



Effects of Ferulic Acid in Rotenone Induced Rat Model of Parkinson's Disease

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ABSTRACT

Parkinson's disease (PD) is an age-related neurodegenerative disorders. In order to explore novel agents for treatment of PD, we have evaluated the neuroprotective efficacy of Ferulic Acid (FA) using rotenone-induced rat model of PD. Rotenone was administered 2.5 mg/kg b.wt to male Wistar rats for four weeks to induce the PD. The paradigm for evaluating FA was based on chronic administration for four weeks at the dose of 50 mg/kg, 30 min prior to rotenone administration. In our study, rotenone administration caused significant reduction in endogenous antioxidant like superoxide dismutase, catalase and glutathione. Rotenone challenge induced lipid peroxidation evidenced by increased malondialdehyde (MDA) following perturbation of antioxidant defense. Apart from oxidative stress, rotenone also activated proinflammatory cytokines and enhanced inflammatory mediators such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). The immunofluorescence analysis revealed a significant increase in the number of activated microglia and astrocytes accompanied by significant loss of dopamine neurons (DA) in the substantia nigra pars compacta (SNc) area upon rotenone injection. However, treatment with FA rescued DA neurons in SNc area and nerve terminals in the striatum from the rotenone insult. FA treatment also restored antioxidant enzymes, prevented depletion of glutathione and inhibited lipid peroxidation. Following treatment with FA, the inflammatory mediators such as COX-2 and iNOS and proinflammatory cytokines were also reduced. Further, the results were supported by a remarkable reduction of Iba-1 and GFAP hyperactivity clearly suggests attenuation of microglial and astrocytic activation. These results suggests that FA has promising neuroprotective effect against degenerative changes in PD and the protective effects are mediated through its antioxidant and anti-inflammatory properties.

1. Introduction

The pathological hallmark of Parkinson's disease (PD) includes intracytoplasmic inclusion known as lewy bodies and consequently leading to dopamine (DA) depletion in the substantia nigra pars compacta (SNc) area [1, 2]. However, the main cause of the disease is poorly understood. Convincing number of pharmacological, genetic and clinical studies including postmortem PD brain shows that mitochondrial defects, increased reactive oxygen species (ROS) and induction of inflammatory mediators play very critical role in developing PD [3, 4]. Oxidative stress and inflammatory processes which contributes loss of dopaminergic neurons in the brain receives enormous attention for therapeutic target in PD. Many pharmacotherapeutic interventions have been tested in order to target oxidative stress and inflammation, an intimately connected process. Till date the available agents only improve the symptoms of PD, therefore there is still need of disease-modifying or preventive agents for PD [5, 6]. In recent years, besides the other pharmacotherapeutic approaches, treatment with antioxidants has gradually getting preference as disease-modifying strategies in the therapy of [7, 8].

It is convincing that the naturally occurring molecules possessing antioxidant and anti-inflammatory activities along with multiple other pharmacological properties could be effective in preventing or halting these neurodegenerative processes [9-12]. In this study, we have chosen ferulic acid (FA), an important component of widely used medicinal herbs and possess several additional benefits. Pure FA is a yellowish powder and belongs to the family of hydroxycinnamic acid. The chemical structure of FA is very similar to that of curcumin, one of the highly studied natural molecules with potent neuroprotective effects (Fig. 1). FA is highly abundant in the leaves and seeds of many plants, but especially in cereals such as brown rice, whole wheat and oats. It has been credited with many

pharmacological properties including neuronal progenitor cell proliferation, anti-inflammatory, antioxidant and neuroprotective activities [13-21]. In the present study, we investigated the effects of FA in rotenone (ROT) induced rat model of PD. Based on the hypothesis that oxidative stress and neuroinflammation underlie neurodegeneration, the results may provide an alternative and early intervention approach to prevent and halt the progression of neurodegenerative changes in PD.

