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ORIGINAL ARTICLE

Anti-inflammatory Activity of Ursolic Acid in MPTP-Induced Parkinsonian Mouse Model

Sachchida Nand Rai[1] · Walia Zahra[1] · Saumitra Sen Singh[1] · Hareram Birla[1] · Chetan Keswani[1] · Hagera Dilnashin[1] · Aaina Singh Rathore[1] · Rajan Singh[1] · Rakesh K. Singh[1] · Surya Pratap Singh[1]

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Abstract

Neuroinflammation plays an important role in the progression of Parkinson's disease (PD) and hence may represent a target for treatment. The drugs used currently for PD only provide symptomatic relief and have adverse effects in addition to their inability in preventing degeneration of neurons. Flavonoids show potent antioxidant and anti-inflammatory activities which is very valuable for the health of human beings. Thus, in the present study, we have tried to explore the anti-inflammatory activity of orally given ursolic acid (UA) (25 mg/kg bwt), a pentacyclic triterpenoid in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-intoxicated mouse model. Significant severe oxidative stress and biochemical alterations have been seen in Parkinsonian mice after MPTP intoxication. Whereas, UA administration has significantly rescued the harmful consequence of MPTP intoxication. Ionized calcium-binding adaptor molecule 1 (Iba1), tumor necrosis factor-alpha (TNF-α), and nuclear transcription factor-κB (NF-κB) were seen to be altered in the substantia nigra pars compacta (SNpc) of MPTP-intoxicated mice through immunohistochemical studies. The changes in the expression level of these parameters primarily suggest increased inflammatory responses in MPTP-intoxicated mice as compared with the control. However, UA have significantly reduced these inflammatory parameters (Iba1 and TNF-α) along with transcription factor NF-κB, which regulates these inflammatory parameters and thus have inhibited MPTP-induced neuroinflammation. The immunoreactivity of tyrosine hydroxylase (TH) was considerably increased by UA treatment in the SNpc of Parkinsonian mice. The neuroinflammation and neurodegeneration along with impairments in biochemical and behavioral parameters were found to be reversed on treatment with UA. Thus, UA has shown potent anti-inflammatory activity by preventing the degeneration of dopaminergic neurons from MPTP-induced Parkinsonian mice.

Keywords Parkinson's disease · Ursolic acid · MPTP · Neuroinflammation

Sachchida Nand Rai and Walia Zahra contributed equally to this work.

✉ Surya Pratap Singh
suryasinghbhu16@gmail.com

Sachchida Nand Rai
raibiochem@gmail.com

Walia Zahra
waliazahra19@gmail.com

Saumitra Sen Singh
saumits77@gmail.com

Hareram Birla
harerambirla76@gmail.com

Chetan Keswani
chetankeswani@rediffmail.com

Hagera Dilnashin
hagerad.1910@gmail.com

Aaina Singh Rathore
aainarathore.ar@gmail.com

Rajan Singh
rajansingh0555@yahoo.com

Rakesh K. Singh
rakesh_bc@bhu.ac.in

[1] Department of Biochemistry, Institute of Science, Banaras Hindu University, Varanasi 221005, India

Introduction

Ursolic acid, (UA; 3b-hydroxy-12-urs-12-en-28-oic acid), a natural pentacyclic triterpenoid carboxylic acid is present in a wide variety of plants, including apples, basil, bilberries, cranberries, peppermint, rosemary, and oregano (Liu 1995). Several biochemical and pharmacological effects of UA such as anti-inflammatory, antioxidative, anti-proliferative, anti-cancer, anti-mutagenic, anti-atherosclerotic, anti-hypertensive, anti-leukemic, and antiviral properties are reported in a number of experimental systems (Ikeda et al. 2008; Tsai and Yin 2008). UA was shown to exhibit anti-inflammatory effects in RAW264.7 cells (mouse monocyte macrophage cell line) by attenuating inducible nitric oxide synthase (iNOS) and cycloxygenase-2 (COX-2) expression (Suh et al. 1998; Ryu et al. 2000). The anti-proliferative, anti-tumor, and anti-leukemic properties have been shown to be mediated via suppression of nuclear transcription factor-κB (NF-κB) activation and inhibiting the expression of NF-κB-regulated genes like lipoxygenase, tumor necrosis factor-alpha (TNF-α), COX-2, matrix metalloproteinase-9 (MMP-9), and iNOS (Shishodia et al. 2003; Ringbom et al. 1998; Cha et al. 1996; Najid et al. 1992). Since dysregulation of the NF-κB function is associated with inflammation, any molecule that interferes with NF-κB activation is a potential candidate for therapeutic strategy in the treatment of inflammatory diseases.

