Quercetin supplementation is effective in improving mitochondrial dysfunctions induced by 3-nitropropionic acid: Implications in Huntington's disease

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ABSTRACT

The study was designed to investigate the beneficial effect of quercetin supplementation in 3-nitropropionic acid (3-NP) induced model of Huntington's disease (HD). HD was induced in rats by administering sub-chronic dose of 3-NP, intraperitoneally, twice daily for 17 days. Quercetin was supplemented at a dose of 25 mg/kg body weight by oral gavage for 21 days. At the end of treatment, mitochondrial bioenergetics, mitochondrial swelling, oxidative stress, neurobehavioral deficits and histopathological changes were analyzed. Quercetin supplementation was able to reverse 3-NP induced inhibition of respiratory chain complexes, restore ATP levels, attenuate mitochondrial oxidative stress in terms of lipid peroxidation and prevent mitochondrial swelling. Quercetin administration also restored the activities of superoxide dismutase and catalase along with thiol content in 3-NP treated animals. Beneficial effect of quercetin administration was observed on 3-NP induced motor deficits analyzed by narrow beam walk and footprint analysis. Histopathological analysis of 3-NP treated rats revealed pyknotic nuclei and astrogliosis in striatum, which were reduced or absent in quercetin supplemented animals. Altogether, our results show that quercetin supplementation to 3-NP induced HD animals ameliorated mitochondrial dysfunctions, oxidative stress and neurobehavioral deficits in rats showing potential of this flavonoid in maintaining mitochondrial functions, suggesting a putative role of quercetin in HD management.

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1. Introduction

Huntington's disease (HD) is a progressive, fatal, neurodegenerative disorder caused by an expanded CAG repeat in the huntingtin gene (Htt), that encodes for an abnormally long polyglutamine tract in a protein termed huntingtin (htt) with a molecular weight of approximately 350 kDa [1]. The disease is inherited in an autosomal dominant manner with age-dependent penetrance. Clinical features of HD include progressive motor dysfunctions, cognitive decline, and psychiatric disturbances, including both neuronal dysfunctions and neuronal cell death [2]. Although, mutation in Htt gene was discovered more than 17 years ago, the role of Htt in the physiology and the pathophysiology is still under investigation [3]. Recent data indicates that the translocation of mHtt into nucleus and transcriptional dysregulation likely play an important role in the pathogenic process and more specifically these events have a significant impact on mitochondrial functions such as electron transport chain (ETC) and reactive oxygen species (ROS) generation leading to bioenergetic failure [4]. Numerous studies in cell and mouse models of HD have revealed mitochondrial impairment [5].

The hypothesis that mitochondrial dysfunctions contribute to the pathogenesis of HD was first tested pharmacologically by using 3-nitropropionic acid (3-NP), an irreversible inhibitor of succinate dehydrogenase [6]. One of the mechanisms following 3-NP administration is the development of mitochondrial dysfunctions leading to generation of a bioenergetic defect which involves three interacting processes such as: energy impairment, oxidative stress and excitotoxicity [7]. 3-NP induced HD model replicates most of the clinical and pathophysiological hallmarks of HD, including spontaneous choreiform and dystonic movements, frontal-type cognitive deficits, and progressive striatal neuronal degeneration [8]. 3-NP administration also results in ATP depletion, which impairs intracellular calcium buffering thereby leading to production of damaging ROS [9].

At present, there are no effective treatments against HD. Current therapies for treating HD are symptomatic; focusing on neurological and psychiatric symptoms that aim at improving the quality of life [10]. Attention has been given on the influence of phytochemical therapeutics on health and mental well-being. Evidence has indicated that a group of plant-derived compounds known as flavonoids exerts particularly powerful action as cardioprotective, chemopreventive and neuroprotective agents [11]. The biological activities of flavonoids have been attributed to their antioxidant, anti-inflammatory and their property to modulate signaling cascades [12]. Within the flavonoid family, quercetin is the most potent scavenger of ROS and

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Abbreviations: 3-NP, 3-nitropropionic acid; CAT, catalase; DTNB, 5,5-dithiobis-2-nitrobenzoic acid; ETC, electron transport chain; HD, Huntington's disease; LMW-SH, low molecular weight thiols; MDA, malondialdehyde; NBT, nitroblue tetrazolium; PSH, protein thiols; ROS/RNS, reactive oxygen and nitrogen species; SOD, superoxide dismutase; SDH, succinate dehydrogenase; TSH, total thiols.
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reactive nitrogen species (RNS). This can be explained by the presence of two antioxidant pharmacophores within the molecule that have the optimal configuration for free radical scavenging [13]. Quercetin has been shown to easily traverse the blood–brain-barrier and acts as promising agents for intervention in neurodegenerative conditions like ischemia [14], Alzheimer's disease [15] and Parkinson's disease [16]. Use of neuroprotective antioxidants is being considered as a promising approach to slow down the disease progression and to limit the extent of functional neuronal loss in chronic neurodegeneration as well as after acute lesions of the brain. However, only a few studies on the use of antioxidants in the management of neurodegenerative conditions have so far been undertaken. Methodological inconsistencies, poor permeation of the blood–brain barrier and lower efficacy per dose are some reasons for the lack of studies in this area [17]. Quercetin exerts its beneficial effect in brain primarily through its antioxidant action. Quercetin has been shown to improve mitochondrial functions in brain by increasing mitochondrial biogenesis [18]. Within the subcellular compartment quercetin shows preferential accumulation in mitochondria [19]. Based on the information in the literature, quercetin appears to be promising agent against HD. Therefore, in the present study, we have evaluated the neuroprotective potential of quercetin against 3-NP induced mitochondrial oxidative stress, mitochondrial dysfunctions and neurobehavioral deficits.

2. Experimental procedures

2.1. Chemicals

All the chemicals used in the present study were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, USA), Merck (Mumbai, India) and Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). Quercetin was purchased from Himedia Laboratories Pvt. Ltd. (Mumbai, India).

2.2. Animals and treatment schedule

Female Wistar rats aged 9–10 weeks, weighing between 200 and 250 g were procured from the Central Animal House facility of Panjab University, Chandigarh, India. The animals were allowed to acclimatize to the local vivarium for 7 days. All the experiments were carried out between 09:00 and 15:00 h. The protocols followed were approved by the Institutional Animal Ethics Committee of the University and were in accordance with the guidelines for humane use and care of laboratory animals. The animals were randomly segregated into the following four groups with each group having 6–8 animals.

Control (vehicle): animals were given vehicle.
Control + quercetin treated: animals were administered with vehicle for 3-NP and quercetin at a dose of 25 mg/kg through oral gavage for 21 days.
3-NP treated: animals were administered 3-NP at a sub-chronic dose twice a day intraperitoneally for 17 days. Twice a day injections were: 7.5 mg/kg for the first 2 days, followed by 3.75 mg/kg for next 7 days, finally a dosage of 2 mg/kg for the last 8 days. The dose of 3-NP used in the study is based on the doses reported in literature and were standardized in our laboratory [20].
3-NP + quercetin treated: One hour before sub-chronic 3-NP treatment, animals were administered with quercetin at a dose of 25 mg/kg by oral gavage for 21 days.

