Rutin Attenuates Acrylamide Induced Neuropathic Pain via Inhibition of Proinflammatory Cytokines, Up-Regulation of Bcl-2 and Down-Regulation of Bax

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Abstract: Rutin is a flavonoid of the flavonol type found in many typical plants, such as buckwheat, passion flower, apple and tea. Acrylamide (ACR) is a known industrial toxic chemical that produces neurotoxicity characterized by progressive neuronal degeneration. Rats were randomly divided into Control, ACR, Pregabalin and Rutin treated groups. Male wistar rats were treated with ACR (50 mg/kg, i.p.) for 4 weeks which produce typical symptoms of neuropathy in rats. Pregabalin (10 mg/kg) and Rutin (50 & 100 mg/kg) were administered orally for 4 weeks after one hour of ACR administration. ACR enhanced the production of reactive oxygen species (ROS). Treatment with Rutin significantly improved neurological score. Rutinsignificantly (p<0.001) attenuated acrylamide induced oxidative stress markers. The expression of Bcl-2 was up-regulated and TNF-α, IL-6 and Bax were down-regulated by rutin treatment. From our results, it can be concluded that rutinshowed an ameliorative effect against ACR induced neurotoxicity in rats through its antioxidant, anti-inflammatory and antiapoptotic actions.

Keywords: Acrylamide, Rutin, Lipid peroxidation, Neurotoxicity, Neuroprotection, Anti-inflammatory action, Anti-apoptotic activity.

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1. INTRODUCTION

Neuropathic pain is linked up with multiple pathological events like oxidative stress, inflammation, and apoptosis. Current drugs available for the effective management of neuropathic pain are tricyclic antidepressants, antiepileptic drugs, cannabinoid receptor agonists and sodium channel blockers but their usage is associated with many side effects. Drugs, cannabinoid receptor agonists and sodium channel blockers are tricyclic antidepressants, antiepileptic phytocannabinoids. Rutin (3,3′,4′,5,7-pentahydroxyflavone-3-rhamnoglucoside) is a flavonoid of the flavonol type found in many typical plants, such as buckwheat, passion flower, apple and tea. It is also an important dietary constituent of foods and plant-based beverages. Rutin has several pharmacological properties, including antiviral, antibacterial, anti-inflammatory, antioxidant, vasoprotective, cardioprotective, and neuroprotective activities. The present work is intended to study neuroprotective function of rutin against ACR induced neurotoxicity and contemplates to establish the possible mechanism of action. ACR is a neurotoxic chemical and causes peripheral and central neuropathy in humans and laboratory animals. It is considered as the important chemical contaminant formed mostly in potato, cereal and bakery products by the heat treatment.

2. MATERIALS AND METHODS

2.1 Materials

Rutin was purchased from Sisco Research Laboratories. Acrylamide was obtained from Merck, India. Antibodies of tumor necrosis factor α (TNF-α), interleukin-6 (IL-6), Bax, Bcl-2, and biotinylated anti rabbit were purchased from Santa Cruz Biotechnology, Inc., USA. All other chemicals used were of analytical grade.

2.2 Animals

Male rats of Wistar strain weighing 280–300 g were used for the study. The rats were maintained under conditions of 12 h light/dark cycle and had free access to food and water. Study protocol was approved by the Institutional Animal Ethics Committee (No. 1529/PO/Re/11/CPCSEA./CHIPS/IAEC7/PRO-7/2019-20).

2.3 Experimental design

Rats were randomly divided into 5 groups (n=6 in each group). Group I rats served as control and received the vehicle normal saline only. Group II, III and IV rats were administered with ACR (50 mg/kg, i.p. thrice a week) for 4 weeks. After one hour of ACR or vehicle administration, Groups III, IV and V rats received pregabalin (10 mg/kg, orally/daily), rutin 50 & 100 mg/kg orally/daily respectively. Rats were monitored on a regular basis for manifestation of neuropathy. All rats were subjected to behavioral tests each week except neurological score which was carried out on 28th day. Finally, rats were sacrificed by cervical dislocation, the sciatic nerves (SN) were isolated and processed for biochemical analysis.

2.4 Behavioral examination

2.4.1 Assessment of behavioral index (neurological scores)

At the end of the treatment, the neurological scores were examined. Rats were placed in a clear plexiglass box and were observed for 3 min, and a neurological score, from 1 to 4, was assigned; where 1= normal, unaffected gait; 2= slightly affected gait (foot splay, slight hind limb weakness and spread); 3= moderately affected gait (foot splay, moderate hind limb weakness, moderate limb spread during ambulation); and 4= severely affected gait (foot splay, severe hind limb weakness, dragging hind limbs, inability to rear).

