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Pharmacology, Biochemistry and Behavior 81 (2005) 805 – 813

PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR

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Neuroprotection by crocetin in a hemi-parkinsonian rat model

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Received 7 March 2005; received in revised form 27 May 2005; accepted 4 June 2005
Available online 6 July 2005

Abstract

Reactive oxygen species (ROS) are implicated as the leading biochemical cause of neuronal death in various neurologic disorders, including Parkinson's disease. In the present study, neuromodulatory effects of crocetin (active constituent of *Crocus sativus*) in a 6-hydroxydopamine (6-OHDA) model of rat Parkinsonism were investigated. Male Wistar rats were pre-treated with crocetin (25, 50 and 75 μg/kg body weight) for 7 days and subjected to unilateral intrastriatal injection of 10 μg 6-OHDA on day 8. Locomotion and rotation were observed on day 23 post-injection, and after 4 weeks, striatum and substantia nigra were dissected out by decapitation. Activity of antioxidant enzymes and content of dopamine (DA) and its metabolites were estimated in striatum, whereas glutathione (GSH) content and thiobarbituric acid reactive substance (TBARS) were evaluated in substantia nigra. Levels of GSH and dopamine were protected, while TBARS content was attenuated in crocetin-treated groups. The activity of antioxidant enzymes was decreased in the lesion group, but protected in the crocetin-treated groups. These findings were supported by the histopathologic findings in the substantia nigra that showed that crocetin protects neurons from deleterious effects of 6-OHDA. This study revealed that crocetin, which is an important ingredient of diet in India and also used in various systems of indigenous medicine, is helpful in preventing Parkinsonism and has therapeutic potential in combating this devastating neurologic disorder.

Keywords: 6-OHDA; Antioxidant enzymes; Dopamine; Glutathione; Lipid peroxidation; Neurobehavior; Neurotoxicity; Oxidative stress; Parkinson disease; Rat model; Saffron

1. Introduction

Parkinson disease (PD) is as a terminal, progressive neurodegenerative disorder marked by primary loss of dopamine (DA) in basal ganglia and physically characterized by debilitating loss of spontaneous and voluntary movements and resting tremor, as well as other behavioral changes. Some of the factors responsible for the arrest of nigrostriatal dopaminergic neurotransmission leading to PD are generation of excessive oxidants (free radicals), auto- and enzymatic oxidation of DA, formation of neuromelanin, and/or high concentration of metals (for review, see von Bohlen und Halbach et al., 2004). The exact mechanism by which free radicals inflict their toxicity is still unclear, although several hypotheses have been proposed (Fernandez-Espejo, 2004; Olanow and Tatton, 1999; Schapira, 1999; Sudha et al., 2003). Reactive oxygen species (ROS) are normal products of cellular metabolism. Overproduction of ROS, loss of antioxidant defenses, or both results in oxidative stress. The glutathione system, responsible for removing free radicals and maintaining protein thiols in their appropriate redox state in the cytosol and mitochondria, is an important protective mechanism for minimizing oxidative stress (Bharath et al., 2002; Dringen, 2000; Mytilineou et al., 2002; Winterbourn and Metodiewa,

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doi:10.1016/j.pbb.2005.06.007

1994). The antioxidant enzymes, such as SOD, catalase, GPx, GR and GST, are also important mediators that reduce the overload of oxidative stress.

6-OHDA, a catecholaminergic neurotoxin, when injected into the nigrostriatal pathway, results in extensive loss of dopaminergic cells in the ipsilateral substantia nigra via production of ROS. Unilateral 6-OHDA models are commonly used to study physiopathologic features responsible for motor impairments in animal models of PD (Cadet et al., 1989; Schober, 2004). These behavioral deficits are confirmed by rotational asymmetry after induction of a dopamine agonist (Henderson et al., 2003) or dopamine-releasing drugs (Hefti et al., 1980).

Recently, more emphasis has been placed on investigating neuroprotective remedies, including healthy diets containing potent antioxidants. The fruits and flowers of *Crocus sativus* (Saffron) are important dietary ingredients in India and other tropical countries. Pistils of Saffron are generally used in traditional Indian medicine as analgesics and cardioprotective agents, as well as in treatment of various kinds of mental illness. A crude extract of pistils of Saffron improves recovery in ischemia/reperfusion injury (unpublished data from our laboratory) and learning and memory (Abe et al., 1998) in rats. It has also been suggested that the crude extract and purified chemicals of Saffron (crocetin and *trans*-sodium crocinate) prevent tumor formation, atherosclerosis and hepatic damage (Gainer and Jones, 1975; Wang et al., 1996; Wang et al., 1991). Crocetin has been known to have free radical scavenging property in various toxic paradigms (Nair et al., 1993; Singer et al., 2000; Tseng et al., 1995). Based on the findings that crocetin and other carotenoids of Saffron protects hippocampal neurons from toxicity in rats (Abe et al., 1998) and improves cerebral oxygenation in hemorrhaged rats (Seyde et al., 1986), we hypothesize that crocetin could be used as a remedy in preventing Parkinsonism in rat models. Our results show that crocetin effectively and significantly protected neurons from 6-OHDA-induced toxicity and suggest that crocetin can be used as a therapeutic tool in manipulating Parkinsonism.

