Research Report

Resveratrol attenuates 6-hydroxydopamine-induced oxidative damage and dopamine depletion in rat model of Parkinson’s disease

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\textbf{ABSTRACT}

The present study was undertaken to investigate the neuroprotective effects of resveratrol (RES) on 6-hydroxydopamine (6-OHDA)-induced Parkinson’s disease (PD) in rats. PD is an age-related neurodegenerative disorder in which the role of reactive oxygen species (ROS) is strongly implicated. RES, a polyphenolic antioxidant compound enriched in grapes, has been shown to have antioxidant and anti-inflammatory actions and thus was tested for its beneficial effects using 6-OHDA-induced PD rat model. Male Wistar rats were pretreated with RES (20 mg/kg body weight i.p.) once daily for 15 days and subjected to unilateral intrastrital injection of 6-OHDA (10 µg in 0.1% ascorbic acid in normal saline). Three weeks after 6-OHDA infusion, rats were tested for neurobehavorial activity and were killed after 4 weeks of 6-OHDA infusion for the estimation of lipid peroxidation, glutathione content, and activity of antioxidant enzymes (glutathione peroxidase [GPx], glutathione reductase [GR], catalase [CAT], and superoxide dismutase [SOD]). RES was found to be successful in upregulating the antioxidant status and lowering the dopamine loss. Conversely, the elevated level of thiobarbituric acid reactive substances (TBARS), protein carbonyl (PC), and activity of phospholipase A2 in 6-OHDA group was attenuated significantly in RES-pretreated group when compared with 6-OHDA-lesioned group. These results were supported by the immunohistochemical findings in the substantia nigra that has shown

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1. Introduction

Parkinson’s disease (PD) is the second most frequent type of neurodegeneration after Alzheimer’s disease (AD), which is accompanied with motor deficit due to degeneration of dopaminergic neurons including striatum, substantia nigra (SN), and nigrostriatal pathway. Recent evidence shows that oxidative stress contributes to the cascade leading to dopaminergic cell degeneration, and it has been intimately linked to other components of neurodegenerative processes, such as inflammation and cell death (Jenner, 2003; Dauer and Przedborski, 2003; Yokoyama et al., 2008).

The brain and nervous system are prone to oxidative stress and are inadequately equipped with antioxidant defense systems to prevent “ongoing” oxidative damage. Oxidant stress to the brain predominantly manifests as lipid peroxidation because of its high lipid content, high concentration of polyunsaturated fatty acids, and low glutathione content that are particularly susceptible to oxidation. Oxidative damage to lipid, fatty acid, and protein (protein carbonyl formation) can lead to structural and functional disruption of the cell membrane, inactivation of enzymes, and, finally, cell death. Thus, it can be speculated that supplemental antioxidant treatment may boost the system to stay normal against the oxidative stress. Earlier, our research group has investigated and reported the preventive effect of certain antioxidants against different experimental models of neurodegeneration (Zafar et al., 2003; Ahmad et al., 2005; Ishrat et al., 2009).

A number of new genetic and toxin models of PD and advances in older models are yielding important new information about the pathogenesis of PD. To understand the pathogenesis of PD and to develop potential therapies for improved symptomatic management, it is important to have relevant disease models. Neurotoxin, 6-hydroxydopamine (6-OHDA) provides useful animal models of PD by inducing the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc), which results in dopamine depletion in the striatum (Kirik et al., 1998; Blum et al., 2001; Deumens et al., 2002).

Since oxidative damage is implicated in the etiology of neurological complications, treatment with antioxidants has been used as a therapeutic approach in various types of neurodegenerative disease. Resveratrol (RES), a polyphenolic compound enriched in grapes and red wine, has attracted wide attention lately because of its antioxidant and anti-inflammatory properties (Fremont, 2000; Tsai et al., 2007; Park et al., 2009; Yousuf et al., 2009; Sebai et al., 2009). RES is a lipophilic potential antioxidant compound; it has been reported to attenuate 6-OHDA-induced neurotoxicity and it up-regulates mitochondrial function and facilitates the synthesis of ATP (Jin et al., 2008; Yousuf et al., 2009). It has been demonstrated that RES is a potent neuroprotective agent (Kumar et al., 2006; Okawara et al., 2007; Jin et al., 2008; Yousuf et al., 2009) and has shown reduction in inflammation via inhibition of prostaglandin production and cyclooxygenase-2 activity (Murias et al., 2004; Jin et al., 2008). RES was found to protect neurons against amyloid toxicity (Marambaud et al., 2003), a prime culprit in the Alzheimer’s disease, and also increases cognitive ability (Luo and Huang, 2006). Recently, our research group has investigated and reported the efficacy of RES on ischemia–reperfusion injury in rat (Yousuf et al., 2009).

RES may have a therapeutic role in the amelioration of oxidative damage and dopamine depletion via its antioxidant potential and modulation of inflammation via inhibition of phospholipase activity and COX-2 expression. This study investigates the pretreatment effects of RES therapy on behavioral dysfunction, biochemical alterations, and histological alterations as well as dopamine level in striatum of our standardized 6-OHDA rat model of Parkinson’s disease.

