0.001$) and OLE pretreatment caused an increase in the level of reduced glutathione ($P<0.01$).

Effect of OLE pretreatment on the activity of antioxidant enzymes (GPx, GR, CAT and SOD)
The effects of OLE on the activity of SOD, GPx, GR and CAT in the striatum of experimental groups are shown in Table 2. The activity of SOD was decreased significantly in lesion group as compared to control group ($P<0.001$), whereas SOD activity was protected significantly when the animals of the lesion group were pretreated with 100 and 150 mg/kg of the OLE as compared to lesion group (respectively $P<0.01$ and $P<0.001$). The activity of CAT was decreased significantly in lesion group as compared to control group ($P<0.001$). A significant increase of the CAT activity was observed in 6-OHDA lesioned groups pretreated with high-dose (150 mg/kg) of the OLE compared to 6-OHDA lesioned group ($P<0.01$). The activity of GR was decreased significantly in lesion group as compared to control group ($P<0.001$), whereas their activity was protected significantly in pretreated lesion group (100 and 150 mg/kg) as compared to lesion group ($P<0.001$). The protective effect of OLE on the activity of GPx in rat brain tissue homogenates of different groups was also assessed. It is apparent from Table 2 that the activity of GPx, was decreased significantly in lesion group as compared to control group ($P<0.001$), whereas a significant increase of the GPx activity was observed when the animals of the lesion group were pretreated with OLE (100 and 150 mg/kg) as compared to lesion group ($P<0.001$). Also, post hoc analyses revealed non-significant statistical differences between 6-OHDA-lesioned groups pretreated with the OLE 50 mg/kg and 6-OHDA-lesioned groups (Table 3).

Discussion

Previous investigations have determined that treatment with 6-OHDA causes neuronal death by the production of reactive oxygen species (Schober, 2004) and damages cell structures (Adly, 2010). Compared to other organs, the brain has been found to be more vulnerable to oxidative stress due to its high lipid content. 6-OHDA is the most common neurotoxin used to produce experimental model of PD manifested by motor imbalance and progressive movement disorder. 6-OHDA shows high affinity for the dopamine transporters and consequently enters dopaminergic neurons. After intrastriatal injection of 6-OHDA, the toxin is retrogradely transported and accumulated in the cytosol and can destroy...
Olive extract reverses oxidative stress

**Fig.1.** Effect of OLE (50, 100 and 150 mg/kg) pretreatment on grip strength test on the 7th (A), 14th (B) and 21st (C) days after the surgery in 6-OHDA-lesioned rats: The OLE has protected the grip of the animals in the OLE pretreatment group as compared to the lesion group, which has shown that the lesion group animals pretreated with OLE has performed better motor coordination as compared to the lesion group. The data are expressed as mean±SEM (n=7), ***P <0.001 versus control group and +++P< 0.001 versus lesioned group (ANOVA and Tukey’s test).
Fig. 2 Effect of OLE (50, 100 and 150 mg/kg) pretreatment on narrow beam test on the 7th (A), 14th (B) and 21st (C) days after the surgery in 6-OHDA-lesioned rats: There was significant difference between the control and lesioned group in the total time to cross the beam and there was also significant difference between the extract-pretreatment lesioned groups and lesioned group. The data are expressed as mean±SEM (n=7), ***P<0.001 versus control group and +++P<0.001 versus lesioned group (ANOVA and Tukey’s test).
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**Table 1:** Net total apomorphine-induced rotations.

<table>
<thead>
<tr>
<th>Group</th>
<th>Average of total net number of rotations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.71 ± 0.99</td>
</tr>
<tr>
<td>Vehicle</td>
<td>13.71 ± 1.14</td>
</tr>
<tr>
<td>6-OHDA (10 µg/rat)</td>
<td>137.85 ± 9.73 ***</td>
</tr>
<tr>
<td>50 mg/kg OLE + 6-OHDA</td>
<td>50.14 ± 3.43 ***</td>
</tr>
<tr>
<td>100 mg/kg OLE + 6-OHDA</td>
<td>17.85 ± 1.22 +++</td>
</tr>
<tr>
<td>150 mg/kg OLE + 6-OHDA</td>
<td>15.71 ± 1.7 +++</td>
</tr>
</tbody>
</table>

The data are expressed as mean±SEM (n=7), ***P<0.001 versus control group, +++P<0.001 versus lesioned group (ANOVA and Tukey’s test).

**Table 2:** Effect of OLE pretreatment on reduced glutathione and TBARS levels in striatum of experimental groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA (µg/mg protein)</th>
<th>GSH (µg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>67.86 ± 1.8</td>
<td>0.59 ± 0.06</td>
</tr>
<tr>
<td>Vehicle</td>
<td>72.94 ± 3.49</td>
<td>0.46 ± 0.03</td>
</tr>
<tr>
<td>6-OHDA</td>
<td>132.98 ± 4.48 ***</td>
<td>0.16 ± 0.04 ***</td>
</tr>
<tr>
<td>50 mg/kg OLE + 6-OHDA</td>
<td>92.14 ± 0.78 ***</td>
<td>0.27 ± 0.02 ***</td>
</tr>
<tr>
<td>100 mg/kg OLE + 6-OHDA</td>
<td>74.33 ± 3.57 +++</td>
<td>0.38 ± 0.007 ** ++</td>
</tr>
<tr>
<td>150 mg/kg OLE + 6-OHDA</td>
<td>72.69 ± 1.58 +++</td>
<td>0.407 ± 0.029 * ++</td>
</tr>
</tbody>
</table>

The data are expressed as mean±SEM (n=7), ***P<0.001 versus control group, **P<0.01 versus control group,* P<0.05 versus control group. +++P<0.001 versus lesioned group. ++ P<0.01 versus lesioned group. +P<0.05 versus lesioned group (ANOVA and Tukey’s test).