2. Materials and methods

2.1. Chemicals

Crocetin (Cr), glutathione (oxidized and reduced), glutathione reductase, nicotinamide adenine dinucleotide phosphate reduced form (NADPH), 1-chloro-2,4-dinitrobenzene (CDNB), 5-5ʟdithio-bis-2-nitrobenzoicacid (DTNB), dopamine (DA), 3,4-dihydroxyphenyl acetic acid (DOPAC), homovanilic acid (HVA), 3,4-dihydroxybenzylamine (DHBA), 6-hydroxydopamine hydrobromide (6-OHDA) and heptane sulphonic acid were purchased from Sigma-Aldrich Foreign Holding Chem. Co. (Bangalore, India). Other chemicals used were of analytical grade.

2.2. Animals and treatment

The animals were used in accordance with NIH guidelines for animal usage and care, and the experimental procedures were approved by the Animal Ethics Committee of Jamia Hamdard (Hamdard University). Male Wistar rats obtained from the Central Animal House of Jamia Hamdard, weighing 180–200 g at the start of the experiment, were maintained on a 12-h dark–light cycle and provided free access to pellet diet and water ad libitum. Rats were divided into three experimental groups as follows:

2.2.1. Experimental group 1

The animals in this group were used to evaluate the pre-treatment effect of crocetin (25, 50, and 75 μg/kg body weight, BW, i.p.) on antioxidant enzymes [glutathione peroxidase (GPx), glutathione-*S*-transferase (GST), glutathione reductase (GR), catalase, and superoxide dismutase (SOD)], GSH and TBARS against 6-OHDA-induced neurotoxicity. Animals were divided into eight sub-groups, 10 animals each: Group 1: vehicle-treated sham group (S); Groups 2–4: sham groups treated with 25 μg/kg BW (S+Cr1), 50 μg/kg BW (S+Cr2) and 75 μg/kg BW (S+Cr3) of crocetin; Group 5: vehicle-treated lesion group (L); Groups 6–8: lesion groups treated with 25 μg/kg BW (L+Cr1), 50 μg/kg BW (L+Cr2) and 75 μg/kg BW (L+Cr3) of crocetin. Rats were pre-treated with vehicle or crocetin for 7 days.

2.2.2. Experimental group 2

The animals of this group were used to study the pre-treatment effects of various doses of crocetin on DA and its metabolites (Dopac and HVA) against 6-OHDA-induced neurotoxicity. Rats divided into 8 sub-groups, 10 animals each, were treated as in experimental Group 1.

2.2.3. Experimental group 3

This experiment was conducted to evaluate the pre-treatment effect of various doses of crocetin for 7 days on histopathologic changes in substantia nigra. The rats were divided into 8 groups, 5 rats each, and were treated as in experimental Group 1.

2.3. Intrastriatal administration of 6-OHDA (lesioning)

On day 8, unilateral lesions were induced in right striatum of rats that were pre-treated with crocetin for 7 days, as described earlier (Ahmad et al., 2005; Zafar et al., 2003). Briefly, each rat was anesthetized with 400 mg/kg chloral hydrate, i.p., positioned in stereotaxic frame. Thereafter, 2 μl vehicle (0.1% ascorbic acid in normal saline) was injected into the right striatum of sham groups S, S+Cr1, S+Cr2 and S+Cr3, while 10 μg 6-OHDA (in 2 μl vehicle) was injected into the right striatum of lesion groups L, L+Cr1, L+Cr2 and L+Cr3 following the protocol described earlier (Ahmad et al., 2005; Zafar et al., 2003).

2.4. Behavioral study

The following behavioral tests were performed on the 23rd day of lesioning. The experiment was carried out under standard optimal conditions. Rats of all experimental groups (1, 2 and 3) were tested for behavioral changes. All tests were performed and analyzed by a subject blind to the experiment.

2.5. Locomotor activities

Observations were recorded for three sessions of 5 min each, as described (Ahmad et al., 2005) on a Video Path Analyzer (Coulbourn Instruments, Allentown, PA, USA). Means of session totals of vehicle and treatment groups were compared for locomotion (s), distance traveled (cm), stereo events (number), and average speed (cm/min).

2.6. Apomorphine-induced rotations

After locomotion activities, rats were subcutaneously injected with 0.5 mg/kg BW apomorphine hydrochloride to investigate the rotational asymmetry (Ahmad et al., 2005). The rats were placed immediately in the open field chamber, rotations were recorded on a Video Path Analyzer, and net rotations towards contra-lateral side were collected at 15-min intervals. The rats were checked and monitored for any basal level of contra-lateral rotations before surgery and data were normalized for these rotations.

2.7. Tissue processing for biochemical studies

After 4 weeks, rats were decapitated and brains were removed quickly. Striatum and substantia nigra were dissected out on ice with the help of brain matrix in the light of rat brain atlas (Paxinos and Watson, 1982). The antioxidant enzymes (GPx, GR, GST, catalase and SOD) were estimated in striatum, while GSH and TBARS were assayed in substantia nigra. Tissues were homogenized in phosphate buffer (10% w/v, pH 7.0), followed by centrifugation at 10,500×g for 20 min at 4 °C to obtain post-mitochondrial supernatant (PMS).

2.8. Estimation of catalase activity

Catalase (EC 1.11.1.6) activity was measured as described earlier (Claiborne, 1985). In brief, the assay mixture consisted of 0.1 M phosphate buffer (pH 7.4), 6.0 mM hydrogen peroxide and 0.10 ml of PMS in a final volume of 3.0 ml. The changes in absorbance were recorded at 240 nm. The catalase activity was calculated in terms of nmol H_2O_2 consumed min^{-1} mg^{-1} protein.