2. Results

2.1. Behavioral observations

2.1.1. Apomorphine-induced circling behavior

The results of apomorphine-induced circling behavior (stereotypy) are presented in Fig. 1a. Apomorphine, a DA receptor agonist, causes contralateral rotations in 6-OHDA-lesioned (L) rats, exhibiting a significant increase \( P < 0.01 \) in circling behavior when compared to sham (S) group. Rats receiving resveratrol (RES) pretreatment before 6-OHDA lesioning (RES+L) exhibited significant attenuation \( P < 0.05 \) in circling behavior. No significant change was observed in the RES alone pretreatment sham group (RES+S) as compared to S group.

2.1.2. Rota rod

A significant depletion \( P < 0.001 \) in muscles coordination in L group as compared to S group was observed (Fig. 1b). RES (20 mg/kg body weight) was found to be effective in partial recovery of muscular in-coordination in RES+L groups as compared to L group. No significant alteration was observed in RES+S group when compared to S group.

2.1.3. Stepping test

Fig. 1c shows a significant impairment in the adjusting steps task in L group as compared to S group. RES-pretreated group subjected to stereotaxic injection of 6-OHDA has a significant \( P < 0.05 \) effect in stepping test. No significant alteration was observed in RES+S group as compared to S group.

2.2. Biochemical observations

2.2.1. Effect of RES on TBARS level

A significant increase \( P < 0.001 \) in TBARS level was observed in L group when compared to S group. Rats of RES+L group exhibited significant attenuation \( P < 0.01 \) in TBARS level in
comparison to L group rats. RES alone pretreated S group exhibited no significant change in TBARS level when compared to S group (Fig. 2a).

2.2.2. Effect of RES on protein carbonyl (PC)
The level of protein carbonyl content was significantly (P<0.01) elevated in L group, which was significantly different (P<0.05) by RES pretreatment. There was no significant alteration in protein carbonyl content in RES+S group animals as compared to S group (Fig. 2b).

2.2.3. Effect of RES on GSH content
A significant decrease (P<0.001) in GSH level was observed in L group when compared to S group, which was restored significantly (P<0.01) in RES+L group as compared to L group rats. The level of GSH was not elevated significantly in RES+S group as compared to S group (Fig. 2c).

2.2.4. Effect of RES on antioxidant enzymes activity in parkinsonian rats
The activity of antioxidant enzymes (GPx, GR, CAT, and SOD) in RES+S group was not attenuated significantly, as compared to S group. But the activity of these enzymes was decreased significantly in L group as compared to S group (Table 1). On the other hand, RES administration in RES+L group reduced the activity of these enzymes significantly as compared to L group.

2.2.5. Na+/K+-ATPase activity and its restoration by RES
The activity of Na+/K+-ATPase was found to be significantly decreased (P<0.05) in L group rats as compared to S group. RES supplementation significantly protected (P<0.01) the activity of Na+/K+-ATPase as compared to 6-OHDA-infused rats (Fig. 3a). Activity was not altered in RES+S group as compared to S group.

2.2.6. Dopamine and DOPAC levels
A significantly decreased (P<0.01) level of DA and DOPAC was observed in striatal region of 6-OHDA-lesioned rats as compared to S group, indicating a significant loss of dopaminergic neurons in L group animals. DA and DOPAC level in RES+L group exhibited more pronounced and significant increase in comparison to L group rats, indicating functional viability of dopaminergic neurons (Table 2). No significant change was observed in the RES alone pretreated sham group (RES+S) as compared to S group.

2.2.7. Dopamine D2 receptor binding
Fig. 3b shows a significant increase (P<0.01) in DA–D2 receptor binding in 6-OHDA-lesioned rats as compared to S group. RES+L group has shown to exhibit attenuated DA receptor binding significantly (P<0.001) when compared to the L group. No significant change was observed in the RES alone pretreated sham group (RES+S) as compared to the S group.

Fig. 1 – (a) Effect of resveratrol (RES) on apomorphine-induced contralateral rotations. 6-OHDA administration significantly increased the rotation in lesion (L) group as compared to sham (S) group. Pretreatment with RES significantly decreased rotations in RES+L group as compared to lesion group. Values are expressed as mean±SEM of eight animals. *P<0.01, L vs. sham; †P<0.05, RES+L vs. L. (b) 6-OHDA administration significantly impaired the stepping task in L group as compared to S group. Pretreatment with RES significantly improved stepping task in RES+L group as compared to L group. Values are expressed as mean±SEM of eight animals. *P<0.01, **P<0.001, L vs. sham; †P<0.05, RES+L vs. L. (c) 6-OHDA administration impaired the stepping task in L group as compared to S group. Pretreatment with RES significantly improved stepping task in RES+L group as compared to L group. Values are expressed as mean±SEM of eight animals. *P<0.05, †P<0.01, L vs. sham; ‡P<0.05, RES+L vs. L.
2.2.8. Effect of RES on phospholipase A2 activity

The activity of PLA2 increased significantly \((P < 0.05)\) in 6-OHDA-lesioned rats as compared to S group rats, and it was significantly \((P < 0.01)\) decreased in RES pretreated 6-OHDA-lesioned animals as compared to L group rats. No significant change was observed in RES alone pretreated sham group (RES+S) as compared to the S group (Fig. 3c).

2.3. Tyrosine hydroxylase (TH) immunohistochemistry

The neuroprotective action of RES and functional viability of dopaminergic neurons in the SNpc were further assessed by mapping the rate-limiting enzyme, tyrosine hydroxylase, for DA biosynthesis. In 6-OHDA-lesioned rats, the expression of TH was less as compared to S group (Fig. 4). Whereas TH expression in RES-pretreated rats was more pronounced as compared to lesioned rats and exhibited a better survival and metabolic activity of DA neurons, RES pretreatment did not show any remarkable effects in the RES+S compared with the S group (data not shown).