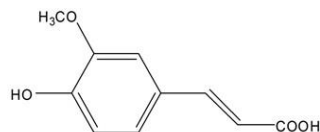


Fig. 1 Chemical structure of Ferulic acid

2. Experimental Methods

2.1 Drugs and Chemicals

Polyclonal rabbit anti cyclo-oxygenase-2 (COX-2), anti-inducible nitric oxide synthase (iNOS) and anti-gial fibrillary acidic protein (GFAP) were purchased from Abcam, Cambridge, MA, USA. Anti-ionized calcium binding adaptor molecule-1 (Iba-1) polyclonal rabbit was purchased from Wako Chemicals, USA. Polyclonal rabbit anti-tyrosine hydroxylase antibody was obtained from Novus Biologicals, USA. Alexa fluor 488/594 conjugated secondary goat anti-rabbit antibodies were purchased from Life Technologies, USA. Rotenone, ferulic acid and the assay kit for reduced glutathione (GSH) and other reagents of analytical grade were purchased from Sigma-Aldrich, St. Louis, MO, USA.

2.2 Experimental Animals

Six- to seven-months old male Wistar rats (280-300 g) bred in the animal research facility of the College of Medicine and Nursing Sciences,

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Huzhou University. A maximum of four rats were housed per cage and were acclimatized for one week to the laboratory conditions prior to the start of the experiment. The animals were housed under standard laboratory conditions of light and dark cycle. The animals had access to commercially available rodent food and water *ad libitum*. All the experiments were carried out between 09:00 and 15:00 h. The experimental protocol for animal experimentation was approved by the Animal Ethics Committee of Huzhou University.

2.3 Experimental Design

Rotenone (ROT) was first dissolved in dimethyl sulfoxide (DMSO) at 50X stock solution and diluted in sunflower oil to obtain a final concentration of 2.5 mg/mL. For the induction of PD in rats, ROT (2.5 mg/kg body weight) was administered intraperitoneally once daily for four weeks. The regimen used in the current study for the induction of Parkinsonism in rats following rotenone administration was adopted with slight modification from the previous report [22]. To test the neuroprotective efficacy of FA, it was dissolved in sterile water and injected i.p. at a dose of 50 mg/kg body weight once daily for four weeks, 30 min prior to ROT administration. The control group received the similar amount of vehicle only. The rats were divided into four experimental groups, each containing eight rats. The experimental groups were as follows:

- Group I: Vehicle-injected control group (C)
- Group II: Rotenone-injected and vehicle-treated group (ROT)
- Group III: Rotenone-injected and FA-treated group (ROT+FA)
- Group IV: FA-only injected group (FA)

2.4 Tissue Preparation for Biochemical Studies

At the end of four weeks, animals were anaesthetized with pentobarbital (40 mg/kg b.wt.) and cardiac perfusion was carried out using 0.01 M phosphate-buffered saline (PBS) pH 7.4 to wash out the blood. The brains were quickly removed and placed on an ice-plate where the two hemispheres were separated. The midbrain and striatum region were dissected out from one hemisphere and immediately frozen in liquid nitrogen for further use. The other hemisphere was post-fixed in 4 % paraformaldehyde solution for 48 hours and subsequently exchanged with 10 % sucrose solution for three times a day for three consecutive days at 4 °C prior to cryostat sectioning.

2.5 Biochemical Studies

Mid brain of animals from each group were collected individually and homogenized in KCl buffer (Tris-HCl 10 mM, NaCl 140 mM, KCl 300 mM, EDTA 1 mM, Triton X-100 0.5 %) at pH 8.0 supplemented with protease and phosphatase inhibitor. The tissue homogenates of each sample were centrifuged at 14,000 g for 20 min at 4 °C to obtain the post-mitochondrial supernatant (PMS) for estimation of antioxidant enzymes, lipid peroxidation and pro-inflammatory cytokines using spectrophotometric measurements and enzyme-linked immunosorbent assay (ELISA).

2.6 Estimation of Lipid Peroxidation

Malondialdehyde (MDA), detection kit was used as per manufacturer's instruction to determine the amount of lipid peroxidation. Briefly, 250 μ L samples or calibrator are incubated in the presence of 250 μ L acid reagent and 250 μ L thiobarbituric acid and vortex vigorously. Samples were incubated for 60 min at 60 °C and then centrifuged at 10,000 Xg for 2-3 min. Reaction mixture was transferred to cuvette and recorded the spectra at 532 nm. The results were expressed as μ molar MDA/mg protein. The MDA kit was procured from North West Life science (Vancouver, WA, USA).