Dying neurons indirectly and toxic, pathogenic, or endogenous proteins directly cause the microglial cells to get activated (Hirsch and Hunot 2009; More et al. 2013). Stimulated microglia produces proinflammatory mediators such as prostaglandin E2, nitric oxide (NO), TNF-α, interleukin (IL)-1β, and IL-6 (Cao et al. 2010; Choi et al. 2009), when intoxicated by lipopolysaccharide (LPS) (Kim et al. 2012a), 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) (Gao et al. 2002; McGeer et al. 2003) and other neurotoxins (Choi et al. 2003; Cicchetti et al. 2002). Microglia on excessive activation leads to the abnormal increase in the production of the chemokines, cytokines, complement cascade proteins, prostaglandins, reactive oxygen species (ROS), and reactive nitrogen species (RNS) in post-mortem Parkinson's disease (PD) brains as revealed through pre-clinical and clinical studies (Barnham et al. 2004; Koppula et al. 2012). Dopaminergic (DA) neurons can get adversely affected by the proinflammatory factors and cytokines, predominantly present in the activated microglial cells (Knott et al. 2000; Nagatsu et al. 2000). Multiple releases of neurotoxic factors from activated microglia cause the damage of DA neurons in vivo (Block et al. 2004; Gao et al. 2003; Qin et al. 2002; Wu et al. 2005).

In neurodegenerative disorders, UA exhibited significant inhibition of dopamine-b-hydroxylase (214 mmol/L), weak inhibition of MAO-B (monoamine oxidase-B) (780 mmol/L), and no inhibition against MAO-A (monoamine oxidase-A) (Kim et al. 2012b). In common forms of familial PD, ursocholanic acid rescues mitochondrial function (Mortiboys et al. 2013).

In our lab, we have shown that UA (25 mg/kg bwt) have potent antioxidative activity in MPTP-induced Parkinsonian mouse model (Rai et al. 2016). So, in this paper, we have tried to investigate the anti-inflammatory activity of UA by assessing various inflammatory parameters.

Material and Method

Chemical Required

Ursolic acid was purchased from MP Biomedicals (Cat No-158253). 1-Methyl-4-phenyl-1,2,3,6-tetra hydropyridine (MPTP) and normal goat serum (NGS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Griess reagent and 1,4-diazabicyclo[2.2.2]octane (DABCO) were procured from HiMedia (Mumbai, India). Protein estimation kit (Bradford) was purchased from GeNei™; hydrogen peroxide (H_2O_2) and potassium dichromate were purchased from Merck (Darmstadt, Germany). Acetic acid, bovine serum albumin (BSA), potassium chloride, disodium hydrogen phosphate, ammonium chloride, and sodium dihydrogen phosphate were procured from Sisco Research Laboratories (SRL; Mumbai, India). Paraformyldehyde and sodium nitrite were purchased from Loba Chemie, India. Primary antibodies for tyrosine hydroxylase (TH; SC-25269) were procured from Santa Cruz, Biotechnology (Santa Cruz, CA, USA), and the primary antibodies for tumor necrosis factor-alpha (TNF-α; ab1793), ionized calcium-binding adaptor molecule1 (Iba1; ab 5076), and nuclear factor-κB (NF-κB; ab16502) were purchased from Abcam Life Science, Biogenuix Medsystems Pvt. Ltd. (New Delhi, India); secondary fluorescent tagged antibodies for IHC cy2 conjugated and cy3 conjugated were procured from Merck Millipore and Chemicon, respectively.

