CNS Neuroscience & Therapeutics

ORIGINAL ARTICLE

CNS Neuroscience & Therapeutics

Quercetin Improves Behavioral Deficiencies, Restores Astrocytes and Microglia, and Reduces Serotonin Metabolism in 3-Nitropropionic Acid-Induced Rat Model of Huntington's Disease

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Keywords

Depression; Gait disturbances; Glial fibrillary acidic protein; Inflammatory mediators; Serotonin metabolism; Striatal dopamine.

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Received 4 July 2013; revision 17 September 2013; accepted 19 September 2013

SUMMARY

Aim: Huntington's disease (HD) is an autosomal dominant disorder, for which clinically available drugs offer only symptomatic relief. These prescription drugs are not free of side effects, and the patients usually suffer from anxiety and depression. We investigated quercetin, a dietary flavonoid with free radical scavenging properties, for its beneficial potential if any, in 3-nitropropionic acid (3-NP)-induced HD in rats where both drugs were administered simultaneously. **Methods:** Performance of rats on beam balancing, elevated plus maze and gait traits were investigated following 3-NP and/or quercetin treatments for 4 days. Striatal biogenic amine levels and monoamine oxidase activity were assayed. Striatal sections were examined for Cd11B and glial fibrillary acidic protein immunoreactivity, and for evidences of neuronal lesion. **Results:** Quercetin significantly attenuated 3-NP-induced anxiety, motor coordination deficits, and gait despair. While the dopaminergic hyper-metabolism was unaffected, quercetin provided a significant reduction of 3-NP mediated increase in serotonin metabolism. Quercetin failed to affect 3-NP-induced striatal neuronal lesion, but decreased microglial proliferation, and increased astrocyte numbers in the lesion core. **Conclusion:** These results taken together suggest that quercetin could be of potential use not only for correcting movement disturbances and anxiety in HD, but also for addressing inflammatory damages.

doi: 10.1111/cns.12189

Introduction

Huntington's disease (HD) is a genetic disorder in which the expansion of CAG repeats in the IT15 gene coding for the protein Huntingtin (Htt) leads to neurodegeneration affecting the striatum initially, and later the cortex and the hippocampus. Patients suffering from this disease exhibit gross motor disability as a major disease syndrome, and to a great extent anxiety, depression, cognitive deficit, and other significant behavioral abnormalities, as the disease progresses. The actual mechanism underlying the site-specific neuronal death in HD is not fully understood, though many hypotheses are put-forth, which partially explain the phenomenon. These are dopamine (DA) toxicity [1,2], calcium imbalance [3,4], oxidative stress [5], and glutamate excitotoxicity [6]. Interrelationship between HD and oxidative stress has been emphasized in many studies. Human HD brains show reduction in complex II/III activity which leads to increased free radical, and reduced ATP generation [6]. Peripheral blood from HD patients exhibited decreased Cu-Zn superoxide dismutase 1 and glutathione peroxidase activities [7]. Cultured HD fibroblasts also showed reduced catalase activity [8]. It has also been speculated that as neurons are more susceptible to oxidative stress, this generalized decrease in antioxidative defense system in the HD brain could be lethal to neurons expressing mutant *Htt*. This probably makes the striatum more specific for neurodegeneration, since involvement of DA in its capacity to generate reactive oxygen species is well established. It has been shown that elevated DA can selectively kill GABA-ergic neurons, enhance early aggregate formation and cause loss of locomotor activity in DA transporter knockout HD mice [9]. Benchoua et al. [10] have shown that DA increases the vulnerability of the striatal cells to mutant *Htt* as well as to 3-nitropropionic acid (3-NP), an irreversible mitochondrial complex II inhibitor, which has been used by many researchers to produce animal models of HD [11,12]. Systemic administration of 3-NP

will cause striatal and cortical lesions, which result in gait abnormalities in animals. 3-NP-induced HD rat model also shows mitochondrial complex I malfunctioning and increased DA levels, which contribute to increased hydroxyl radical generation in the striata [2,12].

The dietary flavonoid, quercetin is shown to cross the blood–brain barrier, and reach brain when administered systemically [13]. Quercetin is suggested to be neuroprotective in MPP^+- or rotenone-induced Parkinson's disease in rats [14,15], and in cellular model of Alzheimer's disease [16]. This neuroprotective effect is said to be mediated partially by its antioxidant capacities, and in part by reinstating mitochondrial functions [15]. Since oxidative stress has been involved in 3-NP model of HD, and as this neurotoxin is an inhibitor of mitochondrial complex-I and -II, we regarded it prudent to assess neuroprotective effects if any, of quercetin in an animal model of HD. In this study, we investigated whether quercetin administration can improve motor coordination dysfunctions and psychological complications seen in this model, and if it can reduce the lesion area in the striatum, and normalize the levels of striatal neurotransmitters.

Materials and methods

Materials

Gelatine, Triton X100, bovine serum albumin, kynuramine, cresyl violet, and L-deprenyl were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3,3′-Diamino benzidine (DAB), acetonitrile and heptane sulfonic acid were procured from MP Biomedicals (Solon, OH, USA). Mouse anti-rat CD11b antibody, rabbit antiglial fibrillary acidic protein (GFAP) antibody, and horseradish peroxidase (HRP)-tagged anti-mouse and anti-rabbit secondary antibodies were procured, respectively, from Chemicon (Billerica, MA, USA), Abcam (Cambridge, UK), and Genie (Bangalore, India). All the other chemicals were of analytical grade and were purchased from SRL (Mumbai, India).

Animals and Drugs Treatment

The animal experimentation procedures were conducted in accordance with national guidelines on the "Care and Use of Animals in Scientific Research", formed by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Animal Welfare Division, under the Ministry of Environment and Forests, Govt. of India. The protocols were approved by the CPCSEA appointed Animal Ethics Committee of Indian Institute of Chemical Biology, Kolkata, India.

Male Sprague Dawley rats of 20–24 weeks old (350–400 g body weight) were housed under standard conditions of temperature (22 ± 2°C), humidity (60 ± 5%), and illumination (12-h light–dark cycle). They were provided with food and water *ad libitum*. Animals were always sacrificed in the mornings to avoid diurnal variations in the levels of neurotransmitters, their metabolites and changes in other neurochemical parameters. The neurotoxin, 3-NP was prepared freshly before each injection in normal saline (0.85% NaCl) and was adjusted to pH 7.4. Male rats were treated with 20 mg/kg (i.p.) once daily, for 4 days as standardized in our laboratory [2,12]. Quercetin was freshly prepared before each injection in minimal amount of DMSO. Rats were treated with 25–50 mg/kg body weight quercetin (i.p.), 6 h before and after 3-NP treatment; for 4 days. Control animals received equal amount of DMSO in saline.