2.3. Mitochondrial respiratory chain enzymes

2.3.1. Isolation of rat brain mitochondria

On day 21, animals were sacrificed by decapitation under mild ether anesthesia. Mitochondria were isolated from striatum by the method described by Puka-Sundvall [21]. Briefly, corpus striatum was dissected, rinsed in ice-cold isotonic saline and homogenized in ice-cold extraction buffer (10 mM Tris–HCl, pH 7.4, 0.44 M sucrose, 10 mM EDTA and 0.1% BSA). The homogenate was centrifuged at 2100 g for 15 min at 4 °C. The pellet was discarded and the supernatant re-centrifuged at 14,000 g for 15 min at 4 °C. The crude mitochondrial pellet was separated, washed with extraction buffer and centrifuged at 7000 g for 15 min at 4 °C. The final mitochondrial pellet was re-suspended in buffer containing 0.44 M sucrose in 10 mM Tris–HCl, pH 7.4.

2.3.2. NADH dehydrogenase (complex I)

Activity of NADH dehydrogenase was measured as described by King and Howard [22]. Requisite amount of mitochondrial preparation was added to the reaction mixture containing 0.2 M glycyl-glycine (pH 8.5), 6 mM NADH, 1 mM oxidized cytochrome c and 0.02 M $NaHCO_3$. NADH dehydrogenase catalyzed reduction of cytochrome c and the increase in absorbance was followed spectrophotometrically at 550 nm for 3 min. Results were expressed as nmol NADH oxidized/min/mg protein. The enzyme activity was normalized to citrate synthase activity.

2.3.3. Succinate dehydrogenase (complex II)

Activity of succinate dehydrogenase was assayed according to the method of King et al. [23]. The reaction mixture contained 0.2 M sodium phosphate buffer (pH 7.8), 1% (w/v) BSA, 0.6 M succinate and 0.03 M potassium ferricyanide. The reaction was initiated by addition of requisite amount of mitochondrial preparation. Succinate dehydrogenase catalyzes the oxidation of succinate to fumarate by potassium ferricyanide, which was measured spectrophotometrically by decrease in absorbance at 420 nm for 3 min. Results were expressed as nmol succinate oxidized/min/mg protein. The enzyme activity was normalized to citrate synthase activity.

2.3.4. Cytochrome oxidase (complex IV)

Activity of cytochrome oxidase was assayed according to the method described by Sottocasa et al. [24]. Oxidized cytochrome c was reduced by adding few crystals of sodium borohydride and then neutralized to pH 7.0 by 0.1 M HCl. 0.3 mM of reduced cytochrome c was added to 0.075 M phosphate buffer and the reaction was initiated by mixing appropriate amount of mitochondrial suspension. The reduced cytochrome c is oxidized in the reaction mixture containing cytochrome oxidase which is measured spectrophotometrically by a decrease in absorbance at 550 nm for 3 min. Results were expressed as nmol cytochrome c oxidized/min/mg protein. The enzyme activity was normalized to citrate synthase activity.

2.3.5. F_1F_0 synthase (complex V)

Mitochondrial F_1F_0 synthase activity was measured as described by Griffiths and Houghton [25]. Reaction was started by adding appropriate amount of mitochondrial suspension in ATPase buffer [50 mM Tris and 5 mM $MgCl_2$, pH 7.5] at 37 °C with 5 mM ATP for 10 min. The reaction was stopped by adding 10% (w/v) trichloroacetic acid. The contents were centrifuged at 3000 g for 20 min, and an appropriate volume of supernatant was mixed with water. Phosphate produced was measured by the method of Fiske and Subbarow [26]. Results were expressed as nmol of ATP hydrolyzed/min/mg protein. The enzyme activity was normalized to citrate synthase activity.

2.3.6. MTT reduction

The reduction of MTT to blue formazan by dehydrogenases present in the mitochondrial suspension was also monitored to assess mitochondrial functions [27]. To appropriate mitochondrial pellet, MTT (0.1 mg/ml) was added, mixed and incubated at 37 °C for 30 min and then centrifuged to obtain formazan pellet. The pellet was dissolved in absolute ethanol and the mixture was re-centrifuged at 2000 g for 10 min. The absorbance of the supernatant was measured at 595 nm.

Results were expressed as µg formazan formed/min/mg protein and the values were normalized to citrate synthase activity.

2.3.7. Citrate synthase assay

Citrate synthase (CS) was assayed as described by Coore et al. [28], wherein the reduction of 0.2 mM 5,5′-dithio-bis(2-nitrobenzoic acid) in the presence of 0.2 mM acetyl-CoA and 0.1 mM oxaloacetate in a medium with 100 mM Tris–HCl, pH 8.0 and 0.1% Triton X-100 was performed at 412 nm. The CS activity was expressed as nmol/min/mg protein.

2.3.8. Mitochondrial swelling

Mitochondrial swelling and contraction by measurement of light scattering in a spectrophotometer were used as a functional test of mitochondrial membrane integrity as described by Tedeshi and Harris [29]. This method is based upon the increased absorbance of mitochondria in a contracted state and decreased density in a swollen or orthodox configuration due to cation influx that can be detected by measurement of light scattering at 520 nm. To appropriate mitochondrial suspension, 0.12 M KCl in 0.02 M Tris–HCl was added and mitochondrial swelling was measured at 520 nm for 6 min. Contraction of mitochondria was initiated after 6 min by adding Mg^{2+}-ATP. Change in absorbance at 520 nm was normalized by the protein concentration.

2.3.9. Adenine nucleotide levels

ATP and ADP levels were determined by the method of Victor et al. [30]. Frozen tissues were transferred to a 1.5-ml micro-centrifuge tube and added with 0.4 M perchloric acid. The tissue was immediately homogenized with a pestle and the homogenate was kept on ice for 30 min, and then centrifuged at 14,000 rpm at 4 °C. The supernatant was neutralized with 4 M K_2CO_3, kept on ice for 10 min and at −80 °C to promote precipitation of the perchlorate, and then centrifuged again. Supernatants were then stored at −80 °C until HPLC assay. Aliquots of the extract and standard were applied separately to the column and the eluate was monitored at 254 nm, at a flow rate of 2 ml/min using supelcosil LC-18, 5 µm (15 cm∗0.46 cm) reversed phase column (Supelco, Crans, Switzerland). An isocratic elution of the samples was performed with 0.52 µm potassium phosphate buffer (KH_2PO_4; pH 4.0), containing 0.04% tetrabutylammonium phosphate and 1.25% (v/v) methanol. Peaks were identified by their retention times and by using cochromatography with standards. Results were expressed as nmol/mg protein.

2.4. Mitochondrial oxidative stress

2.4.1. Lipid peroxidation

Malondialdehyde (MDA), a measure of lipid peroxidation was quantified by reaction with thiobarbituric acid at 532 nm by the method of Ohkawa et al. [31]. The values were expressed as nmol MDA/mg protein, using molar extinction coefficient of chromophore $(1.56 \times 10^5 M^{-1} cm^{-1})$.