2.4.2 Paw Cold Alldynia (Acetone drop test)

Cold chemical thermal sensitivity of the hind paw was assessed using acetone drop method for assessing the reactivity to non-noxious cold chemical stimuli. The rats were placed on the top of a wire mesh grid, allowing access to the hind paws. Acetone (100 µl) was sprayed on the planter surface of the left hind paw of the rat. Cold chemical sensitive reaction with respect to licking, shaking or rubbing the left hind paw was observed and recorded as a paw withdrawal threshold. The cut-off time of 20 sec was maintained.

2.4.3 Motor Coordination Test

Motor coordination was evaluated by a Rota-Rod as described by Jones and Roberts (1968). Rats were placed for 2 min on the rotating rod. The time taken for the falling from the roller, was recorded.

2.4.4 Biochemical Estimations

At the end of the study all the rats were sacrificed by cervical dislocation and the sciatric nerve was isolated immediately from the rats. The sciatic nerve was homogenized in phosphate-buffered saline, pH 7.4, and the homogenates were processed immediately for centrifugation at 1500 rpm, at 4°C to obtain the supernatant for biochemical estimations.

2.4.5 Estimation of superoxide dismutase (SOD)

SOD activity was estimated according to the method of Misra and Fridovich (1972). In brief, the homogenate was centrifuged at 10,000 rpm for the enzyme assay. 100µl of sciatic nerve homogenate was added to 880 µl of carbonate buffer (0.05M, pH 10.2), containing 0.1mM EDTA, and 20 µl of 30 mM epinephrine (in 0.05% acetic acid) was added to the mixture and the optical density values were measured at 480 nm for 4 min on an UV-Vis Spectrophotometer. One unit of activity is expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50%.

2.4.6 Assessment of Lipid Peroxidation (LPO)

LPO was assessed by measuring the formation of thiobarbituric acid reactive substances (TBARS). The reaction mixture contained 0.2 ml of sciatic nerve homogenate, 1.5 ml of acetic acid (pH 3.5, 20 %), 1.5 ml of 0.8 % thiobarbituricacid (0.8 % w/v) and 0.2 ml Sodium dodecyl sulphate (SDS) (8 % w/v). The mixture was heated to boiling for 45 min and TBARS adducts were extracted into 3 ml of 1-butanol and its absorbance was measured at 532 nm and quantified as malondialdehyde (MDA) equivalents using 1,1,3,3-tetramethoxypropane as the standard.
2.4.7 Reduced Glutathione (GSH)
Reduced glutathione was measured according to the method of Ellman (1959). Equal quantity of sciatic nerve homogenate was mixed with 10% trichloroacetic acid and centrifuged to separate proteins. To 0.01 ml of this supernatant, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5, 5-dithio, bis-(2-nitrobenzoic acid) and 0.4 ml double-distilled water were added. Mixture was vortexed and the absorbance was read at 412 nm within 15 min. The concentration of reduced glutathione was expressed as nmol/mg of protein.

2.4.8 Estimation of Total Calcium
0.5 ml of the sample was added to 4.5 ml of deproteinized buffer in a glass centrifuge tube, and placed in a water bath for 3 minutes. Tubes were centrifuged while they were still hot. 0.5 ml of each supernatant and standard were transferred into clean test tubes. For the reagent blank, 0.5 ml of blank solution was prepared by mixing 9 volumes of deproteinization buffer with one volume of water. 5 ml of working colouring reagent was added to each tube, mixed well and then read at 570 nm.

2.4.9 Detection of TNF-α, IL-6, Bcl-2 and Bax expression by Western blotting
Sciatic nerve from each experimental group was minced and homogenized in an ice cold lysis buffer. Homogenates were centrifuged at 4,000×g for 10 min to remove cellular debris. The cytotoxic fractions of the proteins were obtained by collecting the supernatant and centrifuged at 16,000×g for 30 min at 4°C to maximize protein extraction. The membrane fraction was obtained by treating the pellet with alysis buffer supplemented with 1% Triton-X followed by centrifugation at 16,000×g. Protein concentrations were determined using modified Lowry, 1951 method. Proteins were denatured with sodium dodecyl sulfate (SDS) sample buffer and epotopes were exposed by boiling the protein samples at 100°C for 5 min. A 50 µg of protein was loaded and separated by electrophoresis on 12% (w/v) SDS-polyacrylamide gel electrophoresis and proteins were transferred to a nitrocellulose membrane. Immunoblotting was carried out by incubating the membrane in blocking solution (5% dry milk in Tris-buffered saline— Tween 20 (TBST) buffer for 1 h) and then with specific polyclonal antibodies, i.e., TNF-α, IL-6, Bcl-2 and Bax (1:100, Santa Cruz Biotechnology, Inc., USA) for 12 h at 4°C. Membranes were washed three times with TBST buffer and incubated with Horseradish Peroxidase-conjugated secondary antibody (1:5,000, Santa Cruz Biotechnology, Inc., USA) for 1 h at room temperature followed by washing three times with TBST buffer. Bands were visualized on the Odyssey infrared scanner (Biosciences, USA) and quantitatively analyzed by densitometry with Quantity one software (BioRad).