Animal Behaviors

Rats were weighed every morning at the same time throughout the period of study, prior to any treatment. We followed the method of Klapdor et al. [17] to quantify gait abnormalities, as standardized in our laboratory [12]. Rats were acclimatized by making them walk on the platform 3–4 times until they started walking up the slanting platform on their own, once placed at the foot of the ascending pathway. The gangway was lined with white paper for recording the foot impression. Fore- and hind-limbs of the animals were painted with two different non-toxic water-colors so as to get differential footprints (forelimbs in red color and hind limbs in green color). Footprints were analyzed for different parameters, *viz.*, foot length, stride length, and stride width. It was made sure that only those papers were used for measurements where animal has not stopped in between the walk. Measurements were done by millimeter scale manually.

Beam balance test [18] was performed to assess the motor coordination of the animals. Animals were allowed to walk across a narrow wooden beam (2.0 cm in width, and 120 cm in length) elevated 65 cm above the ground. The beam was connected with a platform of 8 cm diameter at the starting point and a dark housing (22 cm × 15 cm × 18 cm) at the other end with a food pellet inside to act as a reward. Before the initiation of treatment, each rat was allowed to explore the beam till it was trained to walk on the narrow beam for acclimatization, which took about three practice sessions conducted on successive days. During the experimental sessions (on 1st day before any treatment, and 5th day), the time taken by each animal to travel the total distance between the platform and the dark housing across the beam was measured.

For elevated plus maze test, we followed the protocol of Walf and Frye [19]. On the 5th day animals were placed on the junction of the open and closed arms, facing the open arm and left on the maze for the next 5 min. Number of entries into each arm, time spent in the arms and numbers of rears were noted down while the animals were exploring the maze. Proper care was taken to avoid any sudden noise or disturbance.

All the behavioral experiments were performed by two blinded persons at the end of the treatment period. Interrater variability was assessed for the data generated, and was found to be not significant.

Determination of the Neurotransmitter Levels

On the 5th day, animals were decapitated and the striata were collected in 10 volumes of ice-cold 0.1 N perchloric acid containing 0.01% EDTA. The tissues were sonicated at 50 Hz for 30 seconds, centrifuged at $12{,}000\times \boldsymbol{g}$ for 10 min and the supernatants were collected. Ten micro liter was injected into a high-performance liquid chromatography (HPLC) system equipped with a Rheodyne injector, glassy carbon working electrode, and Ag/AgCl

reference electrode (Bioanalytical Systems Inc, West Lafayette, IN, USA). A C_{18} ion-pair, reverse phase analytical column (4.6 × 250 mm; Ultrasphere-IP; Beckman, Pasadena, CA, USA) was used for the separation of the biogenic amines [20]. Flow rate was 0.7 mL/min and potential applied was +0.74 V. Composition of the mobile phase was 8.65 mM heptane sulfonic acid, 0.27 mM EDTA, 13% acetonitrile, 0.43% triethylamine and 0.32% phosphoric acid.

Effect of Quercetin on Monoamine Oxidase Activity *in vitro* and *in vivo*

Total monoamine oxidase (MAO) and MAO-A activities were determined in the crude mitochondrial P_2 fraction obtained from the forebrain of normal, saline, 3-NP and 3-NP+quercetin-treated rats, according to the fluorimetric assay procedure of Morinan and Garratt [21], as modified by Mitra et al. [22]. Mitochondrial P_2 fraction was obtained as described previously [12]. The effect of quercetin on MAO activity was determined from the amount of the fluorigenic product, 4-hydroxyquinoline (4-HQ) formed due to oxidation of kynuramine. For estimating MAO-A activity specifically, MAO-B inhibitor L-deprenyl (40 nM) was used. In the *in vitro* studies, quercetin (1–10 μM concentration or appropriate amount of DMSO) was incubated with the mitochondrial fraction obtained from the normal rats for 15 min before the experiment. The enzyme reaction in triplicate was initiated by the addition of 3.07 mM kynuramine and incubated for 15 min at 37°C. Addition of 150 μL of 0.4 M ice-cold perchloric acid terminated the reaction. The reaction mixture was centrifuged (7500× ***g*** for 5 min) and 1 mL of 1 N NaOH was added to 500 μL of the supernatant. From each reaction set, 200 μL of the reaction mixture was taken in microtiter plate for the measurement of fluorescence at E_x/E_m-318/380 nm. Standard curve was prepared by taking 0–5 nmol of 4-HQ in a reaction volume of 500 μL to which 150 μL of 0.4 M ice-cold perchloric acid was added. The enzyme activity is expressed as nmol of 4-HQ formed/h/mg protein. Protein was estimated in the P_2 fraction by the method of Lowry et al. [23]. The final results represent the average of three completely independent sets of experiments.

Histochemical Analysis of Brain Sections

Following the treatments, animals were anesthetized using chloral hydrate (350 mg/kg; i.p.) and perfused transcardially with 4% paraformaldehyde. Brains were dissected out, cleaned by washing in the fixative and cryoprotected in sucrose. Brain sections (20 μm) were taken using a cryotome on gelatin-coated slides. Sections from the same area for all the treatment groups were chosen for immunohistochemistry and cresyl violet staining [24]. For immunohistochemistry, the sections were washed with 0.1 M PBS (three times), incubated with 1% H_2O_2, permeabilized with 0.4% Triton X100, blocked with 8% bovine serum albumin and incubated with the primary antibody overnight at 4°C (1:500 for CD11b and 1:1000 for GFAP). Proper HRP-tagged secondary antibodies (1:500) were used for visualization using DAB. Both the cresyl violet stained and immunohistochemically processed sections were dehydrated in alcohol series and cleared in xylene, mounted on DPX, and photographed.

Statistics

The data were subjected to statistical procedures employing ANOVA followed by appropriate post hoc analyses such as Dunnett test in cases of behavioral test. Student's *t*-test was used for determining significance among neurochemical variables. Results are provided as Mean ± SEM, and the error bars represent the SEM. $P \leq 0.05$ are considered significant.