Experimental Animals

From the animal research facility of Banaras Hindu University, Varanasi, male Swiss albino mice (8–10 weeks, 25–30 g) were purchased. Animals were acclimatized under standard laboratory conditions for about a week by keeping light and dark cycles of 12 h prior to the experiment. Standard rodent food was purchased from the market and was given to the mice along with water ad libitum. Between 12:00 noon and 03:00 pm, all experiments were performed. The experimental protocol used for animals and the test that were carried out in this study was approved and permitted by the Animal Ethical Committee of Banaras Hindu University, Varanasi, India.

Experimental Design

In four experimental groups ($n = 6$), animals were randomly divided as follows;

Group 1: Intraperitoneal (i.p.) injections of normal saline (0.9%) were given to the mice; this served as the control.
Group 2: MPTP (30 mg/kg bwt) were prepared in normal saline (0.9%) and two doses were administered by i.p. injections to mice at 16-h interval.
Group 3: After two doses of MPTP (30 mg/kg bwt), at 16-h interval, from next day, mice were orally treated with UA (25 mg/kg body wt) for 21 days.
Group 4: Mice were orally administered with UA for 21 days which served as the positive control.

Different analyses were performed to study the anti-inflammatory activity of UA at the completion of various treatments.

Neurobehavioral Studies

Rotarod Test

Before starting the experiment, rotarod test was performed in which, at a set speed (5 rpm), each group of animals were trained for 3 consecutive days and the time was noted after the mice fall up to 5 min. For each animal, the experiment was repeated up to four times and the average time was calculated as suggested previously (Manna et al. 2006). For each group, similar process was repeated once the treatment was done and the time taken by the mice to fall down was noted.

Hanging Test

In hanging test, mice were positioned on a grid horizontally and allowed to have a tight grip on it. This horizontal grid was then positioned upside down so that the mouse hangs downwards, gripping or hanging on it until they lose their control and fall down. For each group, the experiment was repeated thrice and the hanging time was noted for the behavioral analysis as described previously (Mohanasundari et al. 2006).

Narrow-Beam Walking Test

In this test, motor coordination in mice was assessed. On a stationary wooden narrow flat beam (1 cm) which was placed at a height of 100 cm above the ground (L 100 cm × W 1 cm), animals from each group were trained to move on this beam. From one end of the beam to another end, time taken was noted and the procedure was repeated three times for each group's animals according to Pisa (1988).

Sample Preparation for Biochemical Studies

Nigrostriatal tissue was collected from each group individually, and they were homogenized at pH 8.0, complemented with phosphatase and a protease inhibitor in the KCl buffer (Tris-HCl 10 mM, NaCl 140 mM, KCl 300 mM, ethylenediaminetetraacetic acid 1 mM, Triton X-100 0.5%). Tissue homogenates were centrifuged for about 20 min at 12,000 × g at a temperature of 4 °C for the estimation of different biochemical parameters.

Biochemical Test

Catalase and Nitrite Test Catalase activity was estimated as described previously (Kumar et al. 2010). 10% *w/v* tissue homogenate was taken in a phosphate buffer (0.01 M, pH 7) and distilled water. Reaction started with the addition of hydrogen peroxide (0.02 M), and then each tube was incubated at room temperature for 1 min. The reaction was stopped by the addition of potassium dichromate and acetic acid (1:3) solution, and it was allowed to boil for 15 min in boiling water bath and absorbance was taken at 570 nm against blank. Finally, the activity of the enzyme catalase was measured in nmoles/min/mg protein. Nitrite level was estimated in the supernatant by using the standard procedures as described previously (Granger et al. 1996). 10% *w/v* tissue homogenate was prepared and supernatant was taken from the homogenate and mixed with ammonium chloride (0.7 mM) and Griess reagent (0.1% N-naphthyl ethylenediamine and 1% sulfanilamide in 2.5% phosphoric acid). This solution was incubated at 37 °C for 30 min, and the supernatant was taken out to take absorbance at 540 nm. The total content of nitrite was calculated in terms of μmoles/ml by using the standard curve for sodium nitrite (10–100 μM).