3. STATISTICAL ANALYSIS
Data were expressed as mean ± S.E.M (n=6) and were analyzed using one way analysis of variance (ANOVA) followed by Dunnet’s T test for behavioral tests using Graph pad prism 8.0. A value of P < 0.05 was considered to be statistically significant.

4. RESULTS

4.1 Effect of Rutin on ACR induced alterations in neurological score
Exposure to ACR (50 mg/kg, i.p) for 4 weeks led to progressive gait abnormalities in rats as shown in Graph 1. ACR treated rats developed characteristic symptoms such as foot splay, twisting of hind-limbs and difficulty in ambulation. At the end of 4 weeks rutin treatment caused a significant reduction in neurological scores (P<0.01) compared to ACR administered rats indicating its protective effect. Pregabalin treated rats also showed significant reduction in neurological scores (P<0.001) compared to ACR administered rats.

4.2 Effect of Rutin on cold allodynia
The ACR administration resulted in a significant (P<0.001) cold allodynia, which was significantly ameliorated (P<0.001) by pregabalin and rutin (50 and 100 mg/kg) (Graph 2).

4.3 Effect of Rutin on Motor coordination test
Administration of rutin significantly attenuated (P<0.001) ACR induced decrease in motor performance in a dose-dependent manner as assessed by time spent on rota rod. Rats treated with rutin (50 and 100 mg/kg) and Pregabalin showed improvement in motor performance (P<0.001) when compared to the control group (Graph 3).

4.4 Effect of Rutin on oxidative stress markers
SOD levels were found to be decreased significantly (P<0.001) in the ACR group as compared with the control group. Rutin 50 and 100 mg/kg treated groups significantly (P<0.001) prevented the ACR induced decrease in SOD levels when compared with ACR group (Graph 4). Lipid peroxidation in the SN was determined by measuring MDA content. ACR treated rats showed a significant increase in the level of MDA when compared to control rats. Treatment with rutin at doses of 50 and 100 mg/kg significantly reversed ACR induced increase in MDA levels in SN (Graph 4). ACR treatment significantly decreased (P<0.01) GSH content in SN. Rutin dose-dependently restored the levels of GSH significantly (P<0.01) compared to ACR treated rats (Graph 5). Effect of Rutin on Calcium levels was found to be increased in the ACR group when compared with the control group. However, rutin treatment significantly (P<0.001) prevented the ACR induced increase in calcium levels when compared with the ACR group and values reached normal (Graph 5). Pregabalin 10 mg/kg group showed the similar results comparable to the control group.

4.5 Effect of Rutin on the expressions of TNF-α, IL-6, Bcl-2 and Bax
The expressions of proinflammatory cytokines TNF-α, IL-6, antiapoptotic protein Bcl-2, and pro-apoptotic protein Bax were evaluated to gather insights into inflammatory and apoptotic signaling. ACR caused substantial increase in TNF-α and IL-6 expression (P<0.001) compared with the control group. Whereas rutin modulated the expressions of TNF-α and IL-6 (P<0.001) (Graph 6) near to normal. ACR treatment reduced Bcl-2 expression compared with the control group, while treatment of rutin markedly restored Bcl-2 expression (P<0.001) (Graph 7). In contrast, Bax content in ACR treated rats showed a significant increase as compared with the control group. And this increase in Bax content was significantly (P<0.001) ameliorated by rutin.
Values were expressed as mean ± SEM (n=6); a (P<0.05) vs Control group; b (P<0.001) vs Acrylamide group.

Graph 1 Effect of Rutin on ACR induced alterations in neurological score

Graph 2 Effect of Rutin on cold alldynia

Graph 3 Effect of Rutin on Motor coordination test

Graph 4 Effect of Rutin on SOD and MDA levels
Graph 5 Effect of Rutin on GSH and Calcium levels

Values were expressed as mean ± SEM (n=6); ** (P<0.01), *** (P<0.001), Vs Control group; # (P<0.05), ## (P<0.01) Vs Acrylamide group.