Results

3-NP Treatment Caused Weight Loss in Animals, Which was Attenuated by Quercetin Administration

We monitored the body weight of the animals for 5 days during the experimental period (Figure 1A). Saline treatment did not show any significant alteration, though 3-NP treatment caused drastic loss in body weight from 3rd day onwards. During the last 5 days, animals lost a total of 45 ± 13.2 g weight. This was also similar in 3-NP-treated rats where DMSO or 25 mg/kg quercetin were administered (Figure 1B). When the animals were treated with 3-NP and quercetin 50 mg/kg they showed insignificant loss of body weight when compared to the saline-treated group (10 ± 5.7 g, Figure 1B), which was significantly less than the 3-NP-treated group only.

3-NP-Induced Decreased Stride Length and Low Performance on Beam Balance were Attenuated by Quercetin

Trained male rats were made to walk through a narrow gangway of a small rodent gait analysis equipment, and the animal's foot impressions were taken on white paper spread over. It was found that in control trained animals there was always a tendency of increased stride length and decreased foot length. This was probably due the animals' tendency to cover the gangway as fast as they can without exploratory behavior due to the training. However, 3-NP treatment caused a significant decrease in the stride length (13 ± 1.3 cm) when compared to the performance of the same group of animals before any treatment (16.05 ± 0.3 cm). Quercetin minimized this effect, but not dose-dependently (15.3 ± 1.3 cm; Figure 1C). The vehicle treatment did not affect stride length in 3-NP-treated rats. The other two parameters, that is, stride width and foot length remained unchanged in the 3-NP-treated rats (data not shown).

We further did beam balance test, another parameter for evaluation of motor coordination activity in animals. 3-NP treatment significantly affected the animals' performance on beam balance; most of the animals were not able to cover the full length of the beam, those which reached the target location took longer period of time (17.2 ± 1.1 seconds), whereas control animals took only 7.1 ± 0.5 seconds. Low doses of quercetin (25 mg/kg)-treated rats did not show any significant recovery in the time taken to cover the distance (12.6 ± 5.2 seconds), whereas 50 mg/kg quercetin-treated rats took significantly less time to reach the end point (10.6 ± 2.2 seconds, Figure 1D), when compared to 3-NP-treated rats. DMSO-treated 3-NP administered rats took similar

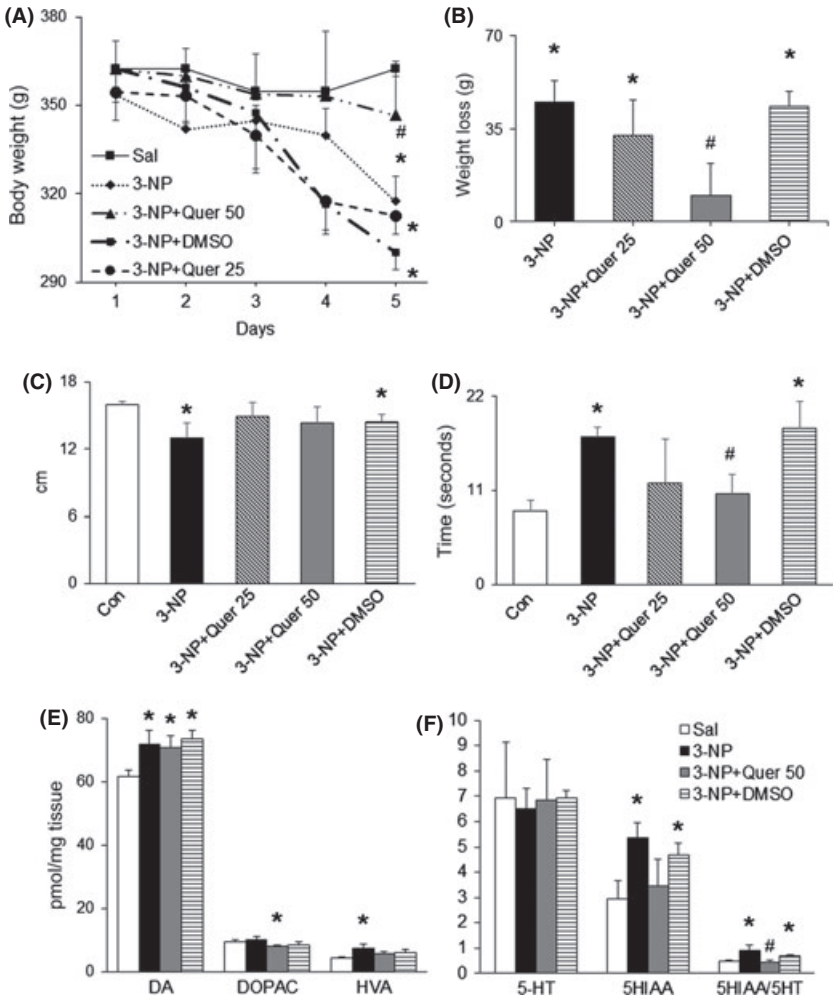

Figure 1 Effects of quercetin administration on 3-NP-induced behavioral dysfunction and striatal biogenic amine levels. Rats were administered with 20 mg/kg 3-NP (i.p.) for 4 days. Quercetin (25 and 50 mg/kg, i.p.) was administered 6 h before and after 3-NP treatment for 4 days. (**A**) Body weight of the animals was monitored for each day for all the treatment groups. (**B**) Average of the total loss of weight during the 5 days was calculated. Saline-treated groups did not show any loss of weight when the average was taken. Animal behaviors were evaluated by two personnel, independently and blind to the treatments, before starting and after completion of the treatments. The interrater variability was statistically insignificant. (**C**) Stride length of animals with different treatment paradigm (n = 4–6). (**D**) Beam balance performance was evaluated in these animals after completing the gait analysis (n = 4–6). (**E**) Striatal levels of dopamine (DA), dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and (**F**) serotonin (5-HT) and its metabolite 5-hydroxyindole acetic acid (5-HIAA) levels were quantified employing high-performance liquid chromatography-electrochemical detection method. The turnover of 5-HT was determined by the metabolite to transmitter ratio (n = 5–6). Results are represented as mean ± SEM; $^{*}P \leq 0.05$ as compared to saline-treated group; $^{\#}P \leq 0.05$ as compared to 3-NP-treated value.

time as 3-NP alone treated animals (18.2 ± 2.2 seconds). As the lower dose of quercetin failed to show recovery in beam balance test, we further proceeded with the 50 mg/kg quercetin dose, for all further assays.