2.4.2. Superoxide dismutase (SOD)

SOD activity was assayed in mitochondrial preparation, wherein the inhibition of nitrozo blue tetrazolium (NBT) reduction was measured at 560 nm [32]. Briefly, the reaction was initiated by the addition of hydroxylamine hydrochloride to the reaction mixture containing NBT and sample. The results were expressed as Units/mg protein, where one unit of enzyme is defined as the amount of enzyme inhibiting the rate of reaction by 50%.

2.4.3. Catalase

Catalase (CAT) activity was assayed in mitochondrial suspension by the method of Aebi [33]. The decomposition of hydrogen peroxide (H_2O_2) by CAT was monitored spectrophotometrically by following the decrease in absorbance at 240 nm. The activity of enzyme was expressed as nmol of H_2O_2 decomposed/min/mg protein, using molar extinction coefficient of H_2O_2 (71 $M^{-1} cm^{-1}$).

2.4.4. Thiols

2.4.4.1. Total thiols.
Total thiols (TSH) were quantified in the mitochondrial preparation according to the method of Sedlak and Hanus [34]. In this method, DTNB is reduced by protein and non-protein free −SH groups to form 1 mol of 5-mercapto-2-nitrobenzoate per mol of −SH. Absorbance was read at 412 nm and the results were expressed as nmol TSH/mg protein.

2.4.4.2. Low molecular weight thiols (LMW-SHs).
LMW-SHs (probably GSH) were estimated in the mitochondrial preparation by the method of Roberts and Francetic [35]. Absorbance was measured at 412 nm and results were expressed as nmol LMW-SH/mg protein.

2.4.4.3. Protein thiols.
Levels of protein thiols (P-SHs) were calculated from the difference between the values of total thiols and LMW-SHs and expressed as nmol P-SH/mg protein.

2.5. Estimation of protein

The protein content was estimated according to the method of Lowry et al. [36].

2.6. Neurobehavioral studies

2.6.1. Locomotor activity

The locomotor activity was measured using actophotometer [37]. The interruption of a beam of light falling on a photocell following the movement of the animal was recorded. Each rat was placed individually in the actophotometer for 3 min and numbers of counts were recorded.

2.6.2. Narrow beam walk test

This behavioral test was used to evaluate motor performance in the treated animals vs. the controls, by progressively increasing the difficulty in the execution of the task as described by Henderson et al. [38]. The animals were trained in crossing a 150 cm long wooden beam, divided into three 50 cm segments, from a platform at one end to the animal's home cage at the other end, placed horizontally 60 cm above the floor. The number of paw slips onto an under-hanging ledge and the time taken to traverse the beam was recorded. The maximum time allowed for the task was 2 min. Occurrence of bradykinesia was quantified by calculating the average velocity of walking for treated and control animals.

2.6.3. Footprint analysis

This test was used to assess gait abnormalities in 3-NP treated animals [20]. After coating the hind feet with a non-toxic green dye and forepaws with a non-toxic red dye, rats were allowed to walk on a beam (100 cm length, 12 cm breadth and 10 cm high walls with an inclination of 30°) leading to a darkened enclosure. The gangway was lined with white paper for recording the feet impressions. Animals in all the groups were tested for footprint length, footprint breadth, and footprint stride length for both left and right paws. Specifically, footprint stride length was quantified as the distance between two subsequent feet. Paws overlap analysis was carried out by measuring the center distance between the anterior paw and rear paw footprints.

2.7. Histochemical and histological analysis

The frozen brain sections were processed for SDH histochemical staining [39]. Animals were anesthetized and transcardially perfused

Table 1
Effect of quercetin administration on the activity of mitochondrial complexes in striatum of 3-NP treated rats.

	Complex I (nmol NADH oxidized/min/mg protein)	Complex II (nmol succinate oxidized/min//mg protein)	Complex IV (nmol cytochrome C oxidized/min/mg protein)	F_1F_0 ATPase (nmol ATP hydrolyzed/min/mg protein)	MTT reduction (μg formazan formed/min/mg protein)
Control	109.23 ± 13.32	147.61 ± 4.54	162.58 ± 6.08	17.69 ± 1.69	5.46 ± 0.2
	(131.6 ± 8.49)	(177.74 ± 4.38)	(195.82 ± 8.19)	(21.22 ± 3.78)	(6.57 ± 0.5)
Quercetin	106.9 ± 16.19	145.9 ± 8.42	145.57 ± 2.77	17.45 ± 1.2	4.56 ± 0.38
	(116.9 ± 11.84)	(159.4 ± 14.11)	(158.65 ± 8.5)	(19.0 ± 3.55)	(4.97 ± 0.7)
3-NP	60.05 ± 7.83[*]	71.37 ± 3.33[*]	112.52 ± 3.28[*]	11.7 ± 0.53[*]	1.64 ± 0.17[*]
	(15.6 ± 1.04)[*]	(18.47 ± 1.95)[*]	(29.39 ± 2.24)[*]	(3.06 ± 0.7)[*]	(0.42 ± 0.02)[*]
3-NP + quercetin	99.11 ± 10.25[†]	91.56 ± 1.05[†]	126.96 ± 3.25[†]	16.96 ± 0.96[†]	2.7 ± 0.1[†]
	(38.91 ± 2.08)[†]	(36.0 ± 2.33)[†]	(49.86 ± 3.76)[†]	(6.47 ± 1.38)[†]	(1.05 ± 0.12)[†]

Values in parenthesis are normalized to citrate synthase activity. All values are expressed as mean ± SEM; n = 6/group.
[*] Significantly different from control group (p < 0.05).
[†] Significantly different from 3-NP treated group (p < 0.05).

with cold 0.1 M PBS, pH 7.4, followed by cold 10% (v/v) glycerol in PBS. Twenty micron thick frozen sections of the brain were dried for 30 min, activated in PBS at 37 °C for 10 min and then incubated with reaction mixture containing 0.3 M NBT, 0.05 M phosphate buffer, pH7.4, and 0.05 M sodium succinate at 37 °C in dark for 30 min. At the end of the reaction, sections were extensively washed with the reaction buffer, examined under the microscope and photographed.

Animals for histology were perfused transcardially with cold saline followed by phosphate buffered 4% paraformaldehyde. The brains were then post-fixed overnight in 4% paraformaldehyde and later brain sections were processed for routine hematoxylin and eosin staining [40].

2.8. Statistical analysis

All values are expressed as mean ± standard error of mean (SEM) of six animals per group. Data was analyzed using one way analysis of variance (ANOVA) followed by Newman–Keuls test for multiple pair-wise comparisons between the various treated groups using SPSS 14 software. Values with p < 0.05 were considered as statistically significant.