2.4. Cyclooxygenase-2 (COX-2) expression

No COX-2-positive expressions are seen in vehicle-treated rat. COX-2 expression is abundant after 6-OHDA insult, which was restored in RES+L group as compared to L group (Fig. 5). These data show the blockade of COX-2 expression in RES+L group, which was attenuated in 6-OHDA-induced SNpc dopaminergic neuronal death. RES pretreatment did not show any remarkable effects on COX-2 expression in the RES+S group compared with the S group (data not shown).

3. Discussion

In this study, to investigate the effects of resveratrol (RES) on dopaminergic neurons, we used unilaterally 6-OHDA-injected rat, which is one of the most popular experimental models of PD (Deumens et al., 2002; Kirik et al., 1998; Warraich et al., 2009). The nigrostriatal damage caused by intrastral infu-
sion of 6-OHDA is associated with oxidative stress and inflammatory response probably by generating free radicals. Oxidative nigrostriatal damage in 6-OHDA-induced rats has consistent with previous studies carried out by us (Zafar et al., 2003; Ahmad et al., 2005) and others (Chaturvedi et al., 2006; Guo et al., 2007; Garcia et al., 2008). Thus, ROS-scavenging antioxidants may play an important role in the prevention of PD and combat against oxidative stress-induced progressive neurodegeneration. Moreover, RES is known to have a potent antioxidant and anti-inflammatory property and significantly prevented all the alterations caused by 6-OHDA infusion in rats. This neuroprotection is consistent with previous reports (Okawara et al., 2007; Jin et al., 2008; Blanchet et al., 2008; Chao et al., 2008).

The behavioral effects are closely linked to the degree of neuronal dysfunction (Schwarting et al., 1991). Apomorphine-induced contralateral rotation in 6-OHDA-lesioned rats is a reliable marker for the nigrostriatal DA depletion. Rotation...
due to apomorphine is only possible when the lesion is complete or nearly complete, whereas the mildly lesioned rats do not rotate significantly (Przedborski et al., 1995). We report here an appreciable decrease in drug induced rotations and a significant restoration of striatal DA and its metabolites following pretreatment with RES. The motor coordination skills observed in 6-OHDA lesioned rats in our study were protected following exposure to RES. The behavioral defects following the lesion may, in turn, be restored by the pool of DA, now made available by this pathway as observed in our results. Our findings correlate well with the earlier studies carried out by us and others, where motor deficits in Parkinsonian rat have been attenuated by selenium, ginkgo biloba, and black tea extract (Zafar et al., 2003; Ahmad et al., 2005; Chaturvedi et al., 2006).

Brain cells are continuously exposed to reactive oxygen species generated by oxidative metabolism, and in certain pathological conditions, defense mechanisms against oxygen radicals may be weakened and/or overwhelmed, which can be effectively protected by the use of various antioxidants. Oxidative stress to dopaminergic neurons of SNpc is believed to be one of the leading causes of neurodegeneration in PD. Oxidative stress promotes lipid peroxidation and alters the antioxidant defense system in the brain. The decreased levels of TBARS in the brain of RES-pretreated lesioned group indicate a

Table 1 – Effect of resveratrol (RES) on activity of antioxidant enzymes in striatum of 6-OHDA-induced rats.

<table>
<thead>
<tr>
<th>Groups</th>
<th>GPx (nmol NADPH oxidized/min/mg protein)</th>
<th>GR (nmol NADPH oxidized/min/mg protein)</th>
<th>SOD (nmol of epinephrine protected from oxidation/min/mg protein)</th>
<th>CAT (nmol H2O2 consumed /min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>383.72 ± 16.86</td>
<td>523.9 ± 20.13</td>
<td>339.01 ± 12.01</td>
<td>7.72 ± 0.71</td>
</tr>
<tr>
<td>Lesion</td>
<td>206.67 ± 15.76 (“−46.07”)</td>
<td>262.27 ± 11.23 (“−49.93”)</td>
<td>168.04 ± 9.94 (“−50.43”)</td>
<td>3.29 ± 0.25 (“−57.38”)</td>
</tr>
<tr>
<td>RES+L</td>
<td>320.79 ± 20.22 (+55.24%)</td>
<td>404.5 ± 24.95 (+54.23%)</td>
<td>272.24 ± 10.78 (“+62.00”)</td>
<td>5.69 ± 0.53 (“+72.94”)</td>
</tr>
<tr>
<td>RES+S</td>
<td>381.69 ± 18.68 (−0.36%)</td>
<td>533.84 ± 21.66 (+1.89%)</td>
<td>344.75 ± 17.09 (−1.59%)</td>
<td>7.46 ± 0.67 (−3.36%)</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. 6-OHDA leads to significant alterations on the activity of antioxidant enzymes (GPx, GR, SOD, and CAT) in L group as compared to S group (*P<0.05, **P<0.01, L vs. S group). Administration of RES significantly attenuated activity of these enzymes in RES+L group as compared to L group (*P<0.05, **P<0.01, ***P<0.001, RES+L vs. L group). Values in parentheses show the percentage increase or decrease with respect to their control.