2.7 Estimation of Reduced Glutathione (GSH)

Commercially available reduced glutathione (GSH) kit was used for the estimation of GSH as per manufacturer's instructions. Briefly, the samples were first deproteinized with 5 % 5-sulfosalicylic acid solution and centrifuged to remove the precipitated protein and then the supernatant was used measure glutathione. Ten microliter (10 μ L) of sample and standard of different concentration were incubated for 5 min with 150 μ L of working mixture (assay buffer+5, 5'-Dithiobis (2-nitrobenzoic acid) +glutathione reductase) in 96 well plate. Diluted 50 μ L NADPH solution was added to each well and mixed it properly. Absorbance of the samples was measured at 412 nm with the kinetics for 5 min by using the micro plate reader. The results were expressed as μ molar GSH/mg protein.

2.8 Estimation of the Activities of Antioxidant Enzymes

Cayman assay kits (Cayman Chemicals Company, Ann Arbor, MI, USA) were used to determine the activities of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) following manufacturer's instructions. Briefly CAT was estimated by adding the 20 μ L samples or standards of different concentration to 100 μ L assay buffer and 30 μ L methanol in 96 well plate. Twenty microliter (20 μ L) H2O2 was added to initiate the reaction and incubated for 20 min at room temperature (RT). Thirty microliter (30 μ L) potassium hydroxide was added to terminate the reaction and subsequently 30 μ L catalase purpald and 10 μ L catalase potassium periodate were added. The plate was incubated for five minutes at RT on shaker and absorbance was read at 540 nm by using the micro plate reader. For SOD measurement, ten microliter (10 μ L) samples or standard were added in the well being used of 96 well plate. Xanthine oxidase 20 μ L was added to each well to initiate the reaction. Plate was shaken for few seconds and then covered with plate cover and incubated for 30 min at RT. Absorbance was read at 450 nm by using the micro plate reader. The CAT activity was expressed as nmol/min/mg protein and the SOD activity was expressed as Units/mg protein.

2.9 Estimation of Pro-inflammatory Cytokines by ELISA Assays

Commercially available ELISA kits for Interleukin-1 β (IL-1 β), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF- α) were purchased from Biosource International, Camarillo, CA, USA. The level of IL-1 β , IL-6 and TNF- α were estimated as per the manufacturer's instructions. Briefly, 96 well plate was coated with the 100 μ L diluted capture antibody for overnight at RT. Aspirate each well and washed with wash buffer (0.05 % tween 20 in PBS 0.01 M pH 7.4). Plate was blocked by adding the 300 μ L reagent diluent (1 % bovine serum albumin in PBS) for 1 hr and washed with wash buffer. Hundred microliter (100 μ L) of samples or standard of different concentration was added to the well and incubated for 2 hrs. Each well was exchanged with 100 μ L detection antibody and then incubated for 2 hrs at RT. The well was then exchanged with 100 μ L working solution (1:200) of streptavidin horse raddish peroxidase and further incubated for 20 min. The wells were exchanged with 100 μ L substrate solution and incubated for 20 min. Fifty microliter (50 μ L) of stop solution (2N H₂SO₄) was added and gently tap the plate to ensure proper mixing. Optical density of each well was read immediately at 450 nm using micro plate reader. The results were expressed as pg/mg protein.

2.10 Immunofluorescence Staining of Tyrosine Hydroxylase (TH) for The Assessment of TH+ Neurons In SNC and TH Immunoreactive (TH-ir) Dopamine Nerve Fibers in the Striatum

Rat brains were collected as mentioned above and sectioned for TH staining. Briefly, 14 μ m thick coronal brain sections were cut at the level of the striatum and SNc using a cryostat (Leica, Germany). Sections were washed twice with 0.01 M Phosphate buffered saline (PBS) pH 7.4 and then incubated with blocking reagent (10 % normal goat serum in PBS 0.3 % Triton-X 100) for 1 hr. Further, the sections were incubated with the primary polyclonal rabbit antibody against TH (1:500) for overnight at 4 °C. Sections were washed and incubated with fluorescent secondary antibody alexa 594 anti-rabbit (1:1000) for 1 hr at RT. Sections were then washed and mounted using mounting media flourosshield (Sigma Aldrich, USA). The images were taken under fluorescent microscope EVOS FL (Life Technologies, USA).

