Rutin a Dietary Flavonoid Protects Against Altered Neurobehavioral, Membrane Bound Enzymes and Striatal Damage Induced by 3-Nitropropionic Acid in Male Wistar Rats

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ABSTRACT
Phytochemicals derived from various plants are gaining more attention owing to their diverse therapeutic potential and less side effects. Rutin (RT) is one of the plant derived flavonoid from buckwheat, citrus fruits and green tea with multiple pharmacological properties. Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder characterized by involuntary choreiform movements and progressive cell damage in basal ganglia, mainly in the striatum. 3-Nitropropionic acid (3-NP), a mycotoxin irreversibly inhibits Succinate dehydrogenase (SDH) in the TCA cycle leads to energy deficient can produce various dyskinetic movements and dystonic postures associated with selective striatal degeneration. The present study investigates the potential effect of RT against 3-NP induced behavioral and neurochemical changes in the striatum. The intraperitoneal administration of 3-NP (10 mg/kg b.w.) to male wistar rats for 14 days caused reduction in motor activities, muscle coordination, non enzymatic antioxidants status, activities of Adenosine triphosphatases (ATPases), increased Lactate dehydrogenase (LDH) activity with striatal degeneration. Pretreatment with RT (25 mg/kg and 50 mg/kg b.w.) orally prior to the administration of 3-NP has restored the biochemical, behavioral and cellular alterations caused by 3-NP induced toxicity in the striatum. The results of the present study indicate that RT (25 mg/kg and 50 mg/kg b.w.) significantly reversed 3-NP induced various behavioral, biochemical parameters and cellular changes. Hence RT could be used as a therapeutic agent for the treatment of Huntington's disease.

Keywords: Rutin, 3-Nitropropionic acid, Neurobehavioral deficits, Antioxidants, Nissl bodies

INTRODUCTION
Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder characterized by psychiatric disturbances, a progression of motor deficits and cognitive decline. The disease is caused by an unstable expansion of CAG repeats (>35) within the coding region of the HD gene, which encodes a protein of approximately 350 kDa, known as mutant huntingtin (mHtt). The abnormal CAG repeats lead to an increase in the glutamine residues at the N-terminal of mHtt. Although mHtt is ubiquitously expressed in human tissues and it initially affects the striatum and later in other brain regions that ultimately leads to neuronal degeneration. Several recent studies have studied in both humans and animal models suggest that the involvement of energy metabolism dysfunction, excitotoxic processes, and oxidative stress might be involved in HD. 3-Nitropropionic acid (3-NP) is a neurotoxin which is produced by various fungal species and it is also naturally present in leguminous plants which are commonly used as a feed for animals. 3-NP was reported for the first time in China in 1980 which is associated with an epidemic of acute encephalopathy in children who ate moldy sugarcane containing 3-NP. The mechanisms by which 3-NP induces neurotoxicity by mitochondrial membrane depolarization, depletion of ATP, dysregulation of intracellular Ca2+ homeostasis, oxidative stress, excitotoxicity and enhanced mitochondrial-dependent apoptosis. 3-NP induces striatal degeneration by upregulating the activation of MAPKs signaling pathways and downregulating the Nrf2-ARE pathways in the striatum. Rutin (3,3',4',5,7-pentahydroxyflavone-3-rhamnogluco side) (RT) is a flavonol composed of quercetin and the disaccharide rutinoside that is found in many typical plants such as buckwheat, apples, onions, passionflower, the rinds of buckwheat, grapes and lime, including cranberries and mulberry. RT can cross the blood-brain barrier and has influence on the central nervous system. It has been reported that RT has several pharmacological properties, including antioxidant, cytoprotective, anti-inflammatory, anti-platelet action, immunomodulator and neuroprotective activities. In humans, it attaches to the iron ion (Fe2+), preventing it from binding to hydrogen peroxide, which would otherwise create a highly reactive free radicals such as superoxide that may damage cells as well as enhance the activity of antioxidant enzymes. Kuan et al. reported that the protective effect of RT against lipopolysaccharide induced acute lung injury by

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MATERIALS AND METHODS

Animals

Male Wistar rats weighing between 200–250 g bred in Central Animal House, Dr. ALMPGIBMS, University of Madras, Taramani campus, Chennai 113, Tamil Nadu, India were used. The animals were housed under standard laboratory conditions and maintained on natural light and dark cycle, and had free access to food and water. Animals were acclimatized to laboratory conditions before the experiment. The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) (IAEC NO. 01/10/2013). Dr. ALMPGIBMS, University of Madras, Taramani campus, Chennai 113, Tamil Nadu, India.

drugs and experimental design

3-NP (sigma chemicals) was diluted with saline (pH 7.4) and administered intraperitoneally at a dose of 10 mg/kg b.w and Rutin (sigma chemicals), was dissolved in DMSO and administered orally at the doses of 25 and 50 mg/kg b.w for 14 days. All other chemicals and solvents used were of analytical grade and highest purity. Animals were divided into six groups and each group comprised of six animals. Group 1: Control rats were given with vehicle. Group 2: Rats were administered with 3-NP (10mg/kg b.w) intraperitoneally for 14 days. Group 3: Rats were administered with Rutin (25mg/kg b.w) orally 1 hr prior to the intraperitoneal administration of 3-NP for 14 days. Group 4: Rats were administered with Rutin (50mg/kg b.w) orally 1 hr prior to the intraperitoneal administration of 3-NP for 14 days. Group 5: Rats were administered with Rutin (25mg/kg b.w) alone orally for 14 days. Group 6: Rats were administered with Rutin (50 mg/kg b.w) alone orally for 14 days.

neurobehavioral studies

Forced swim test (FST)

All rats were subjected to a forced swim test. This test was carried out according to the methods described by Porsolt et al.38 After the open field test, the rats were placed individually in Plexiglas cylinders (height: 40 cm, diameter: 18 cm) containing 25 cm water, maintained at 23–25 °C. Animals were removed from the water cylinder after 15 min and dried before they returned to their home cages. On the next day, they were replaced in the cylinders for 5 min, and the total duration of immobility was measured. A rat was judged to be immobile when it remained floating passively in the water.

Balance beam test (BBT)

The balance beam test was used to measure the ability of rats to traverse a horizontal narrow beam (1cmx100 cm) suspended 1mabove a foam-padded cushion described by Haik et al.36 During testing, the rats were given 2 min to traverse the beam. If they did not complete the task or if they fell off the beam, the trial was ended and the rats were placed back into their home cages. For successful performers, the latency to cross the beam was recorded. Narrow beam walk test (NBWT) 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.37 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.

biochemical study

Tissue preparation

On day 15, the animals were sacrificed and the brain was removed by decapitation. Striatum was separated from each isolated brain. A 10% (W/V) tissue homogenate were prepared in 0.1 M phosphate buffer (pH 7.4). The homogenate were centrifuged at 10,000 x g at 4°C for 15 min. Aliquots of supernatant were separated and used for biochemical estimations.

Assay of Na+/K+ ATPases

The activity of Na+/K+ ATPase was done by Bonting38 to 1.0 ml of Tris buffer and 0.2 ml of each of the above reagents were mixed together. Thus the assay medium in a final volume of 2.0 ml, contained 92mM tris buffer, 50mM MgSO4, 60mM NaCl, 1mM EDTA and 4mM ATP. After 10 minutes, equilibrium at 37°C in an incubator, reaction was started by the addition of 0.1 ml of homogenate. The assay medium was incubated for 15 minutes. After incubation, the reaction was arrested by the addition of 1.0 ml of 10% TCA. The enzyme activity is expressed as micromoles of Pi liberated/min/mg protein.

Assay of Mg2+ ATPases

The activity of Mg2+ ATPases was determined by the method of Ohnishi et al.39 The assay was initiated by the addition of 0.1 ml of homogenate to an incubation medium containing 0.1 ml of water and 0.1 ml of each of the above reagents. The final concentration of tris buffer, MgCl2 and ATP were 75mM, 5mM and 2mM respectively with total incubation volume of 0.5 ml. The reaction was terminated after 15 minutes by the addition of 1.0 ml of 10 % TCA. The enzyme activity was expressed as micromoles of Pi liberated/min/mg protein.

Assay of Ca2+ ATPases

The activity of Ca2+ ATPase was estimated by the method of Ohnishi et al.39 To 0.1 ml of homogenate to an incubation medium containing 0.1ml of water and 0.1ml of each of the above reagents. The final concentration of Tris buffer, CaCl2 and ATP were 75mM, 5mM, 2mM with total incubation volume of 0.5 ml. The reaction was terminated after 15 minutes by the addition of 1.0ml of 10% TCA. The enzyme activity was expressed as micromoles of Pi liberated /min/mg protein. Inorganic phosphorus was estimated by the method of Fiske and Subbarow40.
Figure 1: Effect of RT on 3-NP induced muscular incoordination of control and experimental rats in Forced swim test.

Figure 2: Effect of RT on 3-NP Induced Movement Impairment of control and experimental Rats on Balanced Beam Test.

Figure 3: Effect of RT on 3-NP induced Motor Impairment on Narrow Beam Walk Test of control and experimental Rats.
Activity of Lactate dehydrogenase (LDH)
The Activity of Lactate dehydrogenase was done according to the method of King et al 1965. To 1.0ml of buffered substrate, add 0.1 ml of homogenate, 0.2ml of NAD+ and incubate for 15 mins at 37 °C, then add 1.0ml of DNPH and 0.4N NaOH and read at 420 nm using a UV-VIS Spectrophotometry. The activity of LDH is expressed as Units/ mg protein.

Estimation of Vitamin C
The level of Vitamin C was measured by the method of Omaye et al. To 0.5ml of tissue homogenate, 0.5 ml of distilled water, 1ml % TCA were added and centrifuged for 20 min. To 1.0 ml of supernatant, 0.2 ml of DTC reagent was added and incubated at 37°C for 3 h. Then, 1.5 ml of 65% sulphuric acid was added mixed well and kept at room temperature for another 30 min. The colour developed was read at 520 nm using a UV-VIS Spectrophotometry. Vitamin C level is expressed as μg/g mg protein.

Estimation of Protein
The protein content was measured according to the method of Lowry et al. using bovine serum albumin as standard.

RESULTS

Estimation of Protein

The animals treated with 3-NP failed to pass the beam within the cut-off time of 120 s, (**P< 0.01) as compared to the control group which passed the beam. Pretreatment with RT 25mg/kg b.w (**P< 0.05) and 50 mg/kg b.w (##P< 0.01) significantly improved the performance of 3-NP induced rats on the beam balance test. There was no significant changes observed in RT 25 and 50 mg/kg b.w alone treated rats compared to control rats. Data represents mean± SD of 6 rats in each group. Units were expressed as immobility time in seconds.

Effect of RT on 3-NP induced altered Locomotor activity on Balanced Beam Test

The effect of RT on 3-NP induced changes in balance beam test of control and experimental rats was shown in Figure 2. The animals treated with 3-NP failed to pass the beam within the cut-off time of 120 s, (**P< 0.01) as compared to the control group which passed the beam. Pretreatment with RT 25mg/kg b.w (**P< 0.05) and 50 mg/kg b.w (##P< 0.01) significantly improved the performance of 3-NP induced rats on the beam balance test. There was no significant changes observed in RT 25 and 50 mg/kg b.w alone treated rats compared to control rats. Data represents mean± SD of 6 rats in each group. Units were expressed as time taken to cross the beam in seconds.

Effect of RT on 3-NP induced Motor Impairment on Narrow Beam Walk Test

The Narrow beam walk test was used to assess the hind limb impairment wherein the time taken by the rat to reach the narrow beam was recorded was shown in Figure 3. The maximum time allowed for each rat for traversing the beam was120 s. On day 14, the 3-NP induced rats has taken increased time to traverse the beam and increased paw slips (***P< 0.001) as compared to the control group. Pretreatment with RT 25mg/kg b.w (**P< 0.05) and 50 mg/kg b.w (##P< 0.01) significantly improved the motor activity by maintain the hind limb function and traverse the beam in less time with less paw slips. There was no significant changes observed in RT 25 and 50 mg/kg b.w alone treated rats compared to control rats. Data represents mean± SD of 6 rats in each group. Units were expressed as time taken to traverse the beam in seconds and number of paw slips.

Effect of RT on 3-NP induced changes in muscular incoordination in Forced Swim Test

The effect of RT on 3-NP induced changes in Forced swim test of control and experimental rats was shown in Figure 1. In Forced swim test, an increase (**P<0.01) in total duration of immobility indicating that 3-NP treated animals with locomotor impairments as compared to the control. RT 25mg/kg b.w. (*P<0.05) and 50mg/kg b.w (**P<0.01) treatment showed improvement in mobility on 14th day as compared to the 3-NP administered group. There was no significant changes observed in RT 25 and 50mg/kg b.w alone treated rats compared to control rats. Data represents mean± SD of 6 rats in each group. Units were expressed as immobility time in seconds.
The Effect of RT on 3-NP induced changes in the activities of Lactate dehydrogenase in control and experimental rats were shown in Figure 4. The activity of Lactate dehydrogenase in 3-NP intoxicated rats were significantly increased when compared with normal rats (**P<0.01). RT pretreatment 25mg/kg b.w (*P<0.05) and 50mg/kg b.w (##P<0.01) has restored the activity of Lactate dehydrogenase when compared to 3-NP induced...
The Effect of RT on 3-NP induced alterations in the levels of non enzymatic antioxidants in the striatum

The Effect of RT on 3-NP induced changes in the levels of non enzymatic antioxidants vitamin C and E in control and experimental rats were shown in Figure 6. The levels of non enzymatic antioxidants vitamin C and E in 3-NP intoxicated rats were significantly decreased when compared with normal rats (**P<0.01). RT pretreatment 25mg/kg b.w (*P<0.05) and 50mg/kg b.w (##P<0.01) has protected the levels of vitamin C and E when compared to 3-NP induced group. There was no significant changes observed in RT (25 and 50 mg/kg, b.w.) alone treated rats. Data represents mean± SD of 6 rats in each group. The level of vitamin C and E was expressed as μg/mg protein.

DISCUSSION

The core finding of the present study is that administration of RT is remarkably neuroprotective in rats against 3-nitropropionic acid-induced neurotoxicity. 3-NP is an inhibitor of complex II in the electron transport chain, inhibiting the transfer of electrons leading to energy impairment produces oxidative stress. This oxidative stress has been largely attributed to the production of Reactive oxygen/nitrogen species (ROS/RNS) during energy failure65. The striatal damage and the pathological features produced by 3-NP-induction mimicking the HD like conditions66. Under physiological conditions, the level of ROS is in equilibrium with the antioxidants level; however, when the production of ROS, overwhelms the cellular antioxidant capacity, damage to cellular

Control group, *P<0.05 and ##P<0.01 versus 3-NP group (one-way ANOVA followed by Tukey’s test).

Figure 7: Effect of RT on 3-NP induced cellular alterations in the striatum of control and experimental rats

There was no significant changes observed in RT (25 and 50 mg/kg, b.w.) alone treated rats. Data represents mean± SD of 6 rats in each group. Units were expressed as Units/mg protein. Group 1: Control; Group 2: 3NP (10mg/ kg b.w); Group 3: 3-NP (10 mg/ kg b.w) + RT (25mg/kg b.w); Group 4: 3 NP (10 mg/kg b.w) + RT (50mg/kg b.w); Group 5: RT (25mg/kg b.w) alone; Group 6: RT (50mg/kg b.w) alone. **P<0.01 versus Control group, *P< 0.05 and ##P< 0.01 versus 3-NP group (one-way ANOVA followed by Tukey’s test).

Effect of RT on 3-NP induced cellular alterations in the striatum

In Figure 7 Control rats striatum stained lightly had intact viable neurons. 3-NP-induced rats showed increased number of darkly stained nonviable neurons and pyknotic nuclei in the striatum. RT 25 and 50 mg/kg pretreated rats has lesser nonviable neurons in the striatum indicated the dose dependent protective effect of Rutin against 3-NP toxicity. RT 25 and 50 mg/kg alone treated rats showed similar pattern as that of control. Figure 7 Histological analysis of striatum (Nissl staining 400x). Tissue sections were stained with Nissl stain and visualized under light microscope at an original magnification of 400x. (A) Control rats showing normal striatal histology. (B) 3-NP-induced rats showing striatal damage with nonviable neurons and condensed pyknotic nuclei. (C) Striatum of 3-NP+ RT 25 treated groups showing lesser number of nonviable neurons and pyknotic nuclei. (D) Striatum of 3-NP+ RT 50 treated groups showing few nonviable neurons (E) and (F) Striatum of RT 25 and 50 mg/kg alone treated rats were similar to that of the control.
macromolecules such as lipids, protein and DNA. Such a state of "oxidative/nitrosative stress" is thought to contribute to the pathogenesis of many human diseases including those of the neurodegenerative diseases. It is well established that the main function of the basal ganglia is to control the overall coordination of the body movements, justifying the analysis of movement patterns in relation to striatal degeneration. In this study, we observed disturbances in the locomotor activities and muscular coordination of animals treated with 3-NP was analyzed by Forced swim test, Balanced beam test and narrow beam walk test. 3-NP treated rats showed higher immobility time in FST, failed to pass the beam within the cut off time in BBT and increased time taken to traverse the beam with more paw slips in NBWT indicating motor impairment as well as muscular incoordination. In the present study, it is suggested that rutin which is a potent antioxidant, has reduced neurobehavioral deficits significantly improved the motor impairment as well as muscular coordination by reducing the immobility time in FST; easily cross the beam in BBT and the time taken to traverse the beam with less paw slips in NBWT on 14th day in rutin-pretreated animals by scavenging free radicals, which are thought to cause behavioral deficits in 3-NP induced animals. Earlier studies have shown an improvement in various behavioral outputs like motor coordination skill as a result of antioxidant treatment. The central nervous system (CNS) is highly sensitive to oxidative stress, owing to a high oxygen consumption and enrichment in polyunsaturated fatty acids, making it particularly vulnerable to lipid peroxidation (LPO). The enhanced susceptibility of membrane to LPO can lead to loss of ATPases activity. Any perturbation in the activities of ATPases affects membrane status by inflicting changes in electrophysiological energetics and normal homeostasis. 3-NP depleted the levels of antioxidants, activity of ATPases and enhances ROS levels in the striatum. The inhibition of Na+/K+-ATPases leads to a partial membrane depolarization allowing excessive Ca2+ entry inside neurons with resultant toxic events like excitotoxicity. Oxidative stress is hypothesized to play a vital role in 3-NP-induced neuronal apoptosis. In this study 3-NP-induced rats exhibited decrease in the activities of membrane bound ATPases (Na+/K+-ATPase, Mg2+-ATPase and Ca2+-ATPase) in the striatum. The decrease in the activities of these ATPases could be due to the enhanced lipid peroxidation by free radicals in 3-NP-induced animals which is in correlation with the previous report. Effective protection against decrease in ATPases activity is discernible among Rutin treated animals might be due to the decline in free radical generation and lipid peroxidation. Lactate dehydrogenase activity (LDH) catalyzes the interconversion of lactate and pyruvate. The amount of this enzyme may be used as a marker of tissue breakdown. Systemic 3-NP administration showed significant increase in brain LDH when compared with control rats. However, RT pretreatment significantly reduced the increased LDH activity in 3-NP induced rats. The non-enzymic antioxidants vitamins C and E serve as primary antioxidants that can directly scavenge singlet oxygen, superoxide and hydroxyl radicals. Vitamin C also can scavenge peroxynitrite, an awful nitrating and oxidizing molecule, which itself induce lipid peroxidation, nitration of amino acids and also apoptosis. Dietary supplementation of vitamin E has been shown to serve as a successful therapeutic strategy for the prevention or treatment of neurodegenerative disease. The decline in the levels of vitamins C and E in 3-NP-induced rats might be related to increased utilization of these antioxidants to counteract the high lipid peroxidation and oxidative stress. However, Rutin treatment increases the level of these antioxidants, which might be due to scavenging free radicals and alleviating oxidative stress. The administration of 3-NP produced specific striatal degeneration which is indicated by darkly stained increased nonviable neurons and pyknotic nuclei in the striatum. Pretreatment with RT dose dependently prevented the striatal damage caused by 3-NP. The antioxidant and neuroprotective activity of rutin is in harmony with the earlier studies may contributed to this effect by diminishing oxidative stress. In conclusion, based on this biochemical, behavioral and histological studies, the present data confirmed that Rutin exerts its protective action against 3-NP-induced neurodegeneration in the striatum. RT can therefore potentially used as a neuroprotective agent against HD. Further investigations are in progress to demonstrate the mechanism through which RT exerts its protective effect against 3-NP-induced toxicity.

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CONFLICTS OF INTEREST
The authors declare that there is no conflict of interests.

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