2.9. Estimation of superoxide dismutase activity

SOD (EC 1.15.1.1) activity was measured spectrophotometrically, as described previously, (Stevens et al., 2000) by monitoring the autooxidation of (−)-epinephrine at pH 10.4 for 5 min at 480 nm. The reaction mixture contained 40 mM glycine buffer (pH, 10.4) and 0.2 ml of PMS in a total volume of 1.02 ml. The reaction was initiated by the addition of 20 μl of 20 mg/ml solution of (−)-epinephrine. The enzyme activity was calculated in terms of nmol (−)-epinephrine oxidized min^{-1} mg^{-1} protein.

2.10. Estimation of glutathione peroxidase activity

GPx (EC 1.11.1.9) activity was measured at 37 °C by coupled assay system (Wheeler et al., 1990). The reaction mixture consisted of 0.2 mM H_2O_2, 1.0 mM GSH, 1.4 U of GR, 1.5 mM NADPH, 1.0 mM sodium azide and 0.1M phosphate buffer (pH 7.4) and 0.1 ml PMS in a total volume of 2 ml. The enzyme activity was calculated as nmol NADPH oxidized min^{-1} mg^{-1} protein.

2.11. Estimation of glutathione reductase activity

The assay system to estimate GR (EC 1.6.4.2) activity consisted of 0.1 M phosphate buffer (pH 7.6), 0.5 mM EDTA, 1.0 mM oxidized glutathione, 0.1 mM NADPH and 0.1 ml PMS in a total volume of 2.0 ml (Carlberg and Mannervik, 1985). The enzyme activity was assayed by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized min^{-1} mg^{-1} protein.

2.12. Estimation of glutathione-S-transferase activity

GST (EC 2.5.1.18) activity was measured in a reaction mixture consisting of 0.1 M phosphate buffer (pH 6.5), 1.0 mM reduced glutathione, 1.0 mM CDNB and 0.1 ml of PMS in a total volume of 3 ml (Habig et al., 1974). The changes in absorbance were recorded at 340 nm, and the enzymatic activity was calculated as nmol CDNB conjugate formed min^{-1} mg^{-1} protein.

2.13. Estimation of glutathione content

GSH was estimated using the protocol described earlier (Jollow et al., 1974) with slight modification. PMS 0.3 ml was mixed with 0.3 ml of sulphosalicylic acid (4%). The samples were incubated at 4 °C for 30 min and then subjected to centrifugation at 1200×g for 15 min at 4 °C. The assay mixture contained 0.1 ml filtered aliquot, 0.1 M phosphate buffer (pH 7.4) and 0.2 ml DTNB (4 mg/ml) in a total volume of 2.0 ml. Any yellow color that developed was read immediately at 412 nm, and GSH concentration was calculated as nmol GSH conjugate-formed g^{-1} tissue.

2.14. Estimation of TBARS content

Lipid peroxidation was assayed as described recently (Ahmad et al., 2005). In brief, 0.2 ml PMS was pipetted in an Eppendorf tube and incubated at 37 ± 1 °C in a metabolic

water bath shaker for 60 min; another 0.2 ml was incubated in an Eppendorf tube at 0 °C incubation. After 1 h of incubation, 0.4 ml of 5% TCA and 0.4 ml of 0.67% TBA were added to both samples (i.e., 0 °C and 37 °C). The reaction mixture from the vial was transferred to the tube and centrifuged, and the supernatant was transferred to another tube and placed in a boiling-water bath for 10 min. Thereafter, the test tubes were cooled and the absorbance of the colour was read at 535 nm. The rate of lipid peroxidation was expressed as nmol of TBARS reactive substance-formed h^{-1} mg^{-1} protein.

2.15. Estimation of dopamine and its metabolites

The content of DA, DOPAC and HVA was measured using an HPLC protocol described earlier (De Vito and