2.11 Immunofluorescence Staining of GFAP and Iba-1

Immunofluorescence staining was performed in the striatum to examine the activation of GFAP positive astrocytes and Iba-1 positive microglia. Brain sections at the level of the striatum were washed twice with PBS and incubated with blocking reagent (10 % normal goat serum in PBS 0.3 % Triton-X 100) for 1 hr. The sections were then incubated with the primary polyclonal rabbit antibodies against GFAP (1:1000) and Iba-1 (1:1000) for overnight at 4 °C. The sections were washed and incubated with fluorescent secondary antibody alexa 488 anti-rabbit for 1 hr at RT. Sections were then washed and mounted using mounting media flourosshield (Sigma Aldrich, USA). The images were taken under fluorescent microscope EVOS FL (Life Technologies, USA).

2.12 Assessment of TH-ir Dopaminergic Neurons and TH-ir Dopamine Nerve Fibers Loss

To determine the loss of TH-immuno positive neurons in the SNc area, three different levels of the medial terminal nucleus (MTN) region were counted and the average was presented as percentage. Loss of striatal fibers was evaluated by measuring the optical density of TH-ir dopaminergic fibers in the striatum using NIH Image J software. The

optical density of TH-ir fibers at three different field of each section (3 sections/rat) with equal area with in the striatum was measured for each rats and an average of the 3 areas was calculated and represented as percentage. The optical density of the overlying cortex was taken as background measure and subtracted from the value generated from the striatum. The counting of TH-immuno positive neurons and optical density of the TH-ir fibers were carried out by an investigator blind to the experimental groups.

2.13 Assessment of Activated Astrocytes and Microglia in the Striatum

A minimum of three coronal sections of the similar level of striatum from each animal were used to analyze the number of activated astrocytes and microglia. From each section, activated astrocytes and microglia were counted from randomly chosen three different field of equal area by using the Image J software (NIH, USA).

2.14 Western Blot Analysis of COX-2 and iNOS

Striatal tissue from each experimental group was homogenized in RIPA buffer supplemented with protease and phosphatase inhibitor and centrifuged at 15,000 rpm for 20 min. The samples of cytoplasmic fractions containing equal amounts of protein (35 µg) were separated in 10 % SDS-polyacrylamide gel electrophoresis. The proteins were transferred onto PVDF membrane and incubated overnight at 4 °C with specific primary rabbit polyclonal antibodies against COX-2 (1:1000) and iNOS (1:500) followed by horseradish peroxidase (HRP)-conjugated secondary anti-rabbit antibody. The protein recognized by the antibody was visualized using an enhanced chemiluminescence pico kit (Thermo Scientific, Rockford, IL, USA). The blots were stripped and re-probed for β-actin (1:5000; monoclonal mouse, Millipore, MA, USA) as a loading control. The intensity of the bands was measured by densitometry and quantified (n=2) using Image J software (NIH, USA).

2.15 Protein Estimation

The protein content was estimated using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) following the manufacturer's instructions.

2.16 Statistical Analyses

The data were expressed as the mean value ± SEM. Normal distribution of data was first by Shapiro-Wilk test. The data for all studies were analyzed using One-way analysis of variance (ANOVA) followed by Tukey's test to calculate the statistical significance between various groups using Graph Pad InStat software. In all the tests, the criterion for any statistically significant difference was set at $p < 0.05$.

3. Results and Discussion

3.1 FA Prevents Rotenone-Induced Dopamine (DA) Neurons Loss in SNC and Decreases of Striatal Dopamine (DA) Nerve Terminal Density

Following administration of ROT, it causes a significant ($p < 0.05$) loss of DA neurons in the SNc area when compared to vehicle-injected control rats (Fig. 2). Interestingly, treatment of FA prior to ROT-injection, rats showed significant protection of DA neurons when compared to ROT only injected rats. The dopamine neurons of SNc project their processes to the striatum where the terminal fibers are enriched with the dopamine transporter (DAT). Therefore, we examined whether the loss of dopamine neurons in the SNc area correlated with terminal loss as evaluated by the intensity of TH-ir dopamine fibers of the striatum. As expected, we observed a significant decrease of the intensity of TH-ir fibers in ROT only treated animals when compared to vehicle-treated controls. However, prior to ROT-treatment, the animals that received FA showed significant increase in the intensity of TH-ir fibers suggesting neuroprotective effect mediated by FA (Figs. 2A and C). Therefore, our results suggest that treatment with FA is beneficial to dopamine neurons which protect them from rotenone induced toxicity.