Fig. 3 – (a) The 6-OHDA infusion led to a significant decrease in Na+/K+-ATPase activity in the L group as compared with the S group. Pretreatment with RES significantly protected the activity of Na+/K+-ATPase in the RES+L group as compared with the L group. Values are expressed as mean ± SEM (n=8). *P<0.01, L vs. S; #P<0.05, RES+L vs. L. (b) 6-OHDA infusion led to a significant increased in D2 receptor binding in the L group as compared with the S group. Pretreatment with RES significantly decreased the receptor binding in the RES+L group as compared with the L group. Values are expressed as mean ± SEM of eight animals. *P<0.01, L vs. S; #P<0.001, RES+L vs. L. (c) The 6-OHDA infusion led to a significant increased in phospholipase A2 activity in the L group as compared with the S group. Pretreatment with RES significantly decreased the activity of phospholipase A2 in the RES+L group as compared with the L group. Values are expressed as mean ± SEM of eight animals. *P<0.05, L vs. S; #P<0.01, RES+L vs. L.
decrease in the levels of lipid peroxidation. Also, there was a simultaneous increase in the glutathione levels and antioxidant enzymes. Glutathione is an essential tripeptide, an antioxidant found in all animal cells. It reacts with the free radicals and can protect cells from singlet oxygen, hydroxyl radical, and superoxide radical. GPx plays a predominant role in removing excess free radicals and hydroperoxides and is a major defence system against oxidative stress in the brain (Imam and Ali, 2000).

Table 2 – Effect of resveratrol (RES) on level of dopamine and DOPAC in striatum of 6-OHDA-induced rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>DA (ng/mg tissue)</th>
<th>DOPAC (ng/mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>7.39±0.23</td>
<td>1.45±0.12</td>
</tr>
<tr>
<td>Lesion</td>
<td>2.29±0.21 (−69.01%)</td>
<td>0.63±0.056 (−56.55%)</td>
</tr>
<tr>
<td>RES+L</td>
<td>4.65±0.17 (∆103.05%)</td>
<td>1.05±0.073 (∆66.65%)</td>
</tr>
<tr>
<td>RES+S</td>
<td>7.43±0.42 (∆1.54%)</td>
<td>1.46±0.090 (∆0.68%)</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM of eight animals. 6-OHDA leads to significant decrease in dopamine and DOPAC level in L group as compared to S group. Administration of RES significantly attenuated the level of dopamine and DOPAC in RES+L group as compared to L group. Values in parentheses show the percentage increase or decrease with respect to their control. */P<0.05, /"P<0.01, L vs. sham. /"P<0.01, RES+L vs. L.

SOD converts superoxide into H₂O₂ (Freeman and Crapo, 1982). The catalase, which was found at very low activity in the brain, detoxifies H₂O₂ to H₂O. The loss of GSH and formation of protein glutathione mixed disulfide (PrSSG) in the brain result in various membrane dysfunction, such as inhibition of Na⁺/K⁺-ATPase activity (Reed, 1990), which is essential for cellular excitability and is very susceptible to free radical reaction and lipid peroxidation because it is embedded in cell membrane and requires phospholipids for the maintenance of its activity (Cooper and Winter, 1980; Furui et al., 1990; Ildan et al., 1996). There are several reports about modulatory effect of RES on lipid peroxidation and antioxidant enzymes following injuries such as hypoxia/ischemia and CNS injuries (Andrabi et al., 2004; Kumar et al., 2006; Yousuf et al., 2009). In agreement with these findings, we also found that RES significantly the TBARS and protein carbonyl levels along with increased in the activity of antioxidant enzymes in striatum portion following 6-OHDA induction.

The brain dopamine has long been thought to play a role in neurotoxicity, which undergoes spontaneous oxidation to toxic quinone and other electrophilic species, causing PD (Halliwell et al., 1992; Bing et al., 1994). Administration of antioxidants inhibits reduction in brain dopamine levels (Roghani and Behzadi, 2001; Zafar et al., 2003; Ahmad et al., 2005). Thus, the preventive effect of resveratrol might be due to reduction in

![Fig. 4](image_url) - The effect of 15 days of pre-treatment of RES on TH expression in right SNpc in rats lesioned by a single injection of 10.0 µg 6-OHDA. The expression of tyrosine hydroxylase was almost negligible in L group (c [100×] and d [400×]) compared to S group (a [100×] and b [400×]), while the lesioned group pretreated with RES (RES+L group) has shown a moderate staining of tyrosine hydroxylase (e [100×] and f [400×]). However, the S group has shown no discernible change in tyrosine hydroxylase staining.
auto-oxidation of dopamine by enhancing antioxidant enzymes activity.

Restoration of these antioxidants defense and striatal DA content was further emphasized by the normalization of denervation-related supersensitivity of dopaminergic D2 receptors in striatum by the RES. The denervation-related up-regulation of these receptors is a compensatory mechanism for DA deficiency (Hu et al., 1990; Hudson et al., 1993). In the present study, the increase in the D2 receptor population in striatum due to 6-OHDA infusion was significantly protected by pretreatment with RES.

Tyrosine hydroxylase is a rate-limiting enzyme in the formation of DA, and its expression is the marker for the DA neuron survival. The immunohistochemical localization of tyrosine hydroxylase in ipsilateral SNpc further strengthens the protective action of RES in 6-OHDA-induced parkinsonism.