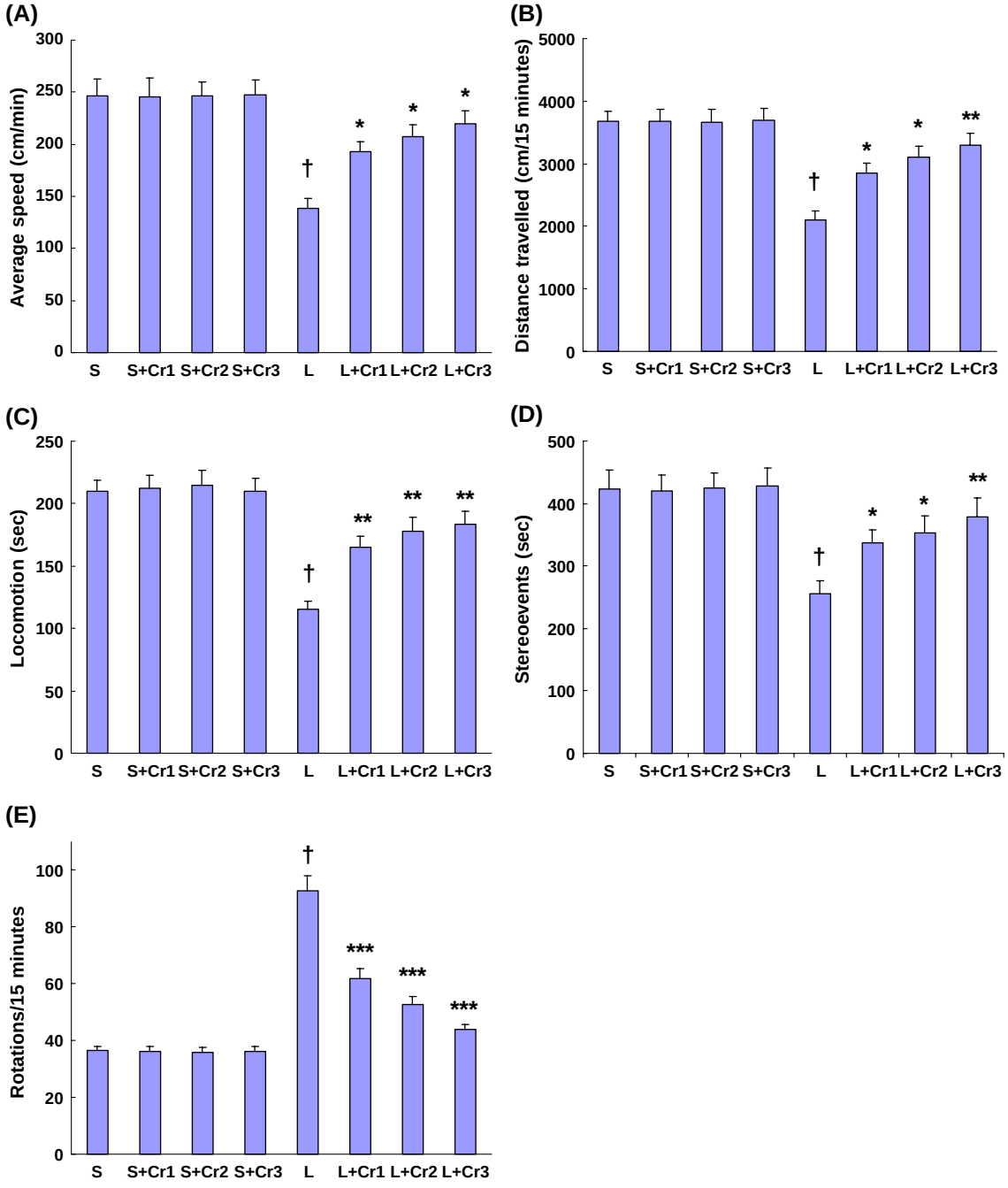

Fig. 1. Effect of crocetin on (A) average speed, (B) distance traveled, (C) locomotion, (D) stereo events, and (E) rotation in sham-operated (S) and 6-OHDA-lesioned (L) rats. Rats were pre-treated with either vehicle (S=sham, L=lesion) or crocetin (Cr1=25 μg/kg BW, Cr2=25 μg/kg BW, Cr3=25 μg/kg BW) for 7 days, followed by unilateral intrastriatal injection of either vehicle (S=sham groups) or 6-OHDA (L=lesion groups). On day 23 of lesioning, rats were observed for average speed, distance traveled, locomotion, and stereo events. Thereafter, they were injected with apomorphine (subcutaneously) to observe rotational asymmetry. Values are expressed as means±S.E.M. †$p<0.01$ vs. S; *$p<0.02$, **$p<0.01$, ***$p<0.001$ vs. L.

Wagner, 1989), with slight modification, as reported earlier (Ahmad et al., 2005; Zafar et al., 2003). Striatum was sonicated (20% w/v) in 0.4 N perchloric acid containing 100 ng/ml of the internal standard, 3,4-dihydroxybenzylamine (DHBA), followed by centrifugation at 25,000×*g* for 10 min at 4 °C. The filtered supernatant was injected manually through a 20-μl loop over the ODS-C_{18} column coupled with a HPLC/Electrochemical detector (Waters, Milford, MA, USA) for separation and quantification. The mobile phase consisted of 0.1 M potassium phosphate (pH 4.0), 10% methanol and 1.0 mM heptane sulphonic acid. The chromatogram was analyzed automatically by Waters 32 Millennium software. The concentration of dopamine and its metabolites was calculated using a standard curve generated by determining the ratio between three known amounts of dopamine or its metabolites and a constant amount of internal standard and represented as ng/mg of tissue.

2.16. Protein estimation

Protein concentration in samples was determined using bovine serum albumin as standard (Lowry et al., 1951).

2.17. Assessment of histopathologic changes in substantia nigra

Brains of rats pre-treated with crocetin were removed quickly and perfusion-fixed according to the method of Nakayama et al. (1988), as described previously (Salim et al., 2003). Coronal sections of 3-mm thickness were made, and its blocks were embedded in paraffin. Sections of 5-μm thickness were cut in the coronal plane and stained with haematoxylin and eosin. Nuclei with characteristic of neuronal death (pycnosis, i.e., condensation or fragmentation of chromatin) were counted. Four fields (×20) were randomly selected in each section and the ratio of the number of normal stained nuclei over pycnotic nuclei was calculated. The ratio of normal nuclei over pycnotic nuclei in sham group was normalized and represented as 100% normal nuclei.

2.18. Statistical analysis

Results are expressed as means±S.E.M. Data were subjected to one-way ANOVA followed by Tukey–Kramer post hoc analysis to analyze differences among the groups. Significance was ascertained at $p<0.05$.