3.2 FA Inhibits Lipid Peroxidation and Preserves Glutathione Level in Rotenone-Treated Animals

The antioxidant property of FA led us to test whether the neuroprotective effects of FA is through alteration of the lipid peroxidation and glutathione level. We observed that rats administered with ROT showed a significant ($p < 0.01$) increase in lipid peroxidation product, known as MDA as compared with the control group (Fig. 3A). Similarly, ROT administration also caused a significant ($p < 0.01$) decrease in GSH levels when compared to the control group (Fig. 3B). As expected, the ROT-

treated rats that received FA significantly ($p < 0.05$) attenuated the rise in MDA level (Fig. 3A) and improved the GSH levels (Fig. 3B) when compared to the ROT group.

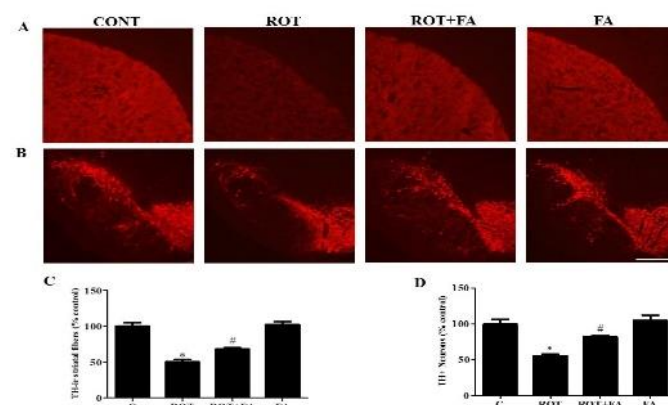


Fig. 2 Immunofluorescence staining of tyrosine hydroxylase (TH) to detect the expression of TH-ir dopamine nerve fibers in the striatum and number of dopaminergic (DA) neurons in the substantia nigra compacta (SNc). The scale bar is 100 µm. (A) The expression of TH-ir fibers in the striatum of CONT, ROT, ROT+FA and FA only group rats. (C) A significant ($*p < 0.05$) decrease in the TH-ir fibers were observed in the ROT group rats as compared to CONT rats. While FA treatment significantly ($#p < 0.05$) attenuated the loss of TH-ir fibers in the ROT+FA group rats as compared to the ROT rats. CONT rats and FA only injected rats did not show any remarkable loss of TH-ir fibers. (B) The number of TH-ir neurons was decreased in the SNc of rotenone (ROT) injected rats as compared to control (CONT) group. While FA treatment shows profound attenuation of TH-ir neurons in ROT+FA injected rats as compared to ROT rats. (D) The number of TH-ir positive DA neurons in the SNc was counted from each group. The number of DA neurons was significantly ($*p < 0.05$) more in the SNc of CONT group when compared to ROT group. FA treatment significantly ($#p < 0.05$) protected the DA neurons from the ROT-induced neuronal death. There is no significant difference were observed in the DA neurons of CONT and FA only group rats. Values are expressed as percent mean ± SEM (n=3).

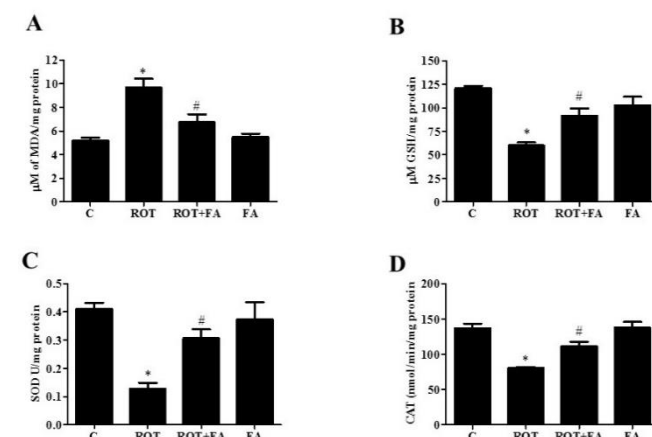


Fig. 3 Rotenone (ROT) injections caused significant ($*p < 0.01$) increase in MDA (A) and decreased level of GSH (B) in the mid brain of ROT rats as compared to control (CONT) group. FA treatment in ROT+FA group significantly ($#p < 0.05$) decreased level of MDA and increased ($#p < 0.05$) the level of GSH. ROT injection also causes significant ($*p < 0.01$) decrease in the activity of SOD (C) and CAT (D) as compared to CONT group. FA treatment significantly ($#p < 0.05$) improved ROT-induced decrease in SOD and CAT activity as compared to the ROT-injected rats. Values are expressed as mean ± SEM (n=6-8).