3. Results

3.1. Effect of quercetin on mitochondrial electron transport chain enzymes, MTT reduction and mitochondrial swelling

Activities of mitochondrial electron transport chain (ETC) enzymes were found to be significantly inhibited by 3-NP administration (Table 1). NADH dehydrogenase activity was inhibited by 45.01% in 3-NP treated animals as compared to control animals. The activity was increased by 39.4% on quercetin supplementation to 3-NP treated animals. Activity of succinate dehydrogenase (complex-II) was found to be significantly lowered by 51.64% in 3-NP treated group as compared to control group. Supplementation with quercetin increased complex-II activity by 22.05% in 3-NP treated animals. Histochemical staining for SDH activity revealed a significant decrease of the enzyme reaction in the striatum of 3-NP treated animals (Fig. 1, C & III) as compared to the control animals (Fig. 1, A & I). Quercetin supplementation alone had SDH staining comparable to that of control striatal tissue section (Fig. 1, B & II). The SDH staining recovered in brain sections on quercetin supplementation to 3-NP treated animals (Fig. 1, D & IV). Activity of terminal enzyme of mitochondrial ETC, cytochrome oxidase was inhibited by 30.79% in 3-NP treated group as compared to control
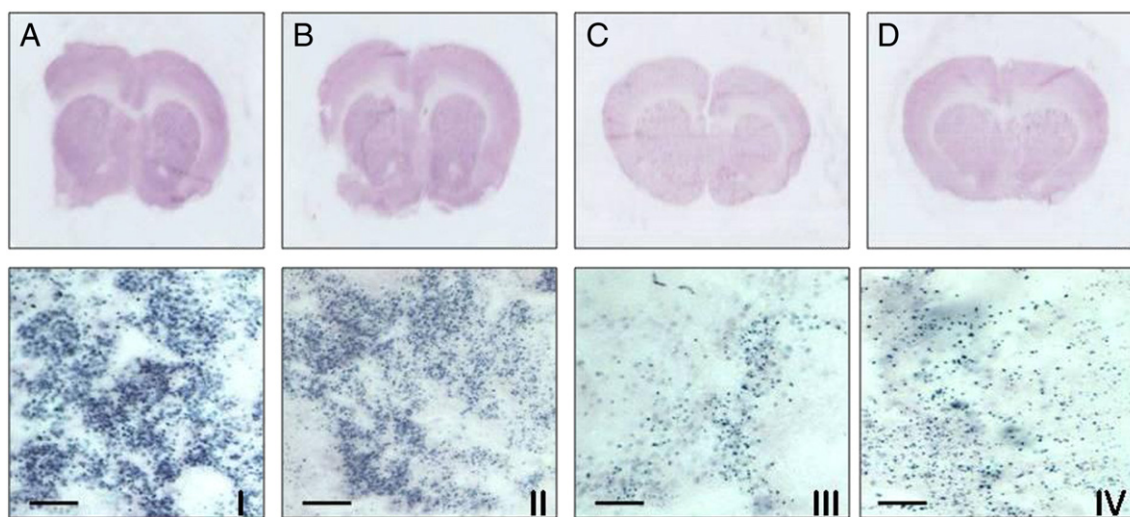

Fig. 1. Effect of quercetin supplementation on SDH histochemical staining in frozen brain sections of 3-NP treated rats. Frozen brain sections were visualized under light microscope at a magnification of 40×. Control sections show dense staining for normal SDH functioning (A & I). Histological appearance of striatal tissue from rats supplemented with quercetin alone shows SDH functioning similar to that of control animals (B & II). SDH functioning was compromised in 3-NP induced rats (C & III). Quercetin + 3-NP administered groups showing improved SDH functioning (D & IV) (scale bar—40 μm).
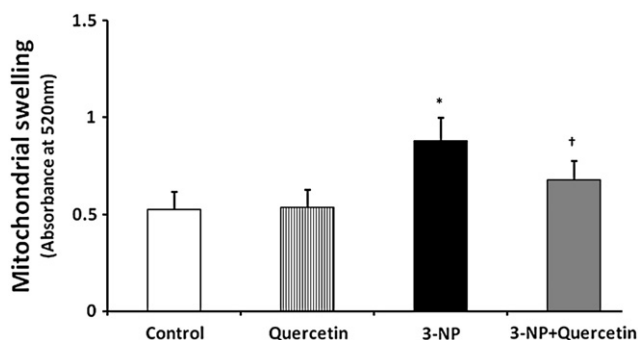
Fig. 2. Effect of quercetin supplementation on mitochondrial swelling in 3-NP treated rats. Values are expressed as mean ± SEM; n = 6/group. * Significantly different from control group (p<0.05), † significantly different from 3-NP treated group (p<0.05).

group. The activity was increased by 11.37% in 3-NP treated animals supplemented with quercetin. Activity of mitochondrial F_1-F_0 synthase (complex-V) was assessed by hydrolysis of ATP to ADP and was found to be inhibited by 33.86% in 3-NP treated group as compared to control group. On quercetin supplementation, the activity was increased by 31.01% in 3-NP treated animals. Mitochondrial functioning was also assayed using MTT reduction (Table 1). MTT reduction was found to be significantly inhibited by 69.96% in 3-NP treated animals as compared to control animals which was increased by 39.25% in 3-NP + quercetin co-supplemented animals. The perturbed activity of ETC enzymes led to the increased mitochondrial swelling in 3-NP treated animals which on quercetin supplementation was found to be lowered (Fig. 2). The data in Table 2 depicts levels of ATP and ADP in striatum following 3-NP and quercetin administration. It is clear from the data that the compromised ETC components following 3-NP administration resulted in reduced levels of ATP with a concomitant increase in ADP levels thereby lowering the ATP/ADP ratio. The effect was reversed on quercetin administration and the ATP/ADP ratio was restored to near controls.

3.2. Effect of quercetin on mitochondrial lipid peroxidation, superoxide dismutase and catalase activity

The data for lipid peroxidation, activity of superoxide dismutase and catalase in mitochondria isolated from striatum are depicted in Table 3. Lipid peroxidation was significantly increased (54%) in 3-NP treated as compared to controls. Quercetin supplementation to 3-NP treated animals lowered mitochondrial lipid peroxides by 38.22% as compared to 3-NP group. The activity of superoxide dismutase (SOD) was inhibited by 32.84% in 3-NP treated animals as compared to control animals. However, quercetin supplementation significantly increased SOD activity by 14.73% in 3-NP animals. Catalase activity was found to be significantly inhibited by 53.48% in 3-NP treated animals as compared to control animals. Quercetin supplementation resulted in a significant increase in catalase activity by 28.6% in 3-NP treated animals.

Table 2
Effect of quercetin administration on adenine nucleotides in striatum of 3-NP treated rats.

	ATP (nmol/mg protein)	ADP (nmol/mg protein)	ATP/ADP ratio
Control	9.15 ± 1.41	4.24 ± 0.39	2.16
Quercetin	10.01 ± 1.29	4.87 ± 0.73	2.07
3-NP	4.83 ± 1.47*	7.87 ± 0.42*	0.61
3-NP + quercetin	8.81 ± 1.06†	4.94 ± 0.38†	1.78

Values are expressed as mean ± SEM; n = 6.
* Significantly different from control group (p<0.05).
† Significantly different from 3-NP treated group (p<0.05).

Table 3
Effect of quercetin administration on lipid peroxidation, superoxide dismutase and catalase activity in mitochondria isolated from striatum of 3-NP treated rats.