Understanding the effects of lipoxidative load on the mechanisms of proinflammatory enzymes, like phospholipase A2 (PLA2), and their modulation for therapeutic purposes has gained significant recent attention (Phillis and O’Regan, 2003; Hoda et al., 2009). This enzyme plays an important role in neuroinflammation due to their nonspecific nature to phospholipid substrate (Adibhatla and Hatcher, 2007). Under normal conditions, the PLA2 enzymes help to maintain membrane composition and thus membrane integrity. However, PLA2 produces excessive amounts of free fatty acid/arachidonic acid (FFA/AA) under pathological conditions. Excessive release of AA by PLA2 leads to inflammation and oxidative stress, which in turn, cause secondary injury. In the present study, we observed the inhibitory effect of RES on PLA2 activity, which is responsible for excessive FFA/AA release during neurodegeneration.

COX-2 is strongly suspected in playing a detrimental role in neurodegeneration and stimulation of an inflammatory process following neuronal death. COX-2 may aggravate the degenerative process through proinflammatory mechanisms and by the formation of reactive oxygen species (Hoozemans et al., 2002; Teismann et al., 2003). It has been demonstrated that COX-2 expression in neurons correlates with their apoptosis and is involved in the neuronal response to stress (Nakayama et al., 1995). Activation of cyclooxygenase-2 leads to the production of prostaglandin E2 (PGE2), which causes inflammation (Liang et al., 2007). Many epidemiological studies showed that nonsteroid anti-inflammatory drugs such as COX-2 inhibitors may reduce the incidence of Parkinson’s disease (Chen et al., 2003; Wahner et al., 2007).

In the present study, we have clearly demonstrated that COX-2 expression was substantially upregulated in vulnerable SNpc after 6-OHDA insult and that pretreatment with RES was
found to be effective in decreasing its expression. Recently, Jin et al. (2008) have reported the inhibition of COX-2 expression in 6-OHDA rat model of Parkinson’s disease by RES treatment.

In conclusion, our results demonstrate a significant neuroprotective effect of RES on the activity of antioxidant enzymes, a subsequent decrease in TBARS content, enhancement in the content of dopamine and its metabolite DOPAC, TH-positive expression and improvement in behavioral activities. Apart from these, RES has been found to suppress COX-2 expression. These beneficial effects of RES may be attributed partially to its antioxidant potential. Thus, these findings suggest that the naturally occurring polyphenol RES is an attractive alternative antioxidant potential. Thus, these findings suggest that the naturally occurring polyphenol RES is an attractive alternative for treating Parkinson’s disease. Further investigation into the role and mechanisms of antioxidant action of RES is needed to determine whether it can be an effective remedy for Parkinson’s disease.

4. Experimental procedures

4.1. Chemicals

Oxidized glutathione (GSSG), glutathione reduced (GSH), glutathione reductase (GR), nicotinamide adenine dinucleotide phosphate reduced form (NADPH), 1-chloro-2,4-dinitrobenzene (CDNB), 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), thiobarbituric acid (TBA), (-) epinephrine, dopamine, 3,4-dihydroxyphenyl acetic acid (DOPAC), 3,4-dihydroxybenzylamine (DHBA), resveratrol (RES), 6-hydroxydopamine (6-OHDA), haloperidol, levapam sulfonic acid, bovine serum albumin (BSA), diaminobenzidine (DAB), antibody of tyrosine hydroxylase expression, phospholipase A2 activity, and cyclooxygenase-2 (COX-2) expression in the substantia nigra. Rats were divided into four groups as in experiment 1, each having 8 animals. The behavioral parameters were performed in all the experiments.

4.2. Animals

Male Wistar rats obtained from Central Animal House Jamia Hamdard (Hamdard University) New Delhi, weighing 230–250 g, aged 16 weeks at the start of the experiment, were used. Rats were housed in groups of four animals per cage, maintained on 12-h dark–light cycle (light on from 06:00 to 18:00 h), and provided free access to rat chow diet and water. The food was withdrawn 12–18 h before the surgical procedure. The experiments were in accordance with the university guidelines and were approved by the animal ethics committee of the university.

4.3. Experimental procedure

4.3.1. Experiment 1

This experiment was carried out to evaluate the pretreatment effect of RES (20 mg/kg body weight) once daily for 15 days on the content of thiobarbituric acid reactive substances (TBARS), GSH, and assays of antioxidant enzymes in striatum. The rats were divided into four groups, each having 8 animals. The first group served as sham (S) and vehicle (20% ethanol, i.p.) was given, group 2 was the vehicle-treated lesioned group (L), group 3 was pretreated with 20 mg/kg body weight of RES once daily for 15 days followed by 6-OHDA injection (RES+L) group, and group 4 was pretreated with 20 mg/kg body weight (i.p.) of RES for 15 days (RES+S) group. The RES dose used in this experiment was determined from previous studies showing that this dose provided the maximal protective effects in the treatment of different types of brain diseases (Sharma and Gupta, 2002; Andrabi et al., 2004).

4.3.2. Experiment 2

This experiment was carried out to evaluate the pretreated effect of RES (20 mg/kg body weight) for 15 days on dopaminergic D2 receptor binding density, content of DA, and its metabolite, DOPAC, in the striatum. The rats were divided into four groups as in experiment 1, each having 8 animals. The behavioral parameters were performed in all the experiments.

4.3.3. Experiment 3

This experiment was carried out to evaluate the pretreated effect of RES (20 mg/kg body weight, i.p.) once daily for 15 days on tyrosine hydroxylase expression, phospholipase A2 activity, and cyclooxygenase-2 (COX-2) expression in the substantia nigra. Rats were divided into four groups as in experiment 1.