Lipid Peroxidation and GSH Test In the nigrostriatal tissue of the mouse brain, lipid peroxidation was estimated as described previously (Ohkawa et al. 1979) with fewer modifications. Lipid peroxidation product known as malondialdehyde (MDA) was estimated. Briefly in 10% SDS solution (0.1 mL), 10% tissue homogenate (0.1 mL) was added and this reaction mixture was kept at room temperature for 5 min. The solution was further incubated for 2–5 min after the addition of 20% acetic acid (0.6 mL). Lastly, solution was kept in a boiling water bath for 1 h after addition of 0.8% thiobarbituric acid (TBA) (0.6 mL). Then reaction mixture was allowed to cool at room temperature. This cooled solution was centrifuged and supernatant was collected; the absorbance of this supernatant was taken at 532 nm against blank. Nanomoles MDA/mg protein unit was used for the expression of LPO levels. In the brain homogenate, reduced glutathione (GSH) level was measured by the method described previously (Moron et al. 1979) and expressed as μM GSH/mg tissue.

Immunofluorescence Staining of TH, NF-κB, TNF-α, and Iba1 in SNpc

In the SNpc of the brain, immunohistochemical expression of TH, NF-κB, TNF-α, and Iba1 was performed (Gorbatyuk et al. 2008). Four percent paraformaldehyde was used for the perfusion of mice, and the brains were post-fixed and collected after perfusion.

Cryomicrotome (Leica, Wetzlar, Germany) was used for the sectioning of the brain, 20-μ-thick coronal sections of the brain were cut at the SN level. Sections were washed twice with 0.01 M PBS at pH 7.4 and then for 1 h, sections were allowed to incubate with blocking reagent (10% normal goat serum in PBS, 0.25% Triton X-100). After blocking, sections were incubated with primary antibodies at 1:1000 dilutions with the polyclonal anti-rabbit against NF-κB p65, monoclonal anti-mouse against TNF-α, polyclonal anti-goat against Iba1, and polyclonal anti-mouse against TH for 16 h at 4 °C. Again, five times washing of the sections was done in PBST and they were incubated with cy3-conjugated secondary antibodies (Ex max 550 nm and Em max 570 nm) donkey anti-rabbit and cy2-conjugated (Ex max 492 nm and Em max 510 nm) donkey anti-mouse for 1 h at room temperature in 1% BSA blocking solution. Again, three times washing of the sections was done and then mounted using mounting media, fluoroshield (Sigma-Aldrich). Fluorescent microscope Nikon (Thermo Fisher Scientific) was used to take the fluorescent image of the sections. Image J software (NIH, United States) was used for the analysis of immunofluorescence images and reported as mean integrated fluorescent value (IFV).

Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction

qRT-PCR analysis was done according to the procedure described by Birla et al. 2019. Briefly, the total RNA was isolated from the SNpc and the RNA yield was quantified. The cDNA was prepared using 2 μg of total RNA and reverse transcription was done (Peinnequin et al. 2004). GAPDH was used as an endogenous control. Calculation of the fold change (ΔCt) was done using $2^{-\Delta\Delta Ct}$ method and reported as an arbitrary unit (Birla et al. 2019).

Statistical Analysis

One-way ANOVA was used for the statistical analysis of differences between means of different groups followed by Student–Newman–Keuls post hoc test using GraphPad Prism 7.0 software. $p < 0.05$ was considered statistically significant.

Result

Behavioral Studies

Our results have suggested that as compared with the control, time taken by MPTP-treated mice for which it remained on the rotarod was significantly reduced ($p = 0.0001$). While on UA supplementation, MPTP-treated mice ($p = 0.0024$) stayed on the rotarod significantly longer than MPTP mice (Fig. 1a).

Our findings have suggested that as compared with the control mice, in MPTP-treated mice, the time of gripping and hanging was significantly poorer ($p = 0.0001$). While as compared with MPTP-treated mice, MPTP+UA mice had shown significantly increased hanging time ($p = 0.0014$) (Fig. 1b).