3.3 Modulation of Antioxidant Enzymes Activity with FA

Since FA normalized the lipid peroxidation, we think that FA also affects the SOD and CAT activities to prevent peroxidation. In this way, we measured the activities of antioxidant enzymes, SOD (Fig. 3C) and CAT (Fig. 3D). We observed that rotenone injection significantly ($p < 0.01$) decreased the SOD and CAT activities to the animals when compared with control animals. However, treatment with FA significantly ($p < 0.05$) increased the activity of SOD (Fig. 3C) and CAT (Fig. 3D) when compared with the ROT treated group. We did not observe any significant changes in SOD (Fig. 3C) and CAT (Fig. 3D) activity between the normal controls and animals injected with FA only.

3.4 Reduced Activation of Iba-1 and GFAP with FA

The expression of GFAP and Iba-1 are considered as markers of ROS production and inflammatory process. In the immunofluorescence

staining, remarkably high activation of GFAP was observed in ROT-injected rats when compared to normal control rats, which indicates increase in number and size of astrocytes (Fig. 4A and C). However, treatment with FA attenuated the activation of hypertrophied astrocytes in rats administered ROT when compared to the animals of ROT group. Similarly, a significant increase in the activation of Iba-1 positive microglia was observed as an indicator of inflammatory response in ROT-injected rats (Fig. 4B and D) whereas treatment with FA significantly prevented the ROT-induced microglial activation represented by Iba-1 expression. These data clearly suggest that FA blocks the activation of microglia and astrocyte activation.

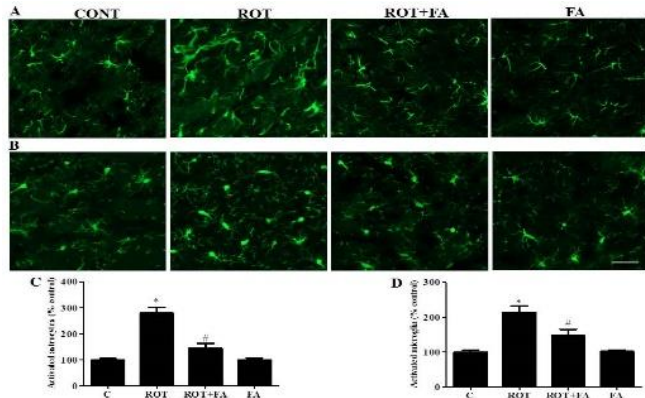


Fig. 4 Immunofluorescence staining to detect the expression of glial fibrillary acidic protein (GFAP) positive astrocyte (green) and ionized calcium binding adaptor molecule-1 (Iba-1) positive microglia (green) in the striatum of CONT, ROT, ROT+FA and FA only rats. Profound expression of GFAP positive astrocytes (A) and Iba-1 (B) was found in the ROT rats as compared to CONT rats. While FA administration to ROT injected rats showed moderate staining of GFAP and Iba-1 in the ROT+FA rats as compared to ROT injected rats (Scale bar 200 μ m). Quantitative analysis of activated astrocytes (C) and microglia (D) revealed that a significant (* $p < 0.05$) increase in number of activated astrocytes and microglia were observed in ROT group rats as compared to CONT rats. Although FA administration significantly (# $p < 0.05$) reduced the number of activated astrocytes and microglia in ROT+FA group rats as compared to ROT rats. CONT rats and FA only injected rats did not show any marked difference in the activation of astrocytes and microglia. Values are expressed as percent mean $\pm$ SEM ($n = 3$).

3.5 Reduced Activation of Pro-Inflammatory Cytokines with FA

We also measured the concentration of proinflammatory cytokines IL-6, IL-1 β and TNF- α in response to ROT challenge. Significant ($p < 0.01$) increase of IL-6 (Fig. 5A), IL-1 β (Fig. 5B) and TNF- α (Fig. 5C) level were observed in the ROT-injected animals when compared to the control group. However, FA treatment significantly ($p < 0.05$) decreased the level of all these cytokines in ROT-treated animals when compared to the ROT control group (Fig. 5A-C). FA treated animals did not show any significant changes in the level of pro-inflammatory cytokines when compared to control group animals.