4.4. Intrastriatal administration of 6-OHDA

Rats were anaesthetized with 400 mg/kg chloral hydrate intraperitoneally (i.p.) and mounted on a stereotaxic stand, and the skin overlying the skull was cut to expose the bregma, and the coordinates of the striatum (Paxinos and Watson, 1982) were measured accurately as anteroposterior 0.5 mm, lateral 2.5 mm and dorsoventral 4.5 mm relative to bregma and ventral from dura with the tooth bar set at 0 mm. Unilateral striatal DA neuronal degeneration was induced in rats by stereotaxic injections of 10-μg 6-OHDA/2 μl in 0.1% in ascorbic acid–saline using 5-μl Hamilton syringe into the right striatum. The sham was treated in the same way except 2 μl of 0.1% ascorbic acid–saline was injected in place of 6-OHDA. The injection rate was 0.5 μl/min, and the needle was kept in place for an additional 5.0 min before being slowly retracted.

4.5. Postoperative care

Recovery of anaesthesia took approximately 4–5 h. The rats were kept in a well-ventilated room at 25±3 °C in individual cages until they gained full consciousness and then were housed together in a group of four animals per cage. Food and water were kept inside the cages for the first week so that animals could easily access it without any physical trauma due to overhead surgery. Then the animals were treated normally; food, water, and the bedding of the cages were changed every day as usual. The dose of buprenorphin used in all studies for postoperative analgesia was 0.05 mg/kg (s.c.).

4.6. Behavioral testing

4.6.1. Apomorphine-induced circling behavior

The effect of 6-OHDA and protection by RES was evaluated on apomorphine-induced rotations in lesioned rats. The animals were given 0.5 mg/kg apomorphine (in ascorbic acid–saline)
subcutaneously to monitor contralateral rotations (Ahmad et al., 2005). Net rotations toward the contralateral side were collected at 5-min intervals. The rats were checked and monitored for any basal level of contralateral rotations before surgery.

4.6.2. Rota rod (muscular coordination)
Omni Rotor (Omnitech Electronics, Inc., Columbus, OH, USA) was used to evaluate the muscular coordination on 21st day of 6-OHDA injection (Rozas et al., 1998). The Rota rod unit consists of a rotating rod, 75 mm in diameter, which was divided into four parts by compartmentalization to permit the testing of four rats at a time. After twice-daily training for 2 successive days (speed 8 rpm on the first day and 10 rpm on second day), the rotational speed of the test was increased to 15 rpm on the third day in a test session. The time for each rat to remain on the rotating bar was recorded for three trials for each rat, at a 5-min interval and a maximum trial length of 180 s per trial. The apparatus automatically records the time in 0.1 s when the rats fall on the rotating shaft.

4.6.3. Stepping test
This is a test for akinesia. The rat was held with one hand by the experimenter fixing the hind limbs (slightly raising the torso) and, with the other hand, fixing the forelimb that was not to be monitored. In this way, the other forepaw had to bear the weight. The rat was moved slowly sideways in both forehand and backhand directions. This was done for both the contralateral and ipsilateral forepaws. The number of adjusting steps for both directions and both paws was counted (Chang et al., 1999).

4.7. Tissue preparation
After 4 weeks of 6-OHDA infusion, the animals were sacrificed, and their brains were taken out to dissect striatum to give 5% (wt/vol.) homogenate (10 mM phosphate buffer, pH 7.0, having 10 μM protease arrests: 5 mM leupeptin, 1.5 mM aprotinin, 2 mM phenylmethylsulfonyl fluoride (PMSF), 3 mM peptatin A, 10 mM EDTA, 0.1 mM EGTA, 1 mM benzamidine, and 0.04% butylated hydroxytoluene) and were centrifuged at 1000×g for 5 min at 4 °C to separate debris. This supernatant was used for the assay of TBARS. The rest of the supernatant was centrifuged at 10,500×g for 20 min at 4 °C to separate postmitochondrial supernatant (PMS), which was used for the estimation of GSH and antioxidant enzymes.

4.8. Biochemical analysis

4.8.1. Assay for thiobarbituric acid reactive substances (TBARS)
The procedure of Utely et al. (1967) as modified by us (Islam et al., 2002) was used for the estimation of the rate of lipid per oxidation. The homogenate 0.25 ml was incubated in a test tube at 37 ± 1 °C in a metabolic shaker (120 stroke to and fro/min) for 1 h. Similarly, the same homogenate was pipetted in an Eppendorf tube and incubated at 0 °C. After 1 h of incubation, 5% chilled TCA was added followed by 0.25 ml of 0.67% TBA in each test tube and Eppendorf tube, and proper mixing was done after each addition. The mixture was centrifuged at 3000×g for 10 min. Thereafter, the supernatant was transferred to another test tube and placed in boiling water bath for 10 minutes. After that, the test tubes were cooled, and the absorbance of the color was read at 535 nm. The rate was expressed as nanomoles of TBARS formed per hour per milligram of protein using a molar extinction coefficient of 1.56×10⁵ M⁻¹ cm⁻¹.