Our data has suggested that as compared with the control mice, in MPTP-treated mice, narrow-beam walking time was increased ($p = 0.0001$). While as compared with MPTP-treated mice, MPTP+UA mice had shown significantly decreased narrow-beam walking time ($p = 0.0006$) (Fig. 1c).

Biochemical Studies

Effect of UA on Catalase, Nitrite, MDA, and GSH

Our result have suggested that, as compared with the control group, MPTP injection has significantly decreased the activity of catalase ($p = 0.0001$) and increased the nitrite content ($p = 0.0001$). While, the MPTP+UA group has shown increased activity of catalase ($p = 0.0001$) (Fig. 2a) and decreased nitrite content ($p = 0.0001$) (Fig. 2b) as compared with the MPTP-treated group.

Our findings have suggested that the mice intoxicated with MPTP showed a significant increment ($p = 0.0001$) in lipid peroxidation product, known as MDA, as compared with the control. On the other hand, as compared with the control group, MPTP intoxication caused a significant decline in GSH content ($p = 0.0026$). While, UA supplementation (MPTP+UA) significantly attenuated the rise in MDA level ($p = 0.0012$) (Fig. 2c) and improved the GSH content ($p = 0.0167$) as compared with the MPTP group (Fig. 2d).

Effect of UA on the Expression of TH, NF-κB, TNF-α, and Iba1 in SNpc

Our results have suggested that immunohistochemical expression of NF-κB ($p = 0.0022$), TNF-α ($p = 0.0007$), and Iba1 ($p = 0.0006$) positive cells was increased in MPTP-treated mice as compared with the control group. After UA supplementation (MPTP+UA), a decrease in NF-κB ($p = 0.0064$) (Fig. 3a), TNF-α ($p = 0.0013$) (Fig. 3b), and Iba1 ($p = 0.0051$) (Fig. 4a) expression was observed as compared with the MPTP-treated group. Also, in comparison with the control group, a reduced ($p = 0.0019$) level of TH-positive dopaminergic neurons was seen in response to MPTP injection. Though, following treatment with UA in the MPTP-intoxicated group, an increase in TH level ($p = 0.0097$) (Fig. 4b) was seen as compared with the MPTP-treated group.

Effect of UA on the Nuclear Translocation of NF-κB in SNpc

Our findings have suggested that, as compared with the control group (Fig. 5A), in MPTP-injected mice, the nuclear translocation of NF-κB has occurred (Fig. 5b). While as