3. Results

A significant decrease in average speed was observed in L group as compared to S group, but crocetin supplementation increased the speed significantly and dose dependently in groups L+Cr1 to L+Cr3 as compared to L group (Fig. 1A). Distance traveled was decreased significantly in L group as compared to S group, and it was increased significantly and dose dependently in crocetin pre-treated groups, L+Cr1 to L+Cr3, as compared to L group (Fig. 1B). Time spent in locomotion was less in L group as compared to S group (Fig. 1C). A significant and dose-dependent recovery in locomotion was observed in L+Cr1 to L+Cr3 groups as compared to L group. However, no change in distance traveled and locomotion was observed in groups S+Cr1 to S+Cr3 as compared to S group. Crocetin supplementation enhanced the stereo-events significantly and dose dependently in L+Cr1 to L+Cr3 groups as compared to L group (Fig. 1D), where these events were decreased significantly as compared to S.

The effect of crocetin and 6-OHDA on apomorphine-induced rotations in rats is presented in Fig. 1E. Rats of L group rotated significantly ($p<0.001$) toward non-lesion side (contralateral rotation) following apomorphine administration, and the rotation was decreased significantly and dose dependently in L+Cr1 to L+Cr3 groups as compared to L group, whereas no significant alteration was observed in S+Cr1 to S+Cr3 groups as compared to S group.

Table 1
Effect of crocetin and 6-OHDA on antioxidant enzyme activity in rat striatum

Groups	GPx	GR	GST	CAT	SOD
S	20.56±0.67	18.25±0.86	32.85±0.86	2.22±0.07	29.10±1.17
S+Cr1	20.62±0.48 (0.29)	18.27±0.95 (0.11)	32.92±0.99 (0.21)	2.23±0.13 (0.45)	29.14±0.14 (0.14)
S+Cr2	20.65±0.53 (0.43)	18.30±0.65 (0.27)	32.96±1.04 (0.33)	2.24±0.05 (0.90)	29.20±0.51 (0.34)
S+Cr3	20.68±0.56 (0.58)	18.40±0.88 (0.82)	33.05±0.81 (0.61)	2.24±0.05 (0.90)	29.26±0.59 (0.55)
L	10.85±0.52†† (−47.23)	10.75±0.46†† (−41.09)	15.36±0.71†† (−53.24)	1.02±0.04†† (−54.05)	14.54±2.67† (−50.03)
L+Cr1	12.58±0.74 (15.94)	13.05±0.64* (21.39)	19.60±0.61** (27.60)	1.73±0.11** (69.61)	15.90±0.64 (9.35)
L+Cr2	15.52±0.81** (43.04)	15.32±0.73** (42.51)	24.02±1.13** (56.38)	1.83±0.09*** (79.41)	22.08±0.70* (51.86)
L+Cr3	16.56±0.65*** (53.97)	16.15±0.92** (50.23)	26.08±1.02*** (69.79)	1.92±0.20** (88.23)	25.03±0.72** (72.14)

Rats were either pre-treated with vehicle (S=sham group; L=lesion group) or different doses of crocetin (Cr1=25 μg/kg BW, Cr2=25 μg/kg BW, Cr3=25 μg/kg BW) for 7 days and subjected to unilateral intrastriatal injection of either vehicle (S+sham group) or 6-OHDA (10 μg/2 μl) (L=lesion group) on day 8. Rats were decapitated after 4 weeks and striatum was dissected out, homogenized and processed for the assay of antioxidant enzyme activity. Activities are reported as nmol min^{-1} mg^{-1} protein. Data were subjected to ANOVA followed by Tukey–Kramer test and values are expressed as mean ±S.E.M. †$p<0.01$, ††$p<0.001$ vs. S, *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. L. Values in parenthesis are percentage increase (+) or decrease (−) as compared to S or L group. Number (n) of rats in each group is 10.

The effect of 6-OHDA and crocetin (25, 50, and 75 μg/kg BW) on the activity of antioxidant enzymes, such as catalase, SOD, GPx, GR and GST, is summarized in Table 1. Significant depletion in the activity of these enzymes was observed in L group as compared to S group, whereas their levels in groups L+Cr1 to L+Cr3 were elevated significantly and dose dependently as compared to L group.

Fig. 2 shows the possible protective effect of crocetin on GSH level. The level of GSH was not altered significantly in S+Cr1 to S+Cr3 groups, but its depletion was significant after lesioning as compared to S group. Pre-treatment with crocetin protected GSH content significantly and dose dependently in L+Cr1 to L+Cr3 groups as compared to L group. The effect of various doses of crocetin on TBARS content in S and L groups is illustrated in Fig. 3. No significant alteration in the level of TBARS was observed in S+Cr1 to S+Cr3 groups, but its level was significantly enhanced in L group as compared to S group. Notably, pre-treatment of the rats with various doses of crocetin resulted in a significant and dose-dependent depletion of TBARS content in L+Cr1 to L+Cr3 groups.

Table 2 shows the content of DA, DPAC and HVA in sham group (S), lesion group (L) and crocetin pre-treated groups L+Cr1 to L+Cr3. A significant depletion in the content of dopamine (68.26%), DOPAC (57.36%), and HVA (58.56%) was observed in L group as compared to S group, while the ratio of DOPAC/DA in L group was increased up to 34.31% as compared to S group. Pre-treatment with crocetin exhibited significant and dose-dependent protection in the level of DA, DOPAC and HVA in L+Cr1 to L+Cr3 groups, while the ratio of DOPAC/DA in L+Cr1 to L+Cr3 groups was depleted significantly and dose dependently as compared to L group.