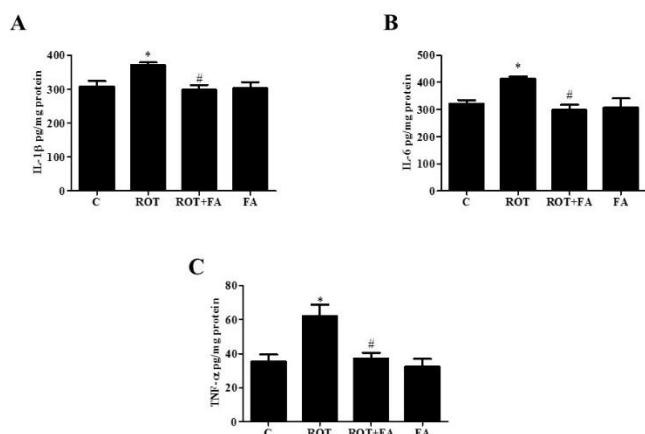


Fig. 5 IL-1 β , IL-6 and TNF- α was measured by enzyme linked immunosorbent assay (ELISA) in the mid brain of CONT, ROT, ROT+FA and FA only group rats. The level of IL-1 β (A), IL-6 (B) and TNF- α (C) was found significantly (* $p < 0.05$) increased in ROT group when compared to CONT rats. While FA treatment significantly (# $p < 0.05$) decreased the ROT-induced increase of these powerful cytokines in ROT+FA rats. There is no significant difference were observed in these cytokines between CONT and FA only group rats. Values are expressed as mean $\pm$ SEM ($n = 6-8$).

3.6 Effect of FA on the Expression of Inflammatory Mediators: COX-2 and iNOS

We further investigated the expression of COX-2 and iNOS using western blots in tissue lysates isolated from striatum region (Fig. 6A-B). An increase in COX-2 expression (Fig. 6C) was observed in response to ROT injection (145.82 %) when compared to the control group (100%). However, following treatment with FA in ROT-administered rats, a reduction in the level of COX-2 (116.51%) was observed when compared to rotenone treated rats. Similarly, we also observed an increase (137.64 %) in iNOS expression (Fig. 6D) in the ROT-injected animals when compared to the control group. Similar to the reduction in COX-2 following treatment with FA, a decrease (28.41 %) in iNOS induction was also observed when compared to rotenone treated.

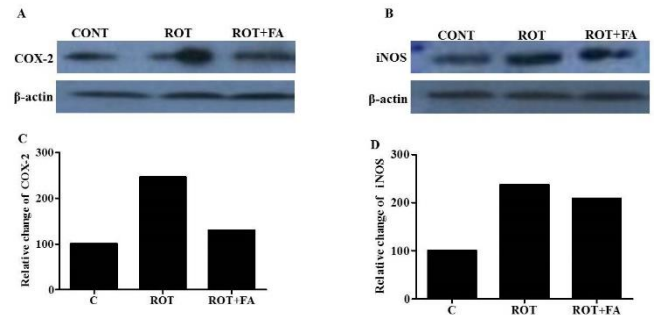


Fig. 6 Expression level of striatum's COX-2 (A) and iNOS (B) were determined by Western blot. COX-2 shows (145.82 %) increase in ROT group as compared to the CONT group. FA treatment followed by ROT injection decreased the expression of COX-2 by 116.51 as compared to ROT group (C). Similarly, iNOS expression was increased 137.64 % in ROT group as compared to CONT group. FA treatment decreased iNOS expression by 28.41% as compared to ROT group (D). ($n = 3$)

In the current study, we have aimed to investigate the effect of FA against a ROT-induced rat model of PD. The immunofluorescence analysis of brain sections revealed significant degeneration of the dopaminergic neurons in the SNc area following chronic challenge of ROT to rats. The results from our study showed that ROT had a significant effect on SNc neurons and consistent with the results from previous studies [23, 24]. TH-neurons in the SNc project their nerve terminal to striatum. Therefore, loss of dopamine neurons in the SNc area will result in the retraction of dopamine-nerve terminal in the striatum region. We observed that ROT-administration significantly reduces the number of TH-immuno-reactive neurons in the SNc area and dopamine nerve terminal density in the striatum. It is noteworthy to mention that the loss of dopamine neurons in the SNc and decrease in the density of nerve terminals in the striatum are considered to be a pathological index of PD. Interestingly, we found that pretreatment with FA to ROT-injected rats provided significant protection to dopamine neurons as well as it preserves nerve terminals integrity (Fig. 2A-D). Recently, FA has been shown to prevent dopamine neurons from MPTP-induced toxicity, inhibits Alzheimer-like pathology, and cerebral ischemia suggesting that this drug has beneficial effect on neurons and it can cross the blood brain barrier to provide such effects to the brain [17, 18, 20, 21].