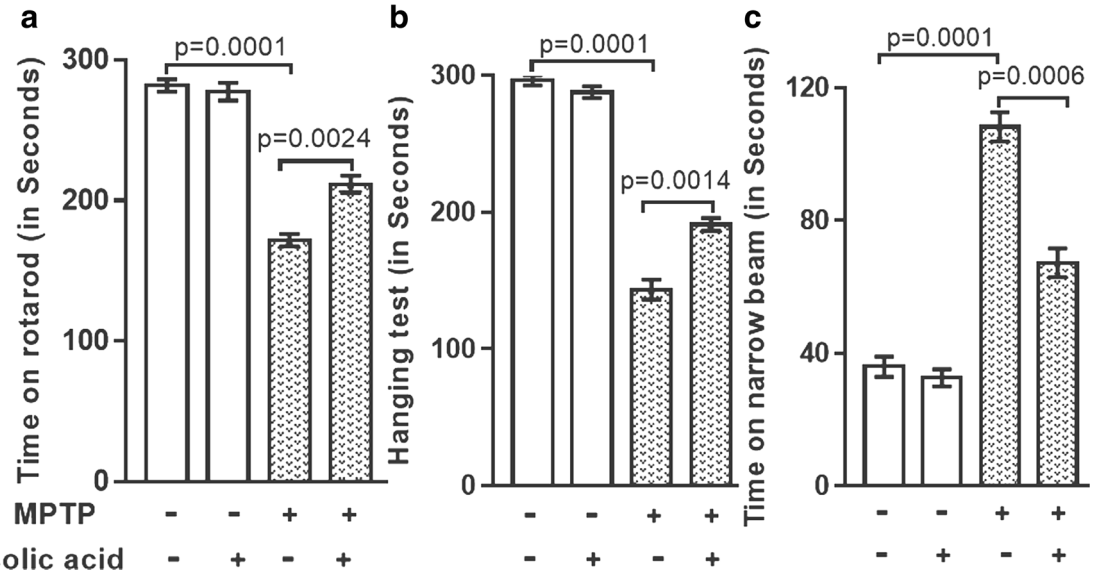

Fig. 1 Effect of UA on behavioral parameters. **a** MPTP-treated mice ($p = 0.0001$) showed significantly reduced time of walking and staying on rotarod as compared with the control group, while UA treatment ($p = 0.0024$) significantly improved the rotarod timing as compared with untreated PD mice. **b** Hanging test showed significant improvement in the UA-treated group ($p = 0.0014$) compared with the MPTP-treated group ($p = 0.0001$). MPTP group mice fall early as compared with the control group. **c** Narrow-beam walking time was significantly increased in the MPTP-treated mice ($p = 0.0001$) in comparison with the control group while UA treatment declines the narrow-beam walking time as compared with the MPTP-induced PD mouse ($p = 0.0006$). ns, non-significant

compared with MPTP-treated mice, UA treatment (Fig. 5c) has significantly inhibited the nuclear translocation of NF-κB.

Effect of UA on Pro- and Anti-inflammatory Cytokines

Using GAPDH as an endogenous mRNA control, the qRT-PCR was done (Fig. 6). The potential role of UA has been proven by qRT-PCR, which has shown its ability to regulate the neuroinflammation in the Parkinsonian mouse model. In MPTP-intoxicated mice, upregulation of IFN-γ (8.3 folds) and IL-12 (8.4 folds) and downregulation of IL-10 (0.85 folds) and IL-4 (0.82 folds) were found through qRT-PCR analysis as compared with the control. While the treatment with UA has significantly reduced IFN-γ (2.9 folds) and IL-12 (4.2 folds) and enhanced the level of IL-10 (4.5 folds) and IL-4 (5.6 folds) as shown in Fig. 6, thereby showing that UA has significantly reduced the inflammation by maintaining the balance between the pro- and anti-inflammatory cytokines.

Discussion

UA has been shown to have many biological activities, for example antioxidative, anti-inflammatory, anti-cancerous, and hepato-protective activities (Ikeda et al. 2008). Currently, researchers have suggested that the anti-carcinogenic and anti-inflammatory activity of UA were due to its capability to inhibit the immunoregulatory transcription factor NF-κB in response to a variety of inflammatory and carcinogenic agents (Shishodia et al. 2003).

In this study, we have tried to examine anti-inflammatory activity of UA against the MPTP-induced mouse model of PD. The nigrostriatal pathway in PD is found to be damaged through MPTP (Watanabe et al. 2005). As shown by evidence, the pathogenesis of PD is finely mimicked by MPTP (Rai et al. 2016; Yadav et al. 2014).

This study has shown that, in mice, MPTP injection causes behavioral impairment which is measured by rotarod, grip strength, and narrow-beam walking test. UA was found to significantly improve the motor abnormalities in MPTP mice. Our behavioral findings are in accordance with the earlier findings (Yadav et al. 2013, 2014; Rai et al. 2017; Singh et al. 2018; Birla et al. 2019).

Recently, the inflammation in the neurons was found to be reduced by ferulic acid and phytic acid in MPTP-intoxicated animal models of PD (Nagarajan et al. 2015; Yuqiang et al. 2015). In our previous studies, it is found that MPTP was capable of inducing oxidative stress and neuroinflammation, two major factors responsible for the pathogenesis of PD (Rai et al. 2016, 2017). In our present study, the dose of UA was chosen according to the data of our previous studies (Rai et al. 2016).

Catalase and GSH provides defense to the cells from oxidative damage (Rai et al. 2016, 2017; Yadav et al. 2014). However, UA has significantly scavenged the free-radical production by restoring the level of catalase and GSH in MPTP mice (Rai et al. 2016, 2017; Yadav et al. 2014). In this study,