4.8.2. Determination of protein carbonyl (PC)
Protein carbonyl content was assayed according to the method of Levine et al. (1990) with slight modification. The tissue homogenate (0.25 ml) was reacted with equal volume of 20% TCA. Thereafter, 0.25 ml of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2.0 M HCl was added and allowed to stand at room temperature for 1 h, with vortexing every 10–15 min and centrifuged at 11,000×g for 5 min after adding 0.5 ml of 20% TCA. The supernatant was discarded, and pellet was washed 3 times with 1 ml of ethanol–ethyl acetate (1:1) to remove the free reagent. The samples were allowed to stand for 10 min before centrifugation, and the supernatant was discarded each time. Precipitated protein was redissolved in 0.6 ml of guanidine hydrochloride solution within 15 min at 37–50 °C and centrifuged at 11,000×g for 5 min to remove any insoluble material. The carbonyl contents were measured at 370 nm in a spectrophotometer (Shimadzu-1601, Japan). The results were expressed as nanomoles of carbonyl per milligram of protein using a molar extinction coefficient of 22×10³ M⁻¹ cm⁻¹.

4.8.3. Glutathione (GSH)
GSH content was determined by the method of Jollow et al. (1974) with slight modification. PMS was mixed with 4.0% sulfosalicylic acid in a 1:1 ratio (vol./vol.). The samples were incubated at 4 °C for 1 h, and later centrifuged at 1200×g for 15 min at 4 °C. The assay mixture contained 0.1 ml of supernatant, 1.0 mM DTNB and 0.1 M PB (pH 7.4) in a total volume of 2.0 ml. The yellow colour developed was read immediately at 412 nm. The GSH content was calculated as nanomoles of GSH per milligram of protein using a molar extinction coefficient of 13.6×10³ M⁻¹ cm⁻¹.

4.8.4. Glutathione peroxidase (GPx)
GPx activity was estimated according to the procedure described by Mohandas et al. (1984). The reaction mixture consisted of phosphate buffer (0.05 M, pH 7.0), EDTA (1 mM), sodium azide (1 mM), glutathione reductase (1 EU/ml), glutathione (1 mM), NADPH (0.2 mM), hydrogen peroxide (0.25 mM), and 0.1 ml of PMS in a final volume of 2 ml. The disappearance of NADPH at 340 nm was recorded at room temperature. The enzyme activity was calculated as nanomoles of NADPH oxidized per minute per milligram of protein using a molar extinction coefficient of 6.22×10⁻³ M⁻¹ cm⁻¹.

4.8.5. Glutathione reductase (GR)
Glutathione reductase activity was assayed by the method of Carlberg and Mannervik (1975) as modified by Mohandas et al. (1984). The assay mixture consisted of phosphate buffer (0.1 M, pH 7.6), NADPH (0.1 mM), EDTA (0.5 mM), oxidized glutathione (1 mM), and 0.05 ml of PMS in a total volume of 1 ml. The enzyme activity was quantitated at room temperature by measuring the disappearance of NADPH at 340 nm and was
calculated as nanomoles of NADPH oxidized per minute per milligram of protein using molar extinction coefficient of 6.22 × 10^3 M^-1 cm^-1.

4.8.6. Catalase activity (CAT)
Catalase activity was assayed by the method of Claiborne (1985). Briefly, the assay mixture consisted of 0.05 M phosphate buffer (pH 7.0), 0.019 M H_2O_2, and 0.05 ml of PMS in a total volume of 3.0 ml. The change in absorbance was recorded at 240 nm. Catalase activity was calculated in terms of nanomoles of H_2O_2 consumed per minute per milligram of protein.

4.8.7. Superoxide dismutase (SOD)
Superoxide dismutase activity was measured spectrophotometrically as described previously by Stevens et al. (2000) by monitoring the auto-oxidation of (-)-epinephrine at pH 10.4 for 5 min at 480 nm. The reaction mixture contained glycine buffer (50 mM, pH, 10.4) and 0.2 ml of PMS. The reaction was initiated by the addition of (-)-epinephrine. The enzyme activity was calculated in terms of nanomoles of (-)-epinephrine protected from oxidation per minute per milligram of protein using molar extinction coefficient of 4.02 × 10^3 M^-1 cm^-1.

4.8.8. Assay for Na^+/K^+-ATPase
Na^+/K^+-ATPase activity was measured by the method of Svoboda and Mossinger (1981) with slight modification. The tissue for Na^+/K^+-ATPase activity was homogenized in 0.01 M Tris–HCl buffer (pH 7.4). The Na^+/K^+-ATPase activity was determined in two reaction media, A and B. The reaction mixture A consisted of 0.2 M KCl, 1.0 M NaCl, 0.1 M MgCl_2, 0.2 M Tris–HCl buffer (pH 7.4), and 0.1 ml of homogenate in a total volume of 2.0 ml. The reaction mixture B consisted of 0.1 M MgCl_2, 10 mM ouabain, 1.0 M NaCl, 0.2 M Tris–HCl buffer (pH 7.4), and 0.1 ml of homogenate in a total volume of 2.0 ml. The enzyme reaction was started by adding 0.2 ml of 25.0 mM ATP at 37 °C and terminated after 15 min by adding 1.0 ml chilled 10% TCA. The mixture was centrifuged, and the supernatant at 37 °C and terminated after 15 min by adding 1.0 ml chilled 10% TCA. The mixture was centrifuged, and the supernatant was used for the estimation of inorganic phosphorous by the addition of (50 mM, pH, 10.4) and 0.2 ml of PMS. The reaction was initiated from oxidation per minute per milligram of protein using molar extinction coefficient of 4.02 × 10^3 M^-1 cm^-1.