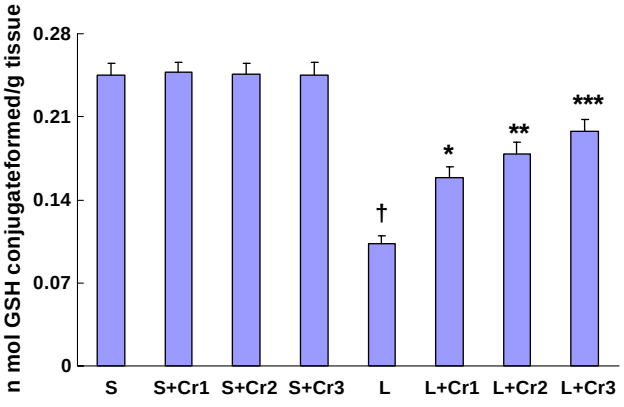

Fig. 2. Effect of crocetin on GSH content in substantia nigra of sham (S) and lesion (L) groups. Rats were pre-treated with either vehicle (S=sham, L=lesion) or crocetin (Cr1=25 μg/kg BW, Cr2=25 μg/kg BW, Cr3=25 μg/kg BW) for 7 days followed by unilateral intrastriatal injection of either vehicle (S=sham groups) or 6-OHDA (L=lesion groups). After 4 weeks of lesioning, rats were decapitated and substantia nigra was dissected and homogenized, and PMS was subjected to standard protocol of GSH estimation. Values are expressed as means±S.E.M. †$p<0.001$ vs. S; *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. L. Number (n) of rats in each group is 10.

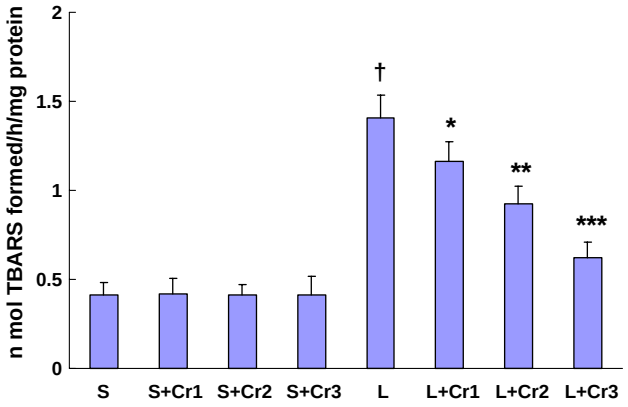

Fig. 3. Effect of crocetin on TBARS content in substantia nigra of sham (S) and lesion (L) group. Rats were pre-treated with either vehicle (S=sham, L=lesion) or crocetin (Cr1=25 μg/kg BW, Cr2=25 μg/kg BW, Cr3=25 μg/kg BW) for 7 days, followed by unilateral intrastriatal injection of either vehicle (S=sham groups) or 6-OHDA (L=lesion groups). After 4 weeks of lesioning, rats were decapitated and substantia nigra was dissected and homogenized, and PMS was subjected to standard TBARS estimation protocol. Values are expressed as means±S.E.M. †$p<0.001$ vs. S; *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. L. Number (n) of rats in each group is 10.

The effect of crocetin and 6-OHDA on neuronal survival is presented in Fig. 4. The representative histopathologic slides show that 6-OHDA caused significant degeneration of cells in substantia nigra of the lesion-group rats, whereas the cells in crocetin pre-treated rats were protected. Moreover, the counting of normal nuclei and pycnotic nuclei shows that the ratio of normal to pycnotic nuclei is significantly decreased in lesion group. Pretreatment of rats with 75 μg/kg BW protected the cells significantly and increased the ratio of normal to pycnotic nuclei (Fig. 4D). The most potent observed dose of crocetin against 6-OHDA neurotoxicity was 75 μg/kg BW; therefore, a photograph of only one dose (75 μg/kg BW) is shown in Fig. 4C.

4. Discussion

Although the etiology of PD has not been fully elucidated, the generation of ROS, leading to oxidative stress, together with a relative paucity of antioxidant defenses in the substantia nigra and nigrostriatal dopaminergic pathway, is widely considered as the final biochemical cause of neuronal death (Fernandez-Espejo, 2004; Gille et al., 2004). The major metabolites of DA (DOPAC and HVA) are subject to the action of monoamine oxidase (MAO), a flavoprotein present in the outer membrane of the mitochondria. Products of MAO reaction include aldehyde, corresponding to the amine substrate, and further dehydrogenation of aldehydic hydrogen forms DOPAC (Sian et al., 1999). Moreover, MAO leads to a high concentration of intracellular H_2O_2, which subsequently leads to oxidative stress (Blum et al., 2001). Auto-oxidation of catechols to

Table 2
Effect of crocetin and 6-OHDA on the level of dopamine, DOPAC and HVA

Groups	DA	DOPAC	HVA	DOPAC/DA
S	9.39±0.20	1.29±0.02	1.11±0.02	0.137±0.01
S+Cr1	9.45±0.27 (0.64)	1.31±0.02 (1.55)	1.12±0.01 (0.90)	0.138±0.01 (0.73)
S+Cr2	9.42±0.47 (0.32)	1.31±0.01 (1.55)	1.12±0.01 (1.63)	0.139±0.01 (1.46)
S+Cr3	9.38±0.44 (−0.11)	1.32±0.01 (2.32)	1.11±0.01 (0.0)	0.141±0.01 (2.92)
L	2.98±0.05† (−68.26)	0.55±0.02† (−57.36)	0.46±0.02† (−58.56)	0.184±0.01† (34.31)
L+Cr1	4.88±0.21* (63.76)	0.83±0.02* (50.91)	0.72±0.020* (56.52)	0.170±0.01* (−7.61)
L+Cr2	6.26±0.32* (110.07)	0.89±0.02* (61.82)	0.82±0.03* (78.26)	0.142±0.01* (−22.83)
L+Cr3	7.89±0.19* (164.76)	0.92±0.02* (67.27)	0.90±0.03* (95.65)	0.116±0.01* (−36.96)

