RUTIN ATTENUATES 3-NITROPROPIONIC ACID INDUCED BEHAVIOURAL ALTERATIONS AND MITOCHONDRIAL DYSFUNCTION IN THE STRIATUM OF RAT BRAIN

Sarumani Natarajan Suganya, Thangarajan Sumathi*

Department of Medical Biochemistry, Dr. ALM Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai 600113, Tamil Nadu, India.

ABSTRACT

3-Nitropropionic acid (3-NP) is a mitochondrial toxin, produced by the plants like Astragalus and also from fungus Arthrium species. Systemic administration of 3-NP in rats produces striatal degeneration serves as an important model of Huntington’s disease (HD). Present study has been designed to explore the neuroprotective effect of Rutin (RT), a bioflavonoid against the 3-NP induced neurotoxicity through mitochondrial dysfunction in male wistar rats. The intraperitoneal (i.p.) administration of 3-NP (10mg/kg) for 14 days elicited marked oxidative stress in striatum as evidenced by significantly enhanced protein oxidation with reduced locomotor activities, grip strength and mitochondrial complex enzymes. Rutin pretreatment 25 mg/kg and 50 mg/kg significantly improved the behavioral alterations and restored the activities of mitochondrial complex enzymes in 3-NP induced group. This study highlights the therapeutic potential of rutin against 3-NP induced Huntington’s like conditions and further indicates that the drug might act through its antioxidant activity.

KEYWORDS: Rutin, 3-Nitropropionic acid, behavior, mitochondrial oxidative stress.

1. INTRODUCTION

Huntington’s disease (HD) is an autosomal-dominant devastating neurodegenerative disorder characterized by lesions in the striatum of the brain, progressive development of involuntary choreiform movements, cognitive impairment, neuropsychiatric symptoms, and premature death. Many evidences suggest that the involvement of mitochondrial dysfunction in the
The pathogenesis of neurodegenerative disorders like Huntington’s disease, Parkinson’s disease, Alzheimer’s disease, Amyotrophic lateral sclerosis. 3-Nitropropionic acid (3-NP), a mitochondrial toxin, an inhibitor of succinate dehydrogenase (SDH), a component of TCA cycle and electron transport chain leads to energy depletion which induces reduced oxidative defense, specific striatal damage results in behavioral alterations like movement and memory impairments, the characteristic features exploited to produce an experimental model of HD. Several invitro and invivo studies has shown that chronic doses of 3-NP cause energy impairment, excitotoxicity (NMDA receptor activation), oxidative/nitrosative stress, release proinflammatory cytokines and apoptotic cell death.

Rutin (3’,4’,5,7-tetrahydroxyflavone-3b-D-rutinoside) is one of the most bioactive flavonoids, known as vitamin P. It was thought to be an activating factor for vitamin C. Rutin, a flavonoid, has shown pharmacological benefits including anti-inflammatory, myocardial protection, immunomodulator, hepatoprotective activities, enzyme-modulating activities and also, they may act as antioxidants to inhibit free-radical mediated cytotoxicity and lipid peroxidation. Rutin is also shown to have antioxidative action in vitro and in vivo. It can act directly by entering the redox reactions, and indirectly by chelation of iron. Rutin has disaccharide sugar molecules as the side chain. Therefore, rutin may exhibit improved antioxidant properties as well as greater bioavailability potential as compared to quercetin.

Hence, the present study was designed to investigate the effects of rutin, a polyphenolic flavonoid on behavioral deficits, mitochondrial dysfunction in control and 3-NP-induced experimental HD in male wistar rats.

2. MATERIALS AND METHODS
2.1 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.
2.2 Chemicals

3-NP (sigma chemicals) was diluted with saline (pH 7.4) and administrated intraperitoneally (10 mg/kg) for 14 days. Rutin (kavins scientifics), was dissolved in saline and administered orally at the doses of (25 and 50 mg/kg) for 14 days.

2.3 Experimental Design

Animals were divided into six groups and each group comprised of six animals.

**Group 1:** Control rats were given saline.

**Group 2:** Rats were administered with 3-NP (10mg/kg) intraperitoneally for 14 days.

**Group 3:** Rats were administered with Rutin (25mg/kg) orally 1hr 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.

2.4 Neurobehavioral Parameters

**Limb Withdrawal Test**

This is an important parameter to measure the functional abnormalities of the hind limbs, an indicator of striatal degeneration. In this behavioral test, the animals was placed on a 20cm high 30cmx30cm perpex platform containing four holes, two holes of 5 cm diameter for the hind limbs and two holes with a diameter of 4 cm for the forelimbs. The rat was placed on the platform by positioning first the hind limbs and then the forelimbs into the holes. The times taken by the animal to retract its first hind limb and the contralateral hind limb were recorded. The difference between the retraction times of both hind limbs was determined. This is considered to be an important parameter to measure functional abnormalities of the hind limbs, which are indicative for the extent of striatal degeneration. The test was performed three times with a 45 min interval and the average values were reported. \[21\]

**String test for Grip strength**

The rat was allowed to hold with the forepaws a steel wire (2mm in diameter and 35 cm in length), placed at a height of 50 cm over a cushion support. The length of time the rat was able to hold the wire was recorded. This latency to the grip loss is considered as an indirect measure of grip strength. \[22\]
2.5 Isolation of Rat Brain Mitochondria
On day 15, the animals were sacrificed and the brain was removed by decapitation. Striatum was separated from each isolated brain and homogenized in isolated buffer. Homogenates were centrifuged at 13,000xg for 5 min at 4 °C. Pellets were resuspended in isolation buffer with ethylene glycol tetra acetic acid (EGTA) and spun again at 13,000xg at 4 °C for 5 min. The resulting supernatants were transferred to new tubes and topped off with isolation buffer with EGTA and again spun at 13,000xg at 4 °C for 10 min. Pellets containing pure mitochondria were resuspended in isolation buffer without EGTA.

2.6 Mitochondrial Dysfunction
Protein oxidative damage was determined by measuring the Protein carbonyl content in supernatants obtained after centrifugation of cytosol/mitochondria at 10,000xg for 15 min by measuring the hydrazone derivatives between 360 and 390 nm according to the method of Levine et al 1990. Total thiols were done according to the method of Ellman (1959) and expressed as nmoles of oxidized DTNB formed/mg protein.

2.7 Respiratory Complex Enzymes
(Complex-I) NADH dehydrogenase activity was measured spectrophotometrically by the method of King and Howard 1967. (Complex-II) Succinate dehydrogenase (SDH) activity was measured spectrophotometrically according to King et al., 1967. The reaction was initiated by the addition of mitochondrial sample and change in absorbance was recorded at 420 nm. (Complex-IV) Cytochrome oxidase assay activity was assayed in brain mitochondria according to the method of Sottocasa et al. 1967. (Complex V) F1F0 synthase activity was measured as described by Griffiths and Houghton et al 1974. Phosphate produced was measured by the method of Fiske and Subbarow 1925. Results were expressed as nmoles of ATP hydrolyzed/min/mg protein.

2.8 MTT Reduction
The reduction of MTT to blue formazan by dehydrogenases present in the mitochondrial suspension was also monitored to assess mitochondrial functions. 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.
2.9 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 et al (1958). [31]

2.10 Statistical Analysis
The data was analyzed by using analysis of variance (ANOVA) followed by Tukey’s test. All the values were expressed as mean±S.D. In all tests, the criterion for statistical significance was $p < 0.05$.

3. RESULTS
3.1 Effect of RT on 3NP Induced Motor Impairment in Control and Experimental Rats
The difference between the retraction times of both the hind limbs were significantly higher ($**p < 0.01$) in 3-NP induced rats as compared to control group rats which were able to quickly retract their hind limbs. However, the pretreatment with RT 25 mg/kg ($p < 0.05$) and 50 mg/kg ($##p < 0.01$) was significantly improved the performance as compared to the 3-NP induced rats. There is no significant change was observed in limb withdrawal test of the RT (25mg/kg and 50mg/kg) alone treated groups as compared to control groups. (Table 1)

3.2 Effect of RT on 3-NP Induced Alterations in String Test of Control and Experimental Rats
The time taken by the rat to hold the wire was recorded. 3-NP administered rats have shown Significantly ($**p < 0.01$) lower latency to hold the wire due to loss of grip strength as compared with control. Thus, the pretreatment RT with 25mg/kg ($p < 0.05$) and 50mg/kg ($##p < 0.01$) significantly increased the latency time to hold the wire as compared to the 3-NP induced rats. There is no significant change in the grip strength of RT (25mg/kg and 50mg/kg) alone treated groups as compared to control groups. (Table 1)

3.3 Effect of RT on 3-NP Induced Protein Oxidation in Striatum of Control and Experimental Rats
The protein oxidation is measured as protein carbonyl levels was significantly ($**p < 0.01$) increased in the striatum of rats which were induced by 3-NP as compared to the control rats. However, the pretreatment with RT 25 mg/kg ($p < 0.05$) and 50 mg/kg ($##p < 0.01$) significantly ($**p < 0.01$)diminished the levels of protein carbonyls as compared to the 3-NP
induced rats. There is no significant change in the oxidation of protein in RT (25mg/kg and 50mg/kg) alone treated groups as compared to control groups. (Table 2)

3.4 Effect of RT on 3-NP Induced Alterations on the Level of Total Thiols in Striatum
3-NP administration Caused a significant reduction (**p<0.01) in the level of total thiols an important antioxidant in mitochondria as compared to the control rats. Thus the RT pretreatment RT 25 mg/kg (*p<0.05) and 50mg/kg (##p<0.01) significantly restored the antioxidant as compared to the 3-NP induced rats. There is no significant change in the levels of total thiols of RT (25 and 50mg/kg) alone treated groups as compared to control groups (Table 2)

3.5 Effect of RT on 3-NP Induced Changes in the Activities of Respiratory Complex Enzymes in the Striatum
Systemic administration of 3-NP significantly (**p<0.01) impaired the mitochondrial complex enzymes (I, II, IV, V) activities as compared to the control rats. RT pretreatment 25 mg/kg (*p<0.05) and 50 mg/kg (##p<0.01) significantly restored the mitochondrial complex enzymes activities as compared to the 3-NP induced group animals. There is no significant change was observed in the activities of complex enzymes of RT (25 and 50mg/kg) alone treated groups as compared to control groups. (Table 3)

3.6 Effect of RT on 3-NP Induced MTT Reduction and Mitochondrial Swelling in 3-NP Treated Rats
Systemic administration of 3-NP significantly (**p<0.01) depleted the mitochondrial redox activity as compared to the control group rats. Further pretreatment with RT 25 mg/kg (*p<0.05) and 50 mg/kg (##p<0.01) significantly restored the mitochondrial redox activity as compared to the 3-NP rats. There is no significant change in the redox potential of mitochondria of RT (25 mg/kg and 50mg/kg) alone groups as compared to control. The reduced activities of respiratory chain enzymes led to the significantly (**P<0.01) increased mitochondrial swelling in 3-NP induced rats as compared to the control group rats. Further pretreatment with RT 25 (*P<0.05) and 50 mg/kg (##P<0.01) significantly lowered the mitochondrial swelling as compared to the 3-NP administered rats. There is no significant change in the mitochondrial swelling of RT (25 and 50mg/kg) alone treated groups as compared to control groups. (Table 4)
Table 1: Effect of RT on 3-NP induced neurobehavioral deficits on control and experimental rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Limb withdrawal test (seconds)</th>
<th>String test (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10±0.17</td>
<td>102±10.7</td>
</tr>
<tr>
<td>3-NP</td>
<td>93±5.68**</td>
<td>20±3.09**</td>
</tr>
<tr>
<td>3-NP+RT25</td>
<td>25±0.26*</td>
<td>50±1.5*</td>
</tr>
<tr>
<td>3-NP+RT50</td>
<td>15±0.16##</td>
<td>34±1.9##</td>
</tr>
<tr>
<td>RT25</td>
<td>13±0.18</td>
<td>92±2.1</td>
</tr>
<tr>
<td>RT50</td>
<td>11±0.76</td>
<td>98±3.3</td>
</tr>
</tbody>
</table>

**p< 0.01 versus control group, *p < 0.05; ##p<0.01 versus 3-NP, (one-way ANOVA followed by Tukey’s test). Data presented are mean ± SD of 6 animals in each group.

Table 2: Effect of RT on 3-NP induced changes in the levels of protein oxidation and in total thiols, in the Striatum of control and experimental rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Protein Carbonyls (nmol/mg protein)</th>
<th>Total Thiols (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.16±0.12</td>
<td>109.98±3.83</td>
</tr>
<tr>
<td>3-NP</td>
<td>45.57±1.04**</td>
<td>50.12±1.62**</td>
</tr>
<tr>
<td>3-NP+RT25</td>
<td>29.01±0.94*</td>
<td>86.02±2.82*</td>
</tr>
<tr>
<td>3-NP+RT50</td>
<td>20.67±0.65##</td>
<td>93.62±3.98</td>
</tr>
<tr>
<td>RT25</td>
<td>18.85±0.58</td>
<td>96.36±2.84</td>
</tr>
<tr>
<td>RT50</td>
<td>16.46±0.41</td>
<td>99.42±0.58</td>
</tr>
</tbody>
</table>

**p< 0.01 versus control group, *p < 0.05; ##p<0.01 versus 3-NP, (one-way ANOVA followed by Tukey’s test). Data presented are mean ± SD of 6 animals in each group.

Table 3: Effect of RT on mitochondrial complex enzymes in striatum of control and experimental rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Complex I (nmolNADH oxidized/min/mg protein)</th>
<th>Complex II (nmolSDH oxidized/min/mg protein)</th>
<th>Complex IV (nmol cytochrome oxidized/min/mg protein)</th>
<th>Complex V (nmolATP hydrolysed/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>112.64±1.31</td>
<td>142.47±1.45</td>
<td>156.61±1045</td>
<td>19.96</td>
</tr>
<tr>
<td>3-NP</td>
<td>65.77±1.33**</td>
<td>45.50±1.11**</td>
<td>78.56±1.03**</td>
<td>09.25**</td>
</tr>
<tr>
<td>3-NP+RT25</td>
<td>93.71±1.43*</td>
<td>98.46±1.27*</td>
<td>126.28±1.86*</td>
<td>14.98*</td>
</tr>
<tr>
<td>3-NP+RT50</td>
<td>99.61±1.31##</td>
<td>109.615±1.13##</td>
<td>139.54±1.28##</td>
<td>17.27##</td>
</tr>
<tr>
<td>RT25</td>
<td>105.23±1.45</td>
<td>130.65±1.32</td>
<td>149.23±1.37</td>
<td>18.11</td>
</tr>
<tr>
<td>RT50</td>
<td>109.32±1.25</td>
<td>148.63±1.30</td>
<td>153.74±1.59</td>
<td>18.63</td>
</tr>
</tbody>
</table>

**p< 0.01 versus control group, *p < 0.05; ##p<0.01 versus 3-NP, (one-way ANOVA followed by Tukey’s test). Data presented are mean ± SD of 6 animals in each group.
Table 4: Effect of RT on 3-NP induced changes in the MTT reduction and Mitochondrial swelling of control and experimental rats

<table>
<thead>
<tr>
<th>Group</th>
<th>MTT reduction (µg farmazon formed/min/mg protein)</th>
<th>Mitochondrial swelling (nM/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.92± 0.41</td>
<td>0.51±0.01</td>
</tr>
<tr>
<td>3-NP</td>
<td>5.13±0.51**</td>
<td>1.29±0.06**</td>
</tr>
<tr>
<td>3-NP+RT25</td>
<td>8.20±0.26*</td>
<td>0.83±0.04*</td>
</tr>
<tr>
<td>3-NP+RT50</td>
<td>11.17±0.19##</td>
<td>0.69±0.04##</td>
</tr>
<tr>
<td>RT25</td>
<td>14.03±0.15</td>
<td>0.64±0.01</td>
</tr>
<tr>
<td>RT50</td>
<td>14.98±0.26</td>
<td>0.59±0.05</td>
</tr>
</tbody>
</table>

**p< 0.01 versus control group, *p < 0.05; ##p<0.01 versus 3-NP, (one-way ANOVA followed by Tukey’s test). Data presented are mean ± SD of 6 animals in each group.

4. DISCUSSION

Present study shows that 3-NP significantly caused impairment in motor activity, grip strength, oxidative defense, mitochondrial complex enzymes activities and protein oxidation suggesting HD like symptoms in rats. Further RT pretreatment significantly attenuated HD like symptoms. striatum, as a central core area in the basal ganglia, controls the locomotor activity, hence the systemic administration of 3-NP induced striatal degeneration which resulted reduced in co-ordination of the motor activities was showed in limb withdrawal test and as well as loss of grip strength in string test. 3-NP administration for 14 days induces motor impairment and striatal toxicity by causing the degeneration of GABAergic medium spiny neurons in the Striatum in a pattern that is similar to the neuronal cell death seen in HD. Further RT pretreatment significantly improved the locomotor and grip strength performance in 3-NP treated animals, which shows the therapeutic potential against these behavioral abnormalities. Many studies has proved the neuroprotective effect of RT against a variety of conditions including Alzheimer’s disease, Parkinson’s disease etc. [33-35]

Several theories and explanations have been proposed that excitotoxicity through the activation of NMDAR which increases ca²⁺ and oxygen influx leading to the production of superoxide and hydroxyl radicals, and also activate the calcium-dependent nitric oxide synthase and also alters the glutathione redox cycle. Thus produced ROS/RNS and mitochondrial dysfunction may be responsible for this striatal damage. [36-41] In this study 3-NP significantly increased the protein oxidation with depletion of endogenous antioxidant defense system such as thiols. We found that pretreatment with RT showed significant decreased in protein carbonlys and increased thiols in striatum.
Yang et al. (2008) [42] investigated the antioxidant mechanism of rutin, including the total antioxidant activity, reducing power, free radical and superoxide anion radical scavenging, hydroxyl radical scavenging activity, and lipid peroxidation assay. Rutin has the ability to donate electron to reactive free radicals by converting them into more stable species and quenching the free radical chain reaction. The administration of rutin was reported to suppress inflammation in neurons by inhibiting the expression of COX-2, which metabolizes arachidonic acid to prostaglandins along with the generation of free radicals and also iNOS, which is responsible for generation of reactive nitrogen species (RNS) and finally leads to inflammation. [43-45]

Mitochondria has easily undergoes diverse assaults either generated in situ or those imposed from extracellular environment. Mitochondrial dysfunction results in decreased cellular energy, a failure in maintaining cellular homeostasis, and activation of cell death pathways. [46] Defects in mitochondrial functions have been proposed to play an important role in most of the neurodegenerative disorders including HD. The activity of mitochondrial complexes I, II, IV, V were found to be significantly inhibited by 3-NP leading to energy failure. In addition, MTT reduction, a marker for mitochondrial function was markedly reduced in the striatum of 3-NP induced rats. We also found increased mitochondrial swelling in 3-NP induced rats which might be attributed to increased production of ROS. Thus rutin pretreatment restores the behavioral changes and mitochondrial dysfunction produced by 3-NP in male wistar rats.

5. CONCLUSION
In conclusion, our results reveal that Rutin prevents mitochondrial dysfunction and neurobehavioral deficits against 3-NP induced HD. Thus suggesting rutin as an antioxidant which can be engaged in the treatment of HD and other neurodegenerative disorders wherein mitochondrial functions are disturbed.

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Conflicts Of Interest
The authors declare that there is no conflict of interests.
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