Rotenone is a known mitochondrial complex I inhibitor that on exposure to cells leads to reactive oxygen generation and reduce ATP production. The brain is highly rich in polyunsaturated fatty acids, susceptible to oxidative damage as a result of mitochondrial dysfunction or electron transport chain inhibition as seen in rotenone exposure. Since FA has antioxidant activity, we tested whether its neuroprotective effect is due to scavenging the reactive oxygen species (ROS) generated by rotenone. We observed a significant increase in the level of MDA suggestive of lipid peroxidation in the brain tissues after 4 weeks of chronic ROT administration. It is noteworthy to mention that the brain is rich in polyunsaturated fatty acids; it is more sensitive to free radical attack. Interestingly, we observed that pre-treatment with FA results in the decrease of elevated MDA levels following ROT administration and clearly suggestive of antioxidant role of FA.

The endogenous antioxidant defense network constitutes enzymatic (SOD and CAT) and non-enzymatic (GSH) molecules to scavenge the oxygen free radicals which otherwise lead to oxidative damage [25, 26]. The reduction in the GSH contents from brain tissues following ROT administration shows its depletion due to its reaction towards the reduction of oxidative stress. In contrast, a significant recovery or the restoration of GSH to control level following FA treatment in ROT-administered rats demonstrates its antioxidant and free radical scavenging activity. The increase in the oxidative damage is often correlated with a simultaneous decline in the activities of the intracellular

antioxidant enzymes SOD and CAT. Following ROT exposure, a significant reduction in the activities of SOD and CAT was observed in the midbrain tissues. However, on simultaneous administration of FA and ROT, a significant improvement in the activities of SOD and CAT demonstrated the antioxidant activity of FA. The enhanced enzyme activity in FA-treated, ROT-induced rats are suggestive of decreased generation of H_2O_2 . Although FA has been shown previously neuroprotective against MPTP [20], this is the first time that evidence has been presented to show that this neuroprotective effect is mediated through its antioxidant activity in a chronic model of PD.

Neuroinflammation plays critical role in the pathophysiology of PD [8]. In our current study, following ROT administration, increased level of pro-inflammatory cytokines, IL-1 β , IL-6 and TNF- α , in the midbrain samples was observed in agreement with previous studies [16, 27]. To explore the possible effects of FA on the inflammatory pathway, levels of various inflammatory molecules and pro-inflammatory cytokines were investigated. Interestingly, FA administration to ROT-treated rats significantly reduced IL-1 β , IL-6 and TNF- α induction along with microglial activation. This result suggested that FA might counteract the activation process of microglia thereby controlling the levels of IL-1 β , IL-6 and TNF- α in agreement with previous studies. It has been reported that pro-inflammatory cytokines cause phosphorylation and degradation of the inhibitory factor I κ B which results in the activation of the NF- κ B signaling cascade [16]. NF- κ B activation also promotes the production of iNOS from the activated microglia causing increased production of nitric oxide which in turn has a detrimental effect on proteins and DNA [28]. In current study, we also observed a remarkable increase in COX-2 and iNOS following ROT challenge that is consistent with earlier reports [27]. However, treatments of FA to ROT-injected rats reduce the up-regulation of COX-2 and iNOS levels. In addition to oxidative stress and inflammation, we also observed microglial activation upon chronic ROT challenge as evidenced by increased expression of Iba-1 and GFAP which are considered to be markers of ROS production and any inflammatory process. In contrast, treatment with FA blocks the activation of microglia and astrocytes evidenced by attenuation of the activation of GFAP and Iba-1 respectively. The data clearly suggest that FA has the potential to protect the dopaminergic neurons and ameliorate the microglial and astrocyte activation, a starting point in dopaminergic neurodegeneration and neuronal loss.

4. Conclusion

Taken together, our present findings suggest that FA may be used as potent neuroprotective agent in the prevention of PD. Though, the exact molecular mechanism by which FA restores the antioxidant capacity or inflammatory response requires further investigation.

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