Rats were either pre-treated with vehicle (S=sham group; L=lesion group) or different doses of crocetin (Cr1=25 μg/kg BW, Cr2=25 μg/kg BW, Cr3=25 μg/kg BW) for 7 days and subjected to unilateral intrastriatal injection of either vehicle (S+sham groups) or 6-OHDA (10 μg/2 μl) (L=lesion groups) on day 8. Rats were decapitated after 4 weeks, and striatum was dissected, homogenized and processed for measuring of DA, DOPAC and HVA content. Content is reported as ng/mg of tissue. Data were subjected to ANOVA followed by Tukey–Kramer test, and values are expressed as means±S.E.M. †$p<0.01$, ††$p<0.001$ vs. S, *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. L. Values in parentheses are percentage increases (+) or decreases (−) as compared to S or L group. Number (n) of rats in each group is 10.

toxic quinones and their subsequent polymerization to neuromelanin may enhance formation of three reactive oxygen species: O_2., H_2O_2, and OH. (Sian et al., 1999). Crocetin is known to have a potent antioxidant property that protects cells from ROS (Tseng et al., 1995; Wang et al., 1996). The experiment presented here demonstrates the ability of crocetin to partially protect degeneration of the nigrostriatal dopaminergic neurons against 6-OHDA-induced Parkinsonism and to maintain the dopamine level closer to basal level (Table 2).

Crocetin reduces the contra-lateral rotations induced by apomorphine (Fig. 1E). Apomorphine-induced rotations in the unilateral 6-OHDA reflect motor deficits in addition to the directional bias related to sensory neglect. Several studies have shown that apomorphine-induced rotations are observed in moderate to severe lesions, but partially lesioned rats do not rotate after such treatment (Fornaguera et al., 1994; Henderson et al., 2003). Thus, a marked decrease in rotation of lesioned groups treated with crocetin might be due to the protective effect of crocetin on

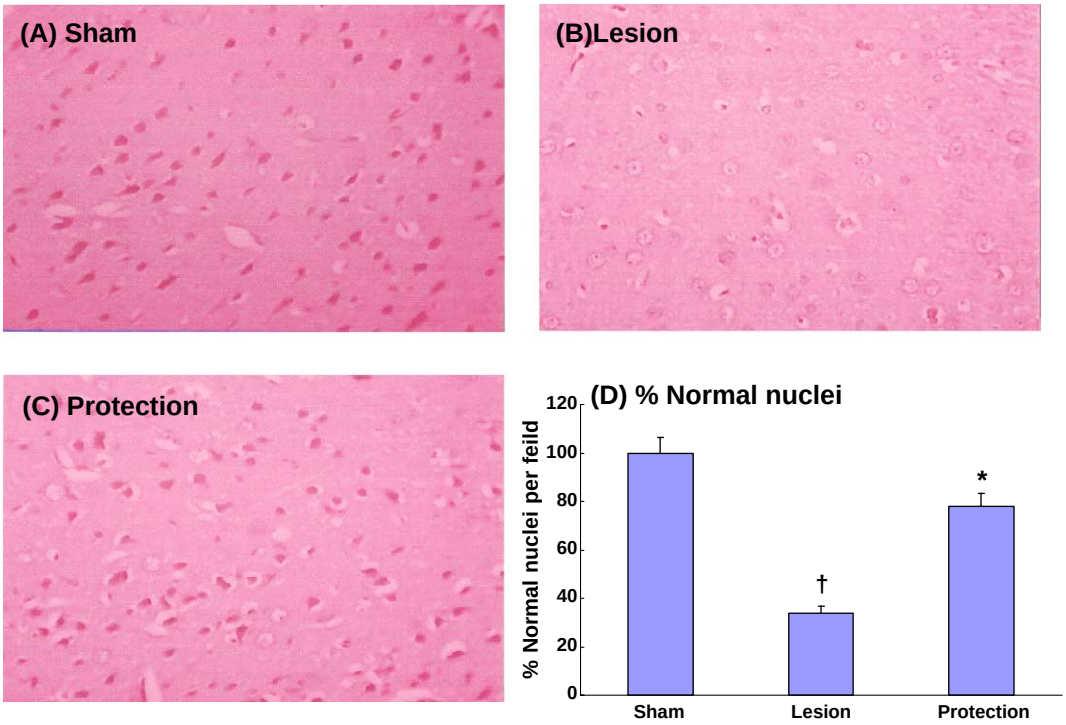

Fig. 4. Representative photograph of coronal sections of substantia nigra, and histogram showing effects of 6-OHDA and crocetin. Rats were pre-treated with either vehicle (sham) or crocetin (25 μg/kg BW, 50 μg/kg BW, and 75 μg/kg BW; protection group) for 7 days, followed by unilateral intrastriatal injection of either vehicle (Sham) or 6-OHDA (Lesion). After 4 weeks of 6-OHDA injection, rats were decapitated and brains were perfusion-fixed, sectioned (5 μm), and stained with haematoxylin-eosin. Magnification (A–C) ×20. (A) Sham group, (B) 6-OHDA-treated lesion group, (C) protection by pretreatment with 75 μg/kg BW crocetin, (D) histogram depicting the ratio of normal nuclei over pycnotic nuclei in sham, lesion and protection groups. Values represents the mean of ratio between normal and pycnotic nuclei in the same field ±S.E.M. †$p<0.001$ vs. S; *$p<0.001$ vs. L.