	Lipid peroxidation (nmol MDA/mg protein)	Superoxide dismutase (Units/mg protein)	Catalase (μmol of H_2O_2 decomposed/min/mg protein)
Control	4.09 ± 0.37	4.42 ± 0.21	2.07 ± 0.17
Quercetin	4.21 ± 0.48	3.81 ± 0.13	1.69 ± 0.06
3-NP	7.91 ± 0.82*	2.99 ± 0.1*	0.96 ± 0.03*
3-NP + quercetin	5.93 ± 0.31†	3.51 ± 0.07†	1.34 ± 0.11†

Values are expressed as mean ± SEM; n = 6.
* Significantly different from control group (p<0.05).
† Significantly different from 3-NP treated group (p<0.05).

3.3. Effect of quercetin on mitochondrial thiols

One of the most important antioxidant present in mitochondria is thiol and its levels were found to be compromised in mitochondria isolated from the striatum of 3-NP treated animals (Fig. 3). Total thiol levels were found to be significantly reduced by 54.22% in 3-NP treated animals as compared to control animals. Quercetin supplementation resulted in increased levels of total thiols (37.46%) in 3-NP treated animals. A significant decrease in low molecular weight (51.39%) and protein thiols (55.03%) was observed in 3-NP treated animals as compared to controls. Quercetin supplementation significantly increased the low molecular weight and protein thiols by 34.52% and 38.32% respectively in 3-NP treated animals.

3.4. Effect of quercetin on neurobehavioral deficits

3.4.1. Locomotor activity

The locomotor activity was assessed in terms of photo beam counts for duration of 180 s using actophotometer (Fig. 4). On day 0, animals in all the four groups had an average count of 166.5. On day 1, the numbers of counts for 3-NP treated animals fell to an average of 125, suggesting a significant impairment in locomotor functions. However, quercetin supplementation to 3-NP treated animals increased the average number of counts to 140. This trend of decline in locomotor functions in 3-NP treated animals was followed for days 7, 14 and 21 following 3-NP treatment with an average of 70, 51 and 80 counts respectively. Quercetin supplementation was able to significantly improved locomotor functions by recording an average of 134, 145 and 150 counts respectively for days 7, 14 and 21 in 3-NP + quercetin animals.
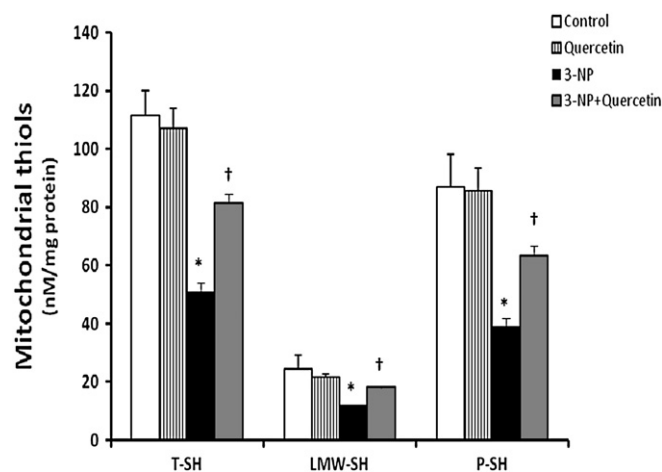

Fig. 3. Effect of quercetin supplementation on mitochondrial total, low molecular weight (LMW) and protein thiols in mitochondria isolated from striatum of 3-NP treated rats. Values are expressed as mean ± SEM; n = 6/group. * Significantly different from control group (p<0.05), † significantly different from 3-NP treated group (p<0.05).
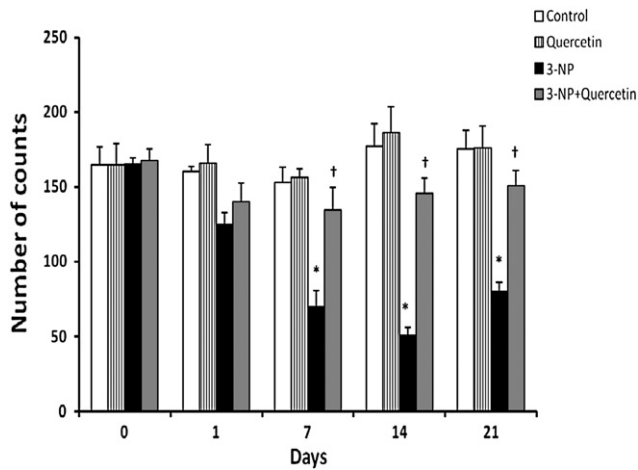
Fig. 4. Effect of quercetin supplementation on locomotor functions assessed using actophotometer in terms of number of counts of 3-NP treated animals. Values are expressed as mean ± SEM; n = 6/group. * Significantly different from control group (p<0.05), † significantly different from 3-NP treated group (p<0.05).

3.4.2. Narrow beam walk test

Narrow beam walk test was used to assess hind-limb impairment, wherein the time taken by the rats to walk across a narrow beam with progressively decreasing width was recorded (Fig. 5A). The maximum time allowed to each animal for traversing the beam was 120 s. On day 0, average time taken by animals in all the four groups was 5.75 s. On day 1 following 3-NP treatment, the average time recorded by 3-NP treated animals increased to 12 s, indicating a significant impairment in hind-limb function. Quercetin supplemented animals on the other hand took an average of 9 s. Hind-limb impairment following 3-NP administration continued to affect the animals having an average of 14, 82 and 32 s on days 7, 14 and 21 respectively. Animals supplemented with quercetin were able to maintain hind-limb function and traversed the beam in less time by recording an average time of 12, 35 and 13 s respectively for days 7, 14 and 21 each, in 3-NP + quercetin group. The results indicate that quercetin administration was effective in improving motor impairments induced by 3-NP.

The average velocity to cross the beam and the number of paw slips for rats in treated groups were also calculated (Fig. 5B & C). On day 0, average velocity by animals in all the four groups was 25 cm/s, whereas the average number of paw slips was 0.125. On day 1, 3-NP treated animals recorded an average velocity of 13 cm/s and the average number of paw slips increased to 1.25. However, quercetin supplementation to 3-NP treated animals increased the average velocity to 17 cm/s and the average number of paw slips was 0.6. Average velocity further decreased to 11 and 2 cm/s on days 7 and 14 with an increase in average number of paw slips to 2.1 and 7.3 respectively. But later on day 21, average velocity increased to 5 cm/s and average paw slips to 2.25 in 3-NP treated animals. Quercetin supplementation was able to maintain the average velocity to 12 and 6 cm/s and the average number of paw slips to 0.7 and 0.6 on days 7 and 14, which were further significantly increased to 11.5 cm/s with a decrease in average number of paw slips to just 0.5 in 3-NP + quercetin animals.