**Table 3:** Effect of OLE pretreatment on antioxidant enzymes activity in striatum of experimental groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>GPx (U/mg protein)</th>
<th>GR (U/mg protein)</th>
<th>SOD (U/mg protein)</th>
<th>CAT (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23.57 ± 0.97</td>
<td>35.82 ± 1.9</td>
<td>63.5±3.25</td>
<td>16.72 ±1.19</td>
</tr>
<tr>
<td>Vehicle</td>
<td>23.16 ± 1.01</td>
<td>32.14 ± 0.32</td>
<td>57.47 ± 2.68</td>
<td>13.74 ± 0.49</td>
</tr>
<tr>
<td>6-OHDA</td>
<td>5.84 ± 0.36 ***</td>
<td>6.81 ± 2.83***</td>
<td>5.48 ± 2.30***</td>
<td>1.51± 0.09 ***</td>
</tr>
<tr>
<td>50 mg/kg OLE + 6-OHDA</td>
<td>8.28 ± 0.20 ***</td>
<td>13.77 ± 0.44***</td>
<td>29.22 ± 3.4**</td>
<td>2.5 ± 0.11 ***</td>
</tr>
<tr>
<td>100 mg/kg OLE + 6-OHDA</td>
<td>14.72 ± 0.38***+++</td>
<td>24.29 ± 0.40***+++</td>
<td>46.8 ± 14.39 ++</td>
<td>5.2 ± 0.96 ***</td>
</tr>
<tr>
<td>150 mg/kg OLE + 6-OHDA</td>
<td>18.68± 0.68***+++</td>
<td>30.31 ± 1.36+++</td>
<td>50.32 ± 1.9+++</td>
<td>10.19± 3.1* ++</td>
</tr>
</tbody>
</table>

The data are expressed as mean±SEM (n=7), ***P<0.001 versus control group,** P<0.01 versus control group,* P<0.05 versus control group. +++P<0.001 versus lesioned group. ++ P<0.01 versus lesioned group. +P<0.05 versus lesioned group (ANOVA and Tukey’s test).

dopaminergic neurons by oxidative stress and apoptosis (Blum et al., 2001; Schober, 2004; Reyhani-Rad et al., 2011; Galindo et al., 2014). Our findings confirm that the increase in apomorphine-induced contralateral rotation in 6-OHDA-lesioned group is a reliable marker to determine the extent of lesion in rats. It was observed that contralateral rotations after treatment with apomorphine are only possible when the lesion is complete or nearly complete (Przedbroski et al., 1995).

This study presents role of OLE in the neuroprotection of PD. Among the various bioactive functions of OLE, it has been known to have antioxidant, anti-inflammatory and anticancer
activities (Omar, 2010; Hassan et al., 2014) and has the ability to cross the blood–brain barrier (Mohagheghi et al., 2011; Rabiei et al., 2012). We report here a significant attenuation of the apomorphine-induced rotational behavior in the OLE pretreated lesion group. Furthermore, we have evaluated the motor disturbance by narrow beam test and grip test in all groups. Narrow beam was provided rich source of information about the motor coordination skill (Shear et al., 1998; Allbutt and Henderson, 2007). We showed that the time taken to cross beam was more in lesion group. This has been further supported by other reports (RajaSankar et al., 2009). Moreover the time was decreased in the OLE pretreated group indicating that the rats have more ability to coordinate their movement on narrow beam. In grip test, animals hold onto a wire. It was observed that the mean time taken on the wire was less in lesion group and this was attenuated by OLE pretreated group.

Previous Study about the causes and mechanisms of neurodegenerative disorders such as Parkinson's disease has led to believe that the therapeutic use of antioxidants might be beneficial in PD (Jenner, 2003; Di Matteo and Esposito, 2003; Zhou et al., 2008). Following this line of evidence, the number of studies with polyphenols has increased considerably during the last years (Ramassamy, 2006; Mandel et al., 2008; Rodriguez-Morato et al., 2015). Our results are in harmony with other reports, where motor deficits in Parkinsonian rat have been attenuated by antioxidant supplementation (RajaSankar et al., 2009; Khan et al., 2010; Khuwaja et al., 2011). 6-OHDA increased lipid peroxidation, along with reduced activities of antioxidant enzymes in the rat brain (Ciobica et al., 2009), as we observed in the current study.

Conclusion

Markers of oxidative stress, such as lipid peroxidation, GSH, SOD, GPx, GR and CAT have increasingly been recognized to play an important role in the pathogenesis of several neurodegenerative disorders, including Parkinson's disease (Gomes et al., 2012). In this respect, we demonstrated that pretreatment of rats with OLE at doses of 50,100 and 150 mg/kg body weight, prior to 6-OHDA-induced oxidative stress, can lead to significant improvement of antioxidants enzymes activity (i.e., SOD, GPx, GR and CAT), increase the level of GSH and reduction in lipid peroxidation in rats brain. Thus based on our findings we can suggest that OLE could be a useful neuroprotective candidate for Parkinson's treatment.

Acknowledgments

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Conflict of interest

The authors declare that there are no conflicts of interests regarding the publication of this paper.

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