dopaminergic neurons against 6-OHDA toxicity. It is evident from our findings that, in a model of in 6-OHDA toxicity, crocetin reduced the increase in dopamine utilization by the tissue. In PD, dopamine metabolism increases with increase in dopamine depletion, which might be due to a compensatory mechanism on the part of the remaining neurons (Agid et al., 1973; Robinson et al., 1994). It is highly significant that, in the present study, pre-treatment with crocetin protected against 6-OHDA-induced Parkinsonism and allowed neurons to function closer to their initial activity.

The protective effect of crocetin might be due to reduction in auto-oxidation of dopamine by enhancement of antioxidant enzyme activity, particularly GPx, which shows relatively high activity in striatum and substantia nigra relative to other brain regions (Brannan et al., 1980). These findings are in agreement with our previous findings, in which in vivo administration of antioxidant protected the level of dopamine in the nigrostriatal system (Ahmad et al., 2005; Zafar et al., 2003).

The brain is thought to be vulnerable to oxidative damage due to its high oxygen consumption, presence of high levels of polyunsaturated fatty acids, and the non-regenerative nature of neurons (Floyd and Carney, 1992; Jenner, 1992). A major consequence of oxidative stress is damage to cellular macromolecules. Auto-oxidation of dopamine and 6-OHDA produces H_2O_2, which is subsequently converted to hydroxyl radical by Fe^{2+}, causing fragmentation of the lipid or alteration of its chemical structure (Floor, 2000). Fatty aldehydes, such as 4-hydroxynonenal, that are generated as a result of lipid peroxidation can react with free thiol groups, such as cysteines on proteins, to produce thioesters, which may affect protein function and stability (Halliwell, 1992; Halliwell and Gutteridge, 1986). This results in the damage of DNA, membrane lipid, carbohydrate, proteins and finally cell damage. Thus, it can be predicted that 6-OHDA potentiates lipid peroxidation in the nigrostriatal system, which might be the result of diminished activity of antioxidant enzymes and a low level of glutathione, whereas our results show that pre-treatment with crocetin partially attenuates lipid peroxidation and protects glutathione content (Fig. 3).

It is reported that the activity of SOD, the enzyme that converts O_2. to H_2O_2, and content of glutathione are decreased in the striatum as a compensatory action against a 6-OHDA-elicited toxic mechanism (Hodgson and Fridovich, 1975), suggesting that oxyradicals can also inhibit the activity of SOD. The level of glutathione and activity of its dependent enzymes (Gpx, GR and GST) and catalase are reported to be lower in PD; hence, rapid formation of H_2O_2 and its insufficient removal have been proposed as the basis of oxidative stress. GPx and catalase detoxify H_2O_2 to H_2O (Halliwell, 1992), and GST catalyses the detoxification of oxidized metabolites of catecholamines (*o*-quinone); thus, these enzymes serve as an antioxidant system that prevents the degenerative cellular process (Baez et al., 1997). Here, increased levels of antioxidant enzymes suggest that crocetin increases the antioxidant potential in brain and helps it to fight against a 6-OHDA-induced neurotoxic paradigm.

Increasing evidence points to a correlation between neurodegenerative disease and reduced activity of mitochondrial oxidative phosphorylation (Beal, 1999; Schapira, 1999). A mitochondrial complex-I deficit could contribute to cell degeneration in PD via a direct generation of ROS, together with a decrease in ATP synthesis, leading to energy failure. These cellular processes result in the decrease of respiratory complex-I activity and generate superoxide radicals, which may elicit cell destruction and exacerbate the complex-I defect. Also, the shift in Fe^{2+}/Fe^{3+} ratio from 2:1 in the normal substantia nigra to 1:2 in that of PD may generate hydroxyl radicals from hydrogen peroxide through the iron-dependent Fenton reaction. These free radical species may ultimately induce cell death via disruption of normal cellular Ca^{2+} homeostasis. It has been reported that pre-treatment of mouse fibroblast cells with crocetin inhibited the 12-*O*-tetradecanonylphorbol-13-acetate (TPA)-induced protein kinase (PKC) activity, thus providing a protective mechanism against toxins and preventing cells from energy deprivation (Wang et al., 1996).

In the present study, an enhanced level or activity of the antioxidant system, depletion of the TBARS level, and a marked elevation in the content of dopamine and its metabolites provide substantial evidence to conclude that crocetin can afford neuroprotection by inhibiting the cascade of events that leads to neurodegeneration. These findings are well supported by our histopathologic findings that show that the lesion group had significant degeneration of neurons, whereas the crocetin-treated groups had neuroprotection (Fig. 4). The biochemical, behavioral, and histopathologic results in this study suggest a neuroprotective role of crocetin in a 6-OHDA model of rat PD. This study can be used as a potential tool to explore the neuroprotective pathways leading to the development of a potential therapeutic remedy in preventing or impeding this devastating neurologic disorder.

Acknowledgements

The technical assistance of Messers Dharamvir, and Anil is greatly acknowledged.

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