4.8.9. Quantification of dopamine and its metabolite
The striatal tissue levels of DA and its metabolite DOPAC were measured by high-performance liquid chromatography (Waters, Milford, MA, USA), using electrochemical detector (Waters 464 detector) by the method of Zafar et al. (2003). The striatum (20% wt./vol) was sonicated in 0.4 N perchloric acid containing 100 ng/ml of the internal standard DHBA (2, 3-dihydroxybenzoic acid), followed by centrifugation at 15,000×g for 10 min at 4 °C and the filtered through a 0.20-µm membrane that was injected manually through a 20-µl loop over the ODS-C18 column for separation and quantification. The mobile phase consisted of 0.1 M potassium phosphate (pH 4.0), 10% methanol, and 1.0 mM heptane sulfonic acid. Samples were separated on an ODS-C18 column using a flow rate of 1.0 ml/min. The concentrations of DA and its metabolite DOPAC were calculated using a standard curve generated by determining ratio between three known amounts of the amine or its metabolites and a constant amount of internal standard DHBA and represented as nanograms per milligram of tissue.

4.8.10. Determination of dopaminergic D_2 receptor binding
Assay of DA-D_2 receptor binding was carried out in right striatum of all experimental groups following the method of Agrawal et al. (1995). The pellet of PMS was resuspended in the same amount of the buffer and homogenized by hand and centrifuged at 10,000 × g for 15 min. The supernatant was discarded, and the pellet was resuspended in the same amount of said buffer. In brief, the incubation mixture of 1.0 ml consisted of synaptic membrane along with 1.0 nM 1-phenyl-4-[H_3] spiperone in 40 mM Tris–HCl (pH 7.4). A parallel incubation was carried out in the presence of 1.0 µM haloperidol to ascertain nonspecific binding. The assay was run in triplicate. Reaction mixture was incubated for 15 min at 37 °C, terminated by cooling at 4 °C, and filtered through glass fiber filters (GF/C, Whatman) through Millipore Filtration Assembly. The filter discs were washed rapidly with 2×5 ml of cold Tris–HCl buffer (40 mM, pH 7.4), transferred to scintillation vials, and dried properly. After adding 10.0 ml of scintillation cocktail to vials, the radioactivity was counted in a β-scintillation counter (WALLAC-1410) with an efficiency of 50% for tritium. Specific binding was calculated by subtracting nonspecific binding from total binding obtained in the absence of haloperidol. Results were expressed as picomoles of [H_3] spiperone-bound per milligram of protein.

4.8.11. Phospholipase A2 (PLA2) assay
Assay of phospholipase A2 was carried out in right striatum of all experimental groups following the method of Kim et al. (1995). PLA2 activity was assayed by measuring the hydrolysis of [1-14C] AA from 1-stearyl-2-[1-14C] arachidonyl-sn-3′-glycero-phosphocholine ([1-14C] AA-GPC). The standard PLA2 assay buffer (200 µl) contained 75 mM Tris–HCl, 5 mM CaCl_2, 1 mg/ml BSA, and 0.45 mM [I-14C] AA-GPC at pH 7.5. The reaction was carried out at 37 °C for 30 min and was stopped by adding 1.25 ml of Dole’s reagent, 78% isopropyl alcohol, 20% n-heptane, and 2% of 0.5 M H_2SO_4 in water. Free fatty acid was extracted in the following manner. An aliquot (0.55 ml) of water was added, and the sample was vortexed and centrifuged for 5 min. Then, 0.75 ml of the upper phase was transferred to a new tube to which 50 mg of silica gel and 0.75 ml of n-heptane were added. The samples were vortexed and centrifuged again for 5 min each. A sample of supernatant was then counted in a liquid scintillation counter. PLA2 activity was expressed as picomoles of arachidonic acid released per minute per milligram of protein.

4.9. Immunohistochemistry
The brain of sham, lesioned, and RES+L groups was perfused as described by us (Ahmad et al., 2005). In brief, 4 weeks after the surgery, the animals were anesthetized with chloral hydrate (400 mg/kg, i.p.) and perfused transcardially with saline followed by 4% paraformaldehyde (PF) in phosphate buffer (PB; 0.1 M, pH 7.2). The brains were removed quickly, postfixed in the PF solution for 48 h, then transferred to 30% sucrose in 0.1 M PB for at least 16 h until they sank for cryoprotection. Finally, the tissues were kept in sucrose solution until sectioning. The fixed tissues were embedded in OCT compound (polyvinyl glycol, polyvinyl alcohol, and water) and frozen at −20 °C. Coronal sections of 10 µm
thicknesses were cut on a Cryostat (Leica, Germany) and transferred to gelatin-coated slides and immersed in wash buffer (sodium phosphate 100 mM, sodium chloride 0.5 M, Triton X-100, sodium azide) pH 7.4 for 20 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide and 10% methanol in PBS and incubated for 30 min at room temperature. Thereafter, the slides were washed with PBS, and these sections were incubated for 48 h in primary antibody anti-TH antibody at 1:100 ratio or anti-COX-2 at 1:200 ratio. After removing the primary antibody, sections were washed three times with PBS and incubated in peroxidase-linked secondary antibody (1: 200) for 2 h at room temperature followed by three washes with PBS. Colour for peroxidase linked antibody was developed with DAB as chromogen. Sections were dehydrated, cleaned, mounted in DPX, and cover slipped. A Nikon Eclipse E-600 microscope was used for microscopic analysis.

4.10. Determination of protein

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as a standard.

4.11. Statistics

Results are expressed as mean±SEM ANOVA with Tukey-Kramer post hoc analysis was used to analyze differences between the groups. P<0.05 was considered as significant.

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