3.4.3. Effect of quercetin on footprint analysis

Paw slips are interpreted as a sign of abnormal gait. To further confirm abnormalities in treated rats, a footprint analysis was performed daily from day 0 through day 21 (Fig. 6). Animals in all the groups
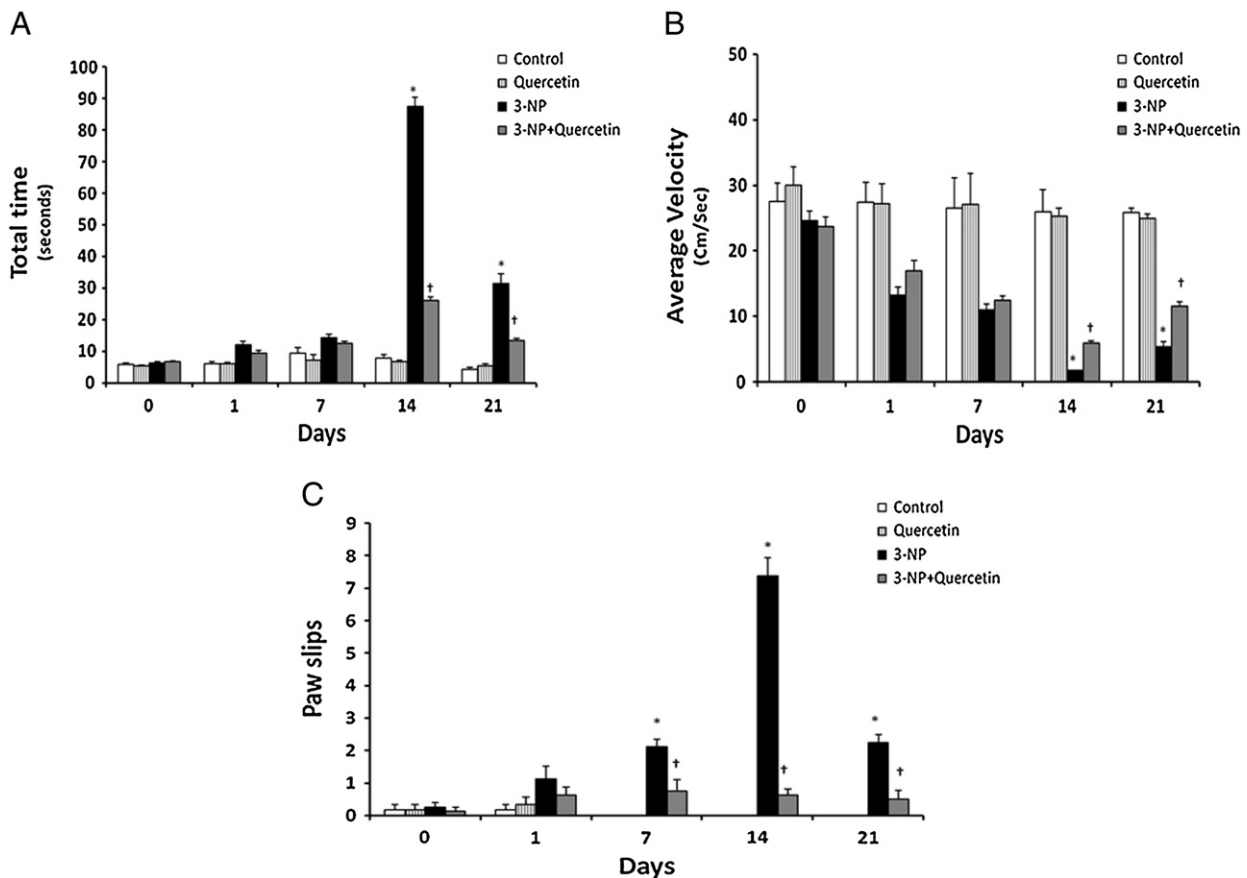

Fig. 5. Effect of quercetin supplementation on narrow beam walk test in terms of total time (A), average velocity (B) and number of paw slips (C) of 3-NP treated animals. Values are expressed as mean ± SEM; n = 6/group. * Significantly different from control group (p<0.05), † significantly different from 3-NP treated group (p<0.05).
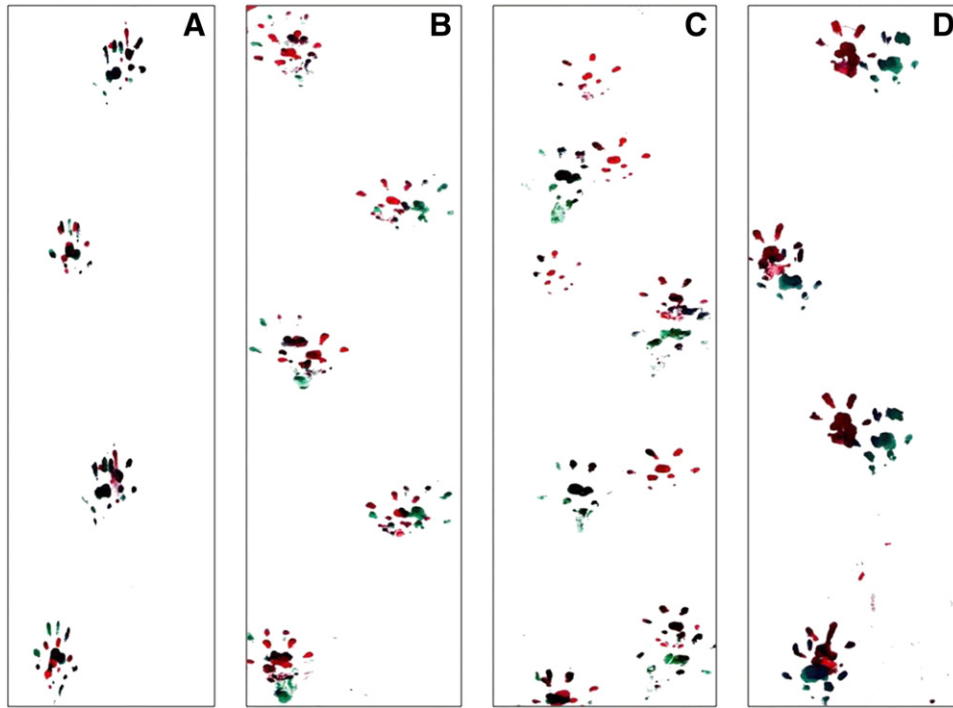
Fig. 6. Effect of quercetin supplementation on footprint test for gait analysis by control (A), quercetin (B), 3-NP (C) and 3-NP + quercetin (D) treated animals.

were tested for footprint length, footprint breadth and footprint stride length for both left and right paws. Out of six parameters that were analyzed only the stride length for both, left and right paws was significantly affected in the animals that received 3-NP + quercetin supplementation in comparison to 3-NP treated animals (data not shown). However, the footprint length and footprint breadth for both left and right paws remained unaffected (data not shown). An additional measurement of walking pattern is the paws overlap which was calculated as the minimum distance (in cm) between left and right paws overlap (Fig. 7). The rationale of this analysis lies on the fact that rodents while walking usually tend to step their hind paw in the same place previously occupied by their front paw. Quantifying this aspect of the motor behavior could then be considered as a measure of locomotor ability. On day 0, the average distance of left paw for animals in all the four groups was 0.75 cm, whereas for right paw it was 0.9 cm. On days 7, 14 and 21, 3-NP treated animals recorded a significant increase in average distance of left paw overlap of 0.9, 0.6 and 0.6 cm respectively. Average overlap distance in right paw for days 7, 14 and 21 was significantly found to be 1.1, 0.66 and 0.61 cm respectively. However, quercetin supplementation in 3-NP + quercetin animals maintained an overall average distance in left and right paws overlap between 0.25 and 0.45 cm for the entire duration of 21 days, suggesting the ability of quercetin in preventing gait abnormalities observed in 3-NP induced HD.

3.5. Effect of quercetin on histopathological changes

Histopathological changes in the striatum of control and 3-NP treated animals are depicted in Fig. 8. The striatum of control rats shows normal morphology. Quercetin supplementation to rats alone exhibited normal appearance of striatum as similar to that of control rats. Striatal sections of rats treated with 3-NP exhibited irregular damaged cells with condensed and pyknotic nuclei (Fig. 8C). Quercetin treatment to 3-NP animals resulted in modulation of the abnormalities in the striatal histopathology near to normal (Fig. 8D).

4. Discussion

Defects in mitochondrial functions have been proposed to contribute substantially to most of the neurodegenerative disorders including HD. The activity of mitochondrial complexes-I, II, IV and F_1F_0 synthase
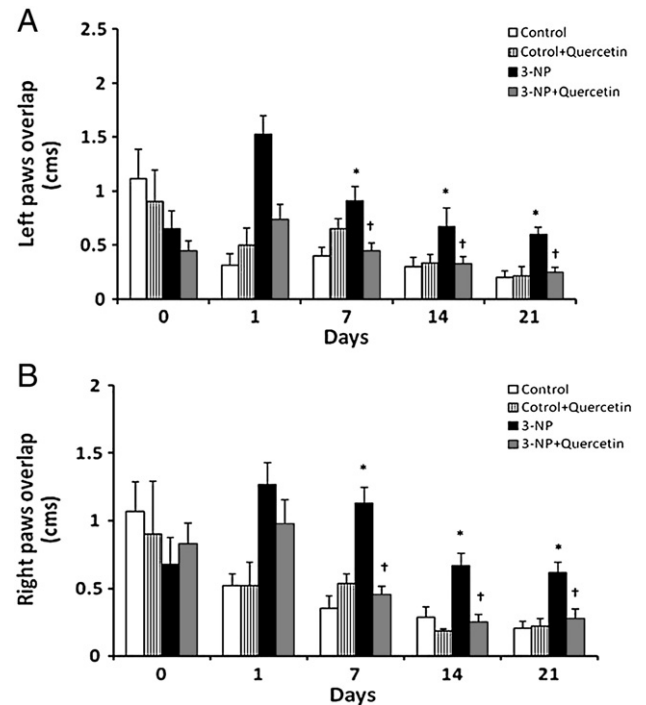

Fig. 7. Effect of quercetin supplementation on gait analysis in terms of left paw (A) and right paws (B) overlap of 3-NP treated animals. Values are expressed as mean ± SEM; n = 6/group. * Significantly different from control group (p < 0.05), † significantly different from 3-NP treated group (p < 0.05).
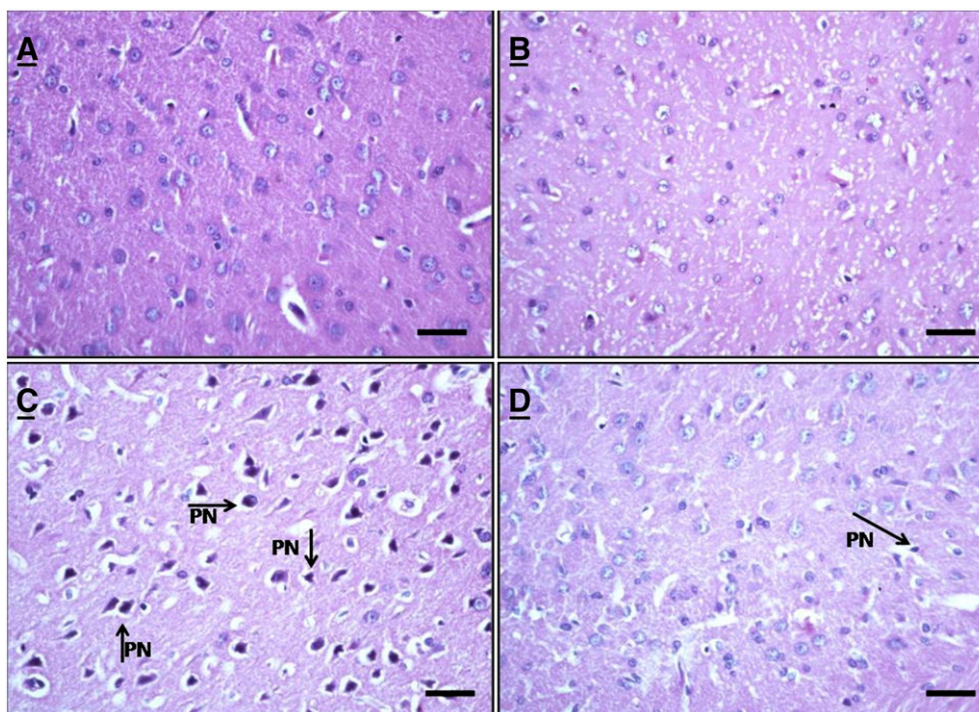
Fig. 8. Effect of quercetin supplementation on histopathological alterations in striatum of control and 3-NP treated rats. Sections were visualized under light microscope at a magnification of 40×. (A) Control rats showing normal striatal histology. (B) Histological appearance of striatal tissue from rats supplemented with quercetin alone shows normal cell nuclei similar to that of control animals. (C) 3-NP induced rats showing damaged and condensed pyknotic nuclei in striatum (PN). (D) Striatum of quercetin + 3-NP administered groups showing lesser number of pyknotic nuclei (scale bar—100 μm).

was found to be significantly inhibited in 3-NP treated animals leading to bioenergetic failure in terms of reduced ATP levels and ATP/ADP ratio. This inhibition in activities might be an effect of hydroxyl radicals generated due to 3-NP mediated SDH inhibition [41]. In addition, MTT reduction (a marker of mitochondrial functioning) was markedly reduced in the striatum of 3-NP treated animals. Mitochondrial complex-II has been reported to, at least partly, involved in MTT reduction by intact astrocytes and neurons, confirming inhibition of SDH by 3-NP [42]. This metabolic defect imposed by 3-NP treatment might be closely associated with ATP depletion, mitochondrial swelling, loss of ionic gradients, and alterations in mitochondrial membrane structure and function [43]. Preferential decrease of SDH activity in the lesion area of 3-NP treated animals in the histochemical assay corroborates the earlier observations [39] and is in accordance with the biochemical findings in the present study showing inhibition in SDH activity. Quercetin supplementation to 3-NP treated animals restored SDH staining in the striatum as compared to 3-NP treated animals. We also found an increase in mitochondrial swelling on 3-NP administration which might be attributed to increased production of harmful ROS. Quercetin supplementation to 3-NP treated animals restored the normal functioning of ETC enzymes along with a significant increase in F_1–F_0 synthase activity, restoration of ATP levels and prevention of mitochondrial swelling. The beneficial effect of quercetin might be attributed to antioxidant activity of this flavonoid. Complex I and cytochrome c are suggested to be molecular targets of quercetin action thereby inhibiting H_2O_2 production [44]. Quercetin has been shown to prevent the opening of MPT pore thereby preventing mitochondrial swelling [19]. Quercetin has been reported to bind to a variety of cytosolic proteins and accumulates in lipid compartments (i.e., membranes), suggesting that, hydrophobic compartments such as mitochondria, in which heme-containing proteins are largely expressed, may represent a site of preferential accumulation of the flavonoid [45]. Since, mitochondria represent a critical source of ROS and their ability to accumulate quercetin may be functional for protection of mitochondrial function and

integrity. Quercetin has recently been reported to be most efficient in protecting against indomethacin-induced mitochondrial dysfunctions and this could be due to its ability to enter cells and accumulate in mitochondria [46]. Furthermore, quercetin supplementation has also been shown to protect mitochondrial integrity and size along with mitochondrial functions by controlling succinate dehydrogenase and NADH oxidase activities [47].

Increased oxidative stress is attributed to alterations in antioxidant defense system which includes lipid peroxidation and antioxidant enzymes. One of the most abundant products of lipid peroxidation is MDA. The values of mitochondrial MDA levels in 3-NP exposed animals were found to be significantly higher. Quercetin supplementation to 3-NP treated animals was able to significantly lower lipid peroxide levels. These findings are supported by a study in which, protective effect of quercetin is correlated with the capacity of this flavonoid to detoxify H_2O_2 and thereby effectively blocking MDA formation in the brain [48]. Also, multiple methylation of the hydroxyl groups present in quercetin increases lipophilic nature of the molecule, thereby increasing availability of the flavonoid in mitochondrial membrane and preventing lipid peroxidation, thus enhancing the pharmacological potential of this flavonoid against such pathological processes [49]. MnSOD is also regarded as a critical component of the enzymatic antioxidant defense system and in the present study SOD activity was found to be significantly lowered in 3-NP treated animals. This could be due to increased generation of superoxide radicals, leading to decrease in the activity of MnSOD. 3-NP administration also resulted in a significant decrease in catalase activity. However, quercetin supplementation to 3-NP induced HD animals for 21 days significantly improved the levels of MnSOD and catalase, suggesting that it is an effective mechanism in ameliorating mitochondrial oxidative stress. In contrast, quercetin possesses both pro- and anti-oxidant properties [50], and it was therefore hypothesized that quercetin effects on MnSOD activity may be due to changes in expression of the MnSOD gene [51]. Quercetin was shown to be a potent inhibitor of H_2O_2 production,

even when production rate of H_2O_2 was stimulated by the mitochondrial inhibitors such as rotenone and antimycin A [45]. Quercetin has also been shown to prevent oxidative nuclear and mitochondrial DNA damage [52].

In biological systems, thiols play a central role in coordinating the antioxidant defense network by their ability to act as reducing agents [53]. Non-enzymatic antioxidant, GSH constitutes the first line of defense against free radicals. Mitochondrial total (TSH), low molecular weight (such as GSH) and protein thiols (PSH) were found to be significantly lowered in 3-NP treated animals. The decrease in TSH content in 3-NP induced HD animals might be due to a reduction in GSH levels, as it has been demonstrated that GSH oxidation causes extensive redox changes to mitochondrial thiol proteins [54]. The probability that a cell will undergo apoptosis and ensue mitochondrial swelling is in part dictated by the cellular redox potential, which is mainly determined by the reduction and oxidation of mitochondrial thiol residues [55]. Thus a decrease in mitochondrial thiol content makes a cell more susceptible to apoptosis causing osmotic swelling of mitochondria, which may be the case in 3-NP induced HD. Evidence of decreased thiol level in 3-NP treated animals suggests that endogenous antioxidants may play a role in protecting against 3-NP induced oxidation [56]. Supplementation with quercetin to 3-NP treated animals was found to be effective in preventing decrease in mitochondrial thiols suggesting direct neutralization of ROS by quercetin.

Neurobehavioral impairment is a characteristic symptom of striatal cell loss and is found in neurodegenerative disorders such as HD. Increased oxidative stress and mitochondrial dysfunctions following 3-NP administration can result in poor cognitive and motor functions [43]. Our results showed consistent decrease in locomotor activity, increased time taken to cross the narrow beam and increased paw slips which resulted in decreased average velocity of 3-NP administered animals. Footprint analysis for gait abnormalities showed reduced step length and significant decrease in stride length in 3-NP treated animals hence, clearly establishing gait abnormalities in the 3-NP model of HD. Among the other parameters of gait abnormalities, stride length has been used as a reliable marker of basal ganglia dysfunction leading to motor abnormalities [57]. In addition, the analysis of the beam test confirmed that a progressive difficulty in accomplishing a task of increasing complexity could be due to 3-NP induced development of gait abnormalities. Quercetin supplementation to 3-NP administered animals significantly improved motor functions and gait in animals, which reflects the ability of quercetin to prevent 3-NP-induced mitochondrial dysfunctions.

3-NP-induced histopathological findings have been well characterized in a number of previous reports [58]. In our study, 3-NP-induced rats exhibited irregular damaged cells with condensed and pyknotic nuclei in striatum, which replicates the histological alterations similar to that in human HD [59]. These histological changes could be attributed to increased oxidative stress following 3-NP administration. Quercetin has shown protective effect by decreasing neuronal death and reducing reactive astrogliosis after ischemia–reperfusion injury, suggesting that quercetin should provide therapeutic benefits for suppression of inflammatory-related neuronal injury in neurodegenerative diseases [60]. The neuroprotective effect of quercetin was observed on animals treated with 3-NP showing only milder degenerative changes compared to 3-NP treated animals. Quercetin improved respiratory chain functions, antioxidant status and neurobehavioral functions in 3-NP treated animals which may be due to the presence of phenolic hydroxyl groups, which act as electron donors and are responsible for free radical scavenging activity of quercetin. In particular, the catechol structure which possesses two hydroxyl groups at neighboring positions is remarkably superior to other dispositions in electron donating ability. The antioxidant activity of quercetin can also be explained by their chelating action and the catechol group is recognized to contribute directly to the chelating action of quercetin, reflecting its natural capability as a therapeutic agent. Furthermore, the beneficial effect of quercetin on

mitochondrial functions might involve increased mitochondrial biogenesis through regulation of sirtuins (SIRT1) or peroxisome proliferator-activated receptor gamma co-activator (PGC-1) [61,62].

5. Conclusions

In conclusion, our results reveal that quercetin prevents mitochondrial dysfunctions, oxidative stress along with motor deficits in 3-NP induced HD. Thus, suggesting the potential of quercetin as an antioxidant which can be engaged in the management of HD and other neurodegenerative disorders wherein mitochondrial functions are perturbed.

Conflict of interest

There is no conflict of interest.

Acknowledgements

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