Quercetin Failed to Affect 3-NP-Induced Increases in Striatal Dopamine, but Serotonin Metabolism was Corrected

Control saline-treated animals' striata contained 61.6 ± 2.03 pmol DA/mg tissue. Sub-acute treatment of 3-NP in rats caused a significant increase in striatal dopamine level (71.7 ± 4.3 pmol/mg tissue). Quercetin administration daily twice for 4 days in 3-NP-treated animals failed to affect the neurotoxin-induced striatal dopamine level (70.8 ± 3.4 pmol/mg tissue). The metabolite of DA, 2,3-dihydroxyphenylacetic acid (DOPAC) level was decreased in quercetin+3-NP-treated group, whereas another metabolite, homovanillic acid (HVA) levels were significantly increased in the striata of 3-NP-treated rats (Figure 1E). Both HVA and DOPAC levels were not statistically different in the 3-NP and 3-NP+quercetin group. Vehicle treatment did not affect DOPAC or HVA levels (Figure 1E), when compared to saline-treated rats which was also not significantly different from the 3-NP-treated group. Similar to 3-NP alone treated animals, the striatal DA level was significantly increased when compared to the saline-treated group (73.3 ± 2.8 pmol/mg tissue), but was not different from 3-NP alone treated group (Figure 1E).

Striatal serotonin (5-hydroxytryptamine or 5-HT) level was unaltered; however, its metabolite 5-hydroxyindole acetic acid (5-HIAA) level was significantly increased in the striata of 3-NP-treated rat (control 2.9 ± 0.7 and 3-NP 5.3 ± 0.5 pmol/mg tissue). The striatal level of 5-HIAA was found to be lowered in quercetin administered, 3-NP-treated rats (3.4 ± 1.04 pmol/mg tissue) as compared to 3-NP alone treated animals, though not statistically significant (Figure 1F). The turnover of the neurotransmitter, as depicted by the metabolite to neurotransmitter ratio was found to be significantly increased in 3-NP-treated animals (Figure 1F). In these animals, when quercetin was administered, the turnover of 5-HT was returned to control level (0.46 ± 0.05; Figure 1F), which was significantly lower than the 3-NP-treated group. The increase in 5-HT turnover in 3-NP-treated rats remained high after DMSO treatment, which was significantly different from 3-NP+quercetin group (Figure 1D).

HD Rats Performed Better in Plus Maze Following Quercetin Treatment

We examined whether the effect of increased 5-HT metabolism is also reflected in the animal's behavior or not and so we conducted the plus maze test, and found that animals treated with saline spent more time in the darkened, closed arm (224 ± 12.59 seconds; Figure 2A). They moved around the maze normally and showed almost equal numbers of entry in both arms (Figure 2C). 3-NP treatment altered this and these animals remained more time in the closed arm (286 ± 4.8 seconds), and lesser time in the open arm, and showed less mobility between the arms, thereby entry into the closed (Figure 2C) or open arm (Figure 2D) was significantly less, a clear indication of anxiety development. Quercetin treated, 3-NP poisoned rats showed reversal of these activities, and the time spent in the closed arm was significantly lesser than the 3-NP-treated rats (249.5 ± 21.2 seconds). These animals also showed much recovery in the anxious behavior, since these animals entered significantly more time into the open arm (Figure 2D). As DMSO treatment did not show any recovery in the other two behavioral parameters, serotonin metabolism or MAO activity (see results in Figure 3), we did not perform plus maze test for 3-NP+DMSO group. The time spent in the junction of two arms during the initial few seconds are not represented here, and so the time spent in the open and close arm do not add up to the total time period of the experiment.

Quercetin is an MAO-A Inhibitor *in vitro* and *in vivo*

Quercetin exhibited dose-dependent inhibition in the activities of total MAO (Figure 3A), as well as of MAO-A *in vitro* (Figure 3B), and *in vivo* (Figure 3D). Interestingly, 3-NP caused a significant inhibition in the total MAO activity and this decrease was unaffected in the animals that received quercetin (Figure 3C). 3-NP did not affect MAO-A activity *in vivo*, but quercetin caused a significant decrease in MAO-A activity in the 3-NP-treated animals (Figure 3D). Highest concentration of the vehicle did not affect MAO activity (data are not shown).

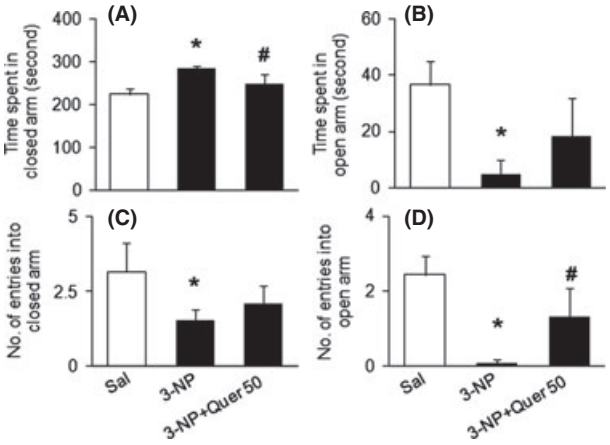

Figure 2 Quercetin treatment improves 3-NP-induced behavior deficiency on plus maze. Animals treated with 3-NP and 3-NP+quercetin were examined on the 5th day for their performance on an elevated plus maze. Each animal was tested for 5 min on the maze. Time (seconds) spent in the closed arm (**A**) or open arm (**B**), and number of entries into the closed arm (**C**) or open arm (**D**) were assessed. Results are represented as mean ± SEM (n = 9–10). $*P \leq 0.05$ as compared to saline-treated group; and $^{\#}P \leq 0.05$ as compared to 3-NP-treated group. n = 5–6.

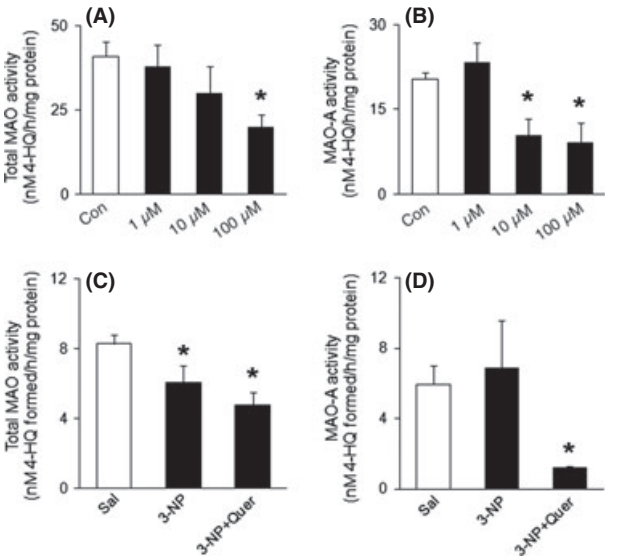

Figure 3 Quercetin inhibits monoamine oxidase (MAO)-A activity *in vitro* and *in vivo*. Mitochondria rich fraction isolated from forebrain of normal rat was incubated with 1, 10, and 100 μM of quercetin for 15 min, washed, and then incubated with the substrate, kynuramine for determining total MAO activity (**A**) and MAO-A activity (**B**). Specific activity of MAO-A was measured in presence of L-deprenyl, the specific inhibitor of MAO-B at a concentration of 40 nM (**B**) & (**D**). Total MAO (**C**) and MAO-A (**D**) *in vivo* activities were estimated in mitochondrial fractions obtained from the forebrain of saline, 3-NP and 3-NP+quercetin-treated (as explained under legends for Figure 1) animals on the 5th day. Data are represented as mean ± SEM of three separate independent experiments. $*P \leq 0.05$ as compared to control. n = 3.

Quercetin Failed to Protect Lesion Formation in Striatum but Reduced Microglial Activation along with Increased Astrocytosis inside the Lesion Core

Striatum and cortices of the control animal stained with cresyl violet showed staining of neurons uniformly (Figure 4A). Striatum of 3-NP-treated animals stained with cresyl violet showed large area without staining (asterisk), which was affected by the neurotoxin, but the cortex was stained appropriately (Figure 4B). Quercetin treatment in 3-NP administered rats failed to offer any improvement in the lesioned (asterisk) area (Figure 4C). Higher magnification of the sections from control animals showed normally stained pyramidal neurons (arrows) in the striatum (Figure 4D). 3-NP-treated animals revealed vacuoles (arrows), and the surviving cells lost their pyramidal

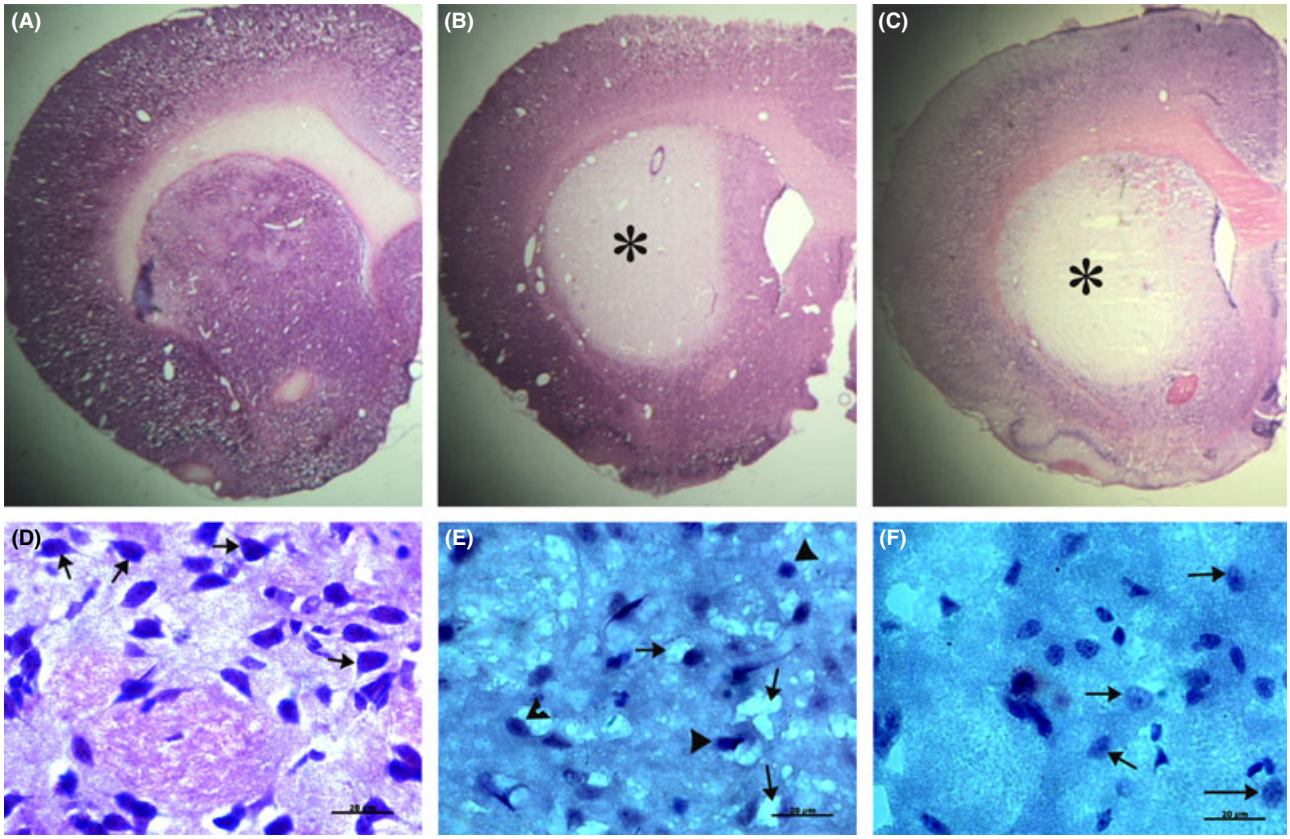

Figure 4 Quercetin fails to protect against striatal lesions caused by 3-NP treatment. On the 5th day, the animals were perfusion fixed in 4% PFA, the section passing though the striatum were cut (20 μm), stained with cresyl violet and were observed under microscope. Figures (**A**), (**B**) and (**C**) are representative sections of brains from three animals treated with saline, 3-NP and 3-NP+quercetin, respectively. A clear lesion area is visible in figure (**B**) and (**C**), as demarcated by asterisks. Control striatum showed clear healthy neurons, which contained pyramidal shaped, oval shaped, and a few elongated ones (**D**; arrows). 3-NP treatment caused formation of vacuoles (**E**; arrows), and the neurons have lost their shapes (**E**; arrowheads). Quercetin and 3-NP-treated rat striata looked no different than 3-NP alone treated striatum, but for lack of vacuoles. Neurons were rounded in shape and looked unhealthy (**F**; arrows). While (**A**–**C**) are sections with 1× magnification, (**D**–**F**) are at 100× magnification. Scale bars in **D**–**F** are 20 μm.

shape (arrowheads) and neuropils (Figure 4E). Quercetin treated, 3-NP administered animals' striatum revealed rounded dying neurons (arrows), but the vacuoles were absent (Figure 4F).

GFAP is a marker for astrocytes, and GFAP immunoreacted cells showed homogeneous distribution of the astrocytes in the control striatal sections (Figure 5A,D,G). 3-NP-treated brain striatal sections showed less number of astrocytes inside the lesion core (asterisk, Figure 5E,H) and most of the astrocytes remained outside the lesion area making a clear boundary (Figure 5B,E,H). Quercetin treatment in 3-NP administered animals showed several infiltrating astrocytes inside the lesion area (asterisk) with their morphology being normal (Figure 5C,F,I).

Immunostained sections for the microglial marker, CD11b showed hardly any staining in control brain sections (Figure 6A, D,G), but 3-NP-treated animals' striata showed numerous microglia, cells with ameboid shape (Figure 6B,E,H). Quercetin was able to reduce the number of microglia, although reactive microglia still persisted (Figure 6C,F,I).

Discussion

The major finding of this study is the potential of quercetin to significantly attenuate several behavioral, neurochemical, and neuropathological outcome of sub-acute treatment of 3-NP in rats that represents HD syndromes. Equally important information coming out of the study is the failure of quercetin to affect the increased dopamine levels in the striatum following 3-NP toxicity. The motor coordination deficit, locomotor insufficiency, and the acute anxiety caused by 3-NP, together with increased level of striatal dopamine, severe loss of neurons from the striatum and the heightened effect of astrogliosis are very relevant to the human disease conditions. Therefore, the observed effects of quercetin to make it better have also significant bearing to therapeutic possibilities for the disease, and therefore are important in the present context of unavailability of better treatment options for HD.

In HD, because of the dominant nature of the mutant protein genetic screening can easily suggest the probability of expressing the phenotype in susceptible individuals. Unlike other neurode-

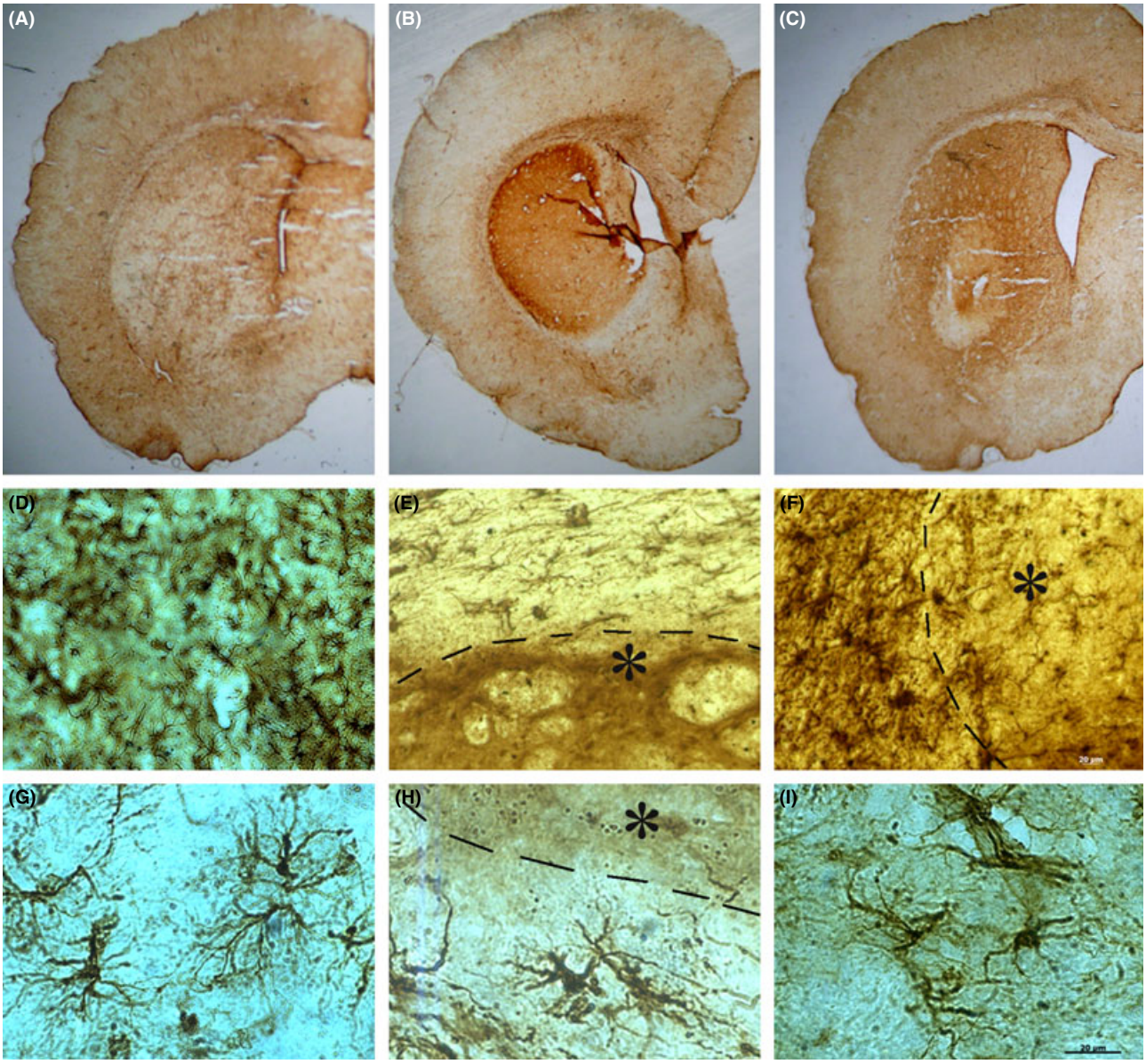

Figure 5 Astrocytosis in the striatum of 3-NP-treated rats. Brain sections passing through striatal region were immunostained for glial fibrillary acidic protein (GFAP) and were observed under microscope. (**A**), (**B**), and (**C**) represent photographs of brain sections from animals treated with saline, 3-NP and 3-NP+quercetin, respectively. In figures (**E**) and (**F**), the boundary of lesion core is demarcated with dotted lines. Asterisk represents the center of lesion in the striatum. The infiltrating astrocytes inside 3-NP+quercetin-treated striatal lesion core is clearly visible in figure (**F**). Magnification for figures **A**–**C** is at 1×, **D**–**F** at 32×, and **G**–**I** at 100×. Scale bars in (**F**) and (**I**) are 20 μm. In case of 3-NP and 3-NP+quercein, 100× photographs were taken from the lesion boundary.

generative diseases, which are sporadic and the time of onset is not well defined, HD has the advantage where the patient can be under therapeutic aid long before the onset, thereby delaying the appearance of signs and symptoms of the disease, and prevent behavioral abnormalities. In the 3-NP-mediated HD model, on the 5th day of treatment, when the behavioral and neurochemical abnormalities are at their peak, it has been found that almost 90% of the striatal cells are already dead [2,12], as visible in Figure 4. The rest also show abnormal features and eventually start to degenerate. So, other than the transplantational options, it is hardly possible to regenerate the neurons by pharmacological intervention after the 5th day. Our main aim was to reduce the behavioral and neurochemical abnormalities in this model by the use of this flavonoid.

One of the hallmarks of HD is involuntary movements that include chorea, bradykinesia, and dystonia, which are progressive in HD patients [25]. These motor abnormalities are believed to be resulting from an excessive increase of striatal DA level,

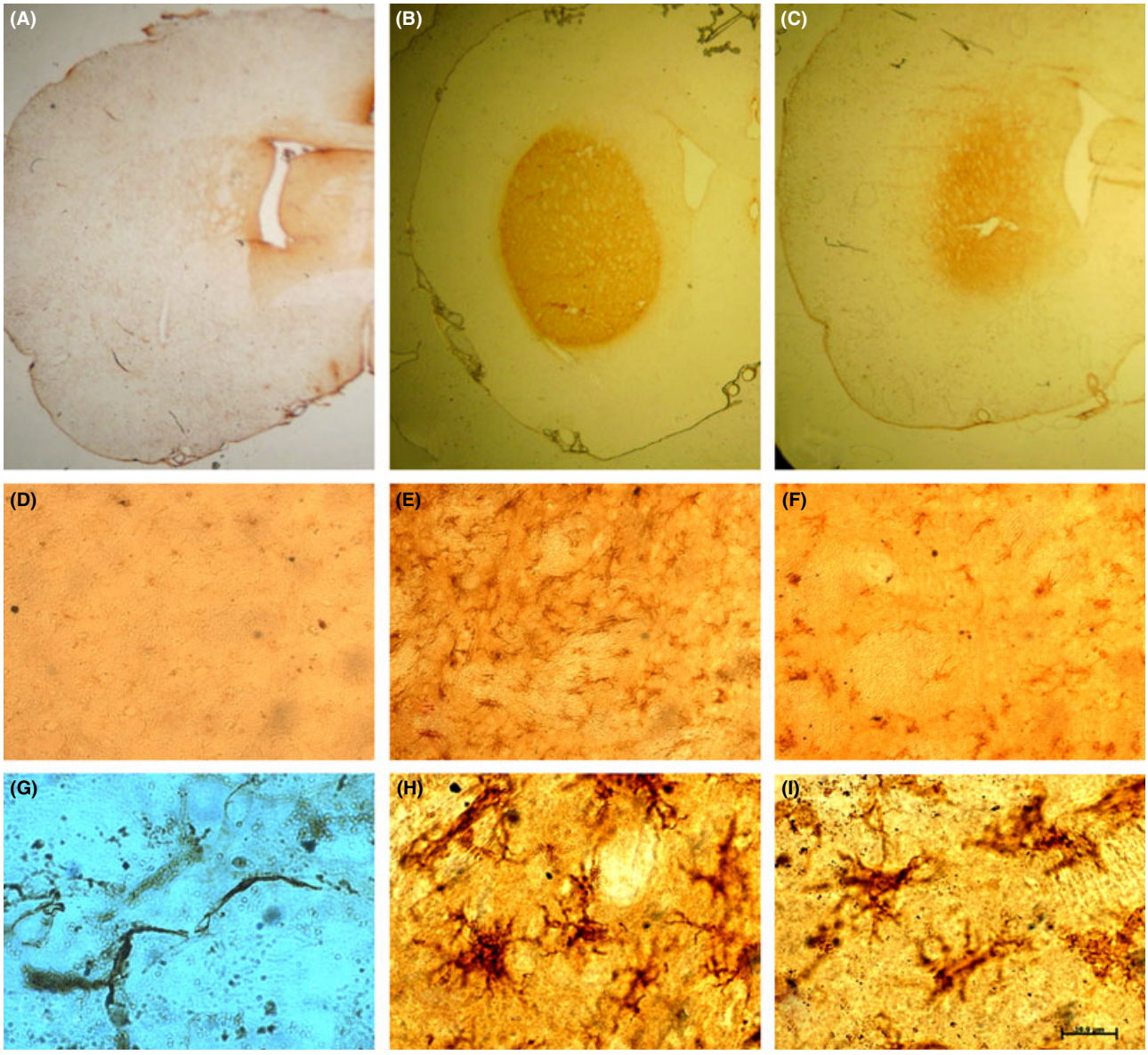

Figure 6 Quercetin treatment attenuates 3-NP-mediated microglial activation. Brain sections from saline (**A**), 3-NP (**B**), and 3-NP+quercetin (**C**) treated animals were immunostained for CD11b. Hardly any microglia is seen in control section at any magnification (**A**,**D**,**G**), but a high proliferation of microglia is observed in 3-NP-treated striatum (**B**,**E**,**H**). Comparatively, fewer microglia are seen in 3-NP+quercetin-treated striatum (**C**,**F**,**I**). **D** & **G**, **E** & **H** and **F** & **I** are, respectively, higher magnification figures from part of figures **A**,**B**, and **C**. Magnifications are - (**A**–**C**) at 1×, (**D**–**F**) at 32×, and (**G**–**I**) at 100×. Scale bar in (**I**) is 19.9 μm.

and probably due to damage of GABA-ergic neurons in the striatum that regulate the total striatal output. Severe motor abnormalities including significant decrease in stride length following 3-NP treatment is reported in rats [12,26]. In this study, we also show a significant inability of the rats to balance themselves on a narrow beam and to take longer time to cover a specified distance, signifying a serious motor in-coordination in these animals. While these abnormalities may be a consequence of a consistently enhanced striatal DA levels, the basis by which quercetin could render a significant relief to these motor abnormalities is hard to explain, since striatal DA level could not be lowered by its treatment.

Literature reveals varied reports on striatal DA levels in animal models of HD. Intrastriatal infusion of 3-NP decreased striatal DA levels, while chronic subcutaneous injections caused no effect [11]. In our previous studies, we found a dose-dependent increase in striatal DA levels [2,12]. Similar to experimental HD results, reports on postmortem human HD brains are also contradictory [27,28]. Although a relationship between the increase in striatal DA level and behavioral deficiencies is quite hard to draw, there

exists a direct, unequivocally accepted correlation between increased striatal DA and striatal cell loss [2,9,29,30]. Since quercetin failed to affect DA level in the striatum, it is a possibility that this polyphenol failed to decrease the 3-NP-induced neuronal lesion. Magnified pictures of the striatum from 3-NP and 3-NP+quercetin also showed more or less same number of surviving cells.

The other aspect of HD is severe anxiety, depression, and irritability [31,32]. It is found that anxiety and depression in stage 2 HD patients is more when compared to the stage 1, which gradually increases in severity in the later stages [32]. The patient's inability to adapt to the increasing motor problems and loss of independence and self-reliance may contribute to this situation. Severe neurochemical changes resulting from progressive neurodegeneration that take place during the disease progression may also be contributory. Brain serotonergic function is known to regulate the level of anxiety and depression, and serotonin transporter blockers are well recognized antidepressants [33,34]. R6/1 HD mice showed depression related behavior and it was found that hippocampus and cortex showed less 5-HT receptor expression [35]. 5-HT turnover was also found to be increased in asymptomatic R6/2 HD mice [36]. In HD patients reduced 5-HT binding with receptors is reported together with an increased blood MAO activity [37]. In our study, we found no change in striatal 5-HT levels following 3-NP treatment, but significantly enhanced 5-HT turnover and 5-HIAA levels. The possibility of this steady level of 5-HT could be because of increased serotonin production by the neurons as a compensatory mechanism to reduce the effect of higher breakdown. Quercetin treatment significantly reduced the 5-HIAA level and 5-HT turnover, as compared to the 3-NP group, clearly resulting from its inhibitory effect on MAO-A activity, as seen in the present *in vitro* and *in vivo* studies. Interestingly, 3-NP treatment seems to have little effect on MAO-A, whereas along with quercetin it showed heightened inhibitory activity. MAO-A inhibitory action of quercetin is reported in literature [38,39]. It was found to be a competitive inhibitor of the enzyme MAO-A, which is more potent against MAO-A when compared to MAO-B [39]. We also did not get any clear indication whether quercetin is able to block MAO-B, as the total MAO activity after 3-NP and 3-NP+quercetin was not significantly different, though both were less than the control, and quercetin treatment blocked MAO-A activity. So, the loss of total MAO activity in 3-NP+quercetin group could be a cumulative effect of 3-NP (blockage of MAO-B) and quercetin (blockage of MAO-A). The foregoing account clearly suggests that the anxiolytic effect of quercetin observed in the present elevated plus maze study could be deriving from its MAO-A inhibitory action, and the resulting normal maintenance of brain serotonergic metabolism in the striatum.

Immunohistochemistry showed profound gliosis in the striatum of 3-NP-treated rats. There was a profound increase in the number of astrocytes and microglia compared to the saline-treated brain, which is in accordance with the earlier studies [12,30]. We were particularly interested to see the effect of quercetin on microglial activation in 3-NP model, because quercetin is reported to possess antiinflammatory activity [40,41], and therefore its treatment may reduce microglial activation. It was found that some of the MAO inhibitors have some impact on the glial population, like safinamide which is a MAO-B inhibitor with the capability of blocking sodium channels as well as free radical scavenging action [42]. Administration of this drug was found to reduce microglial activation. Another MAO inhibitor, phenelzine was reported to protect neurons and astrocytes from formaldehyde induced toxicity by enhancing glutamate transport [43], but by activating microglial proliferation [44]. So, the impact of the MAO inhibitors on glial population vary, and mostly dependent on the associated properties of the molecule. The most likely mechanism by which quercetin exerts it antiinflammatory effect could be by activating the AMP-activated protein kinase pathway, which reduces CD11b expression and down-regulate iNOS in high cholesterol fed mice [40]. Quercetin also decreased IL-1β, IL-6, and TNF-α expression in these mice [40]. As expected our study showed a perceptible decrease in the number of microglia in the striatal lesion core, although some remained. GFAP positive cells remained at the periphery of the lesion core in the striatum of 3-NP-treated rats, but quercetin treatment caused infiltration of astrocytes inside the lesion core. Very few astrocytes remaining in the striatum may further aggravate the disease in 3-NP-treated rats, but quercetin may help to deliver growth factors released by the astrocytes in the lesion core, to support the (remaining?) neuronal population within the lesion center.

Conclusion

There are some far-reaching implications of our study in the treatment of HD. First, quercetin can be a supporting therapy for improving the motor coordination, locomotor functions, and anxiety. According to the study by Paulsen et al. [32], more than 50% of HD patients suffer from severe anxiety and depression and the percentage of suicide attempt is 14. Most commonly used drugs, such as tetrabenazine, haloperidol, and valproic acid, target DA receptors or DA transporters, and provide behavioral relief. However, these are with side effects like severe drowsiness, insomnia, anxiety, and depression [45,46]. None of these molecules improves anxiety, depression, and irritability, which are the common problems in HD. Therefore, quercetin could be used along with the dopamine antagonists to improve the anxiety-related complexities in the treatment of HD. Furthermore, our result on its antiinflammatory effects, specifically on the microglial inactivation suggests its additional benefit. This is relevant, since Cicchetti et al. [47] have found that transplanted neurons in human HD brains die in a faster rate than the host cells mainly because of microglial cuffing even when immunosuppressant are administered. The effect of quercetin on motor disabilities suggests that it may elicit beneficial effect on HD when supplemented with other neuroprotectants as was also found with coenzyme Q_{10}. This molecule protected against the behavior and neurochemical abnormalities in transgenic HD mice when supplemented with other neuroprotectants [48–51].

Acknowledgments

J.C., R.S., D.D., and A.N. are Senior Research Fellows of the Council of Scientific and Industrial Research (CSIR), Government of India. The study was partially funded by the Neurodegenerative Diseases: Causes and Corrections (miND; Grant # BSC-0115),

funded by the CSIR as one of the planned projects under 12th FYP. A part of the work is financed by the major laboratory program of CSIR-IICB (MLP-0115).

Conflict of Interest

The authors declare no conflict of interest.

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