Graph 6a Effect of Rutin on the expressions of TNF-α, IL-6

Graph 6b Western blot image of Effect of Rutin on the expressions of TNF-α, IL-6

Graph 7a Effect of Rutin on the expressions of Bcl-2 and Bax

Values expressed as mean ± SEM (n=6); *** (P<0.001) Vs Control group, # (P<0.01) Vs Acrylamide group.
5. DISCUSSION

The present study investigated the mechanism of the neuroprotective effect of rutin against ACR induced neuropathic pain. Acrylamide has been proved to induce a central and peripheral neuropathy in laboratory animals including rats and monkeys as well as in humans. Furthermore, acrylamide induced neuropathy and neuronal loss which leads to behavioral abnormalities in early development. Acrylamide consumption impairs motor coordination and motor control and reduces motor neurons’ ability to generate action potentials, it causes dysfunction of limbs and abnormal behavior. Neurologic score, Acetone drop test, and motor coordination tests were performed to measure the extent of impairment of motor functions and abnormal behavior of rats. Dose dependently, rutin ameliorated the neuropathological cascade in ACR induced neuropathy, indicating its ameliorative potential. In-vitro and in-vivo studies showed critical role of oxidative stress in ACR-induced neurotoxicity and indicated oxidative stress, down-regulating pro-apoptotic and anti-apoptotic proteins that modulate apoptosis significantly, which supports the anti-inflammatory potential of rutin against ACR-induced neurotoxicity. On par with this, previous studies showed that Ferulic acid and Selenium nanoparticles showed neuroprotective effect by inhibiting the proinflammatory cytokines in ACR induced neurotoxicity in rats. Another major mechanism of ACR-induced neurotoxicity is apoptosis, which is induced by oxidative stress. Bcl-2 is a family of regulatory proteins which include pro-apoptotic and anti-apoptotic proteins that modulate apoptosis. The mechanism underlying acrylamide-induced neuronal injury is through elevated expression of apoptotic markers as Bcl-2 and Bax in the cerebral cortex of rats. The main action of the Bcl-2 family of proteins is the regulation of mitochondrial membrane permeability. Resistance to apoptosis can be by the up-regulation of anti-apoptotic proteins such as Bcl-2 or by the down-regulation of pro-apoptotic proteins such as Bax. Thymoquinone showed neuroprotective effects in ACR induced peripheral nervous system toxicity through modulating MAPK kinase and apoptosis pathways in rats. Taurine attenuated acrylamide-induced apoptosis via PI3K/AKT-dependent manner. ACR downregulated Bcl-2 protein expression while upregulated Bax protein and potentiated apoptosis in PC12 cells. Administration of ACR to rats markedly increased the late apoptosis ratio in neutrophils. The results of our study showed that exposure to ACR reduced the level of Bcl-2 protein & increased Bax protein expression. Rutin up-regulated anti-apoptotic protein Bcl-2 and down-regulated pro-apoptotic protein Bax. These observations clearly demonstrated that rutin offered a significant protection against ACR induced neurotoxicity, possibly due to its anti-apoptotic potential as well.

6. CONCLUSION

In conclusion, targeting oxidative stress, inflammation and apoptotic cascade seems to be promising therapeutic interventions for ACR induced neurotoxicity. Our results clearly indicated that rutin rendered a remarkable protection by reducing the oxidative stress, down-regulating proinflammatory cytokines IL-6, TNF-α, apoptotic mediator directly in chronic hyperalgesia and allodynia. Anti-inflammatory effect of rutin was observed in dexamethasone treated mice. Rutin prevented cognitive impairments by ameliorating oxidative stress and neuroinflammation in the rat model of Alzheimer type. In the present study, ACR administration upregulated the expressions of proinflammatory cytokines IL-6 and TNF-α. Administration of rutin downregulated the expressions of IL-6 and TNF-α significantly, which supports the anti-inflammatory potential of rutin against ACR-induced neurotoxicity. On par with this, previous studies showed that Ferulic acid and Selenium nanoparticles showed neuroprotective effect by inhibiting the proinflammatory cytokines in ACR induced neurotoxicity in rats.

Graph 7b Western blot image of Effect of Rutin on the expressions of Bcl-2 and Bax
Bax proteins and up-regulating anti-apoptotic Bcl-2 protein, thereby decreasing neurological severity, and prevented associated neuronal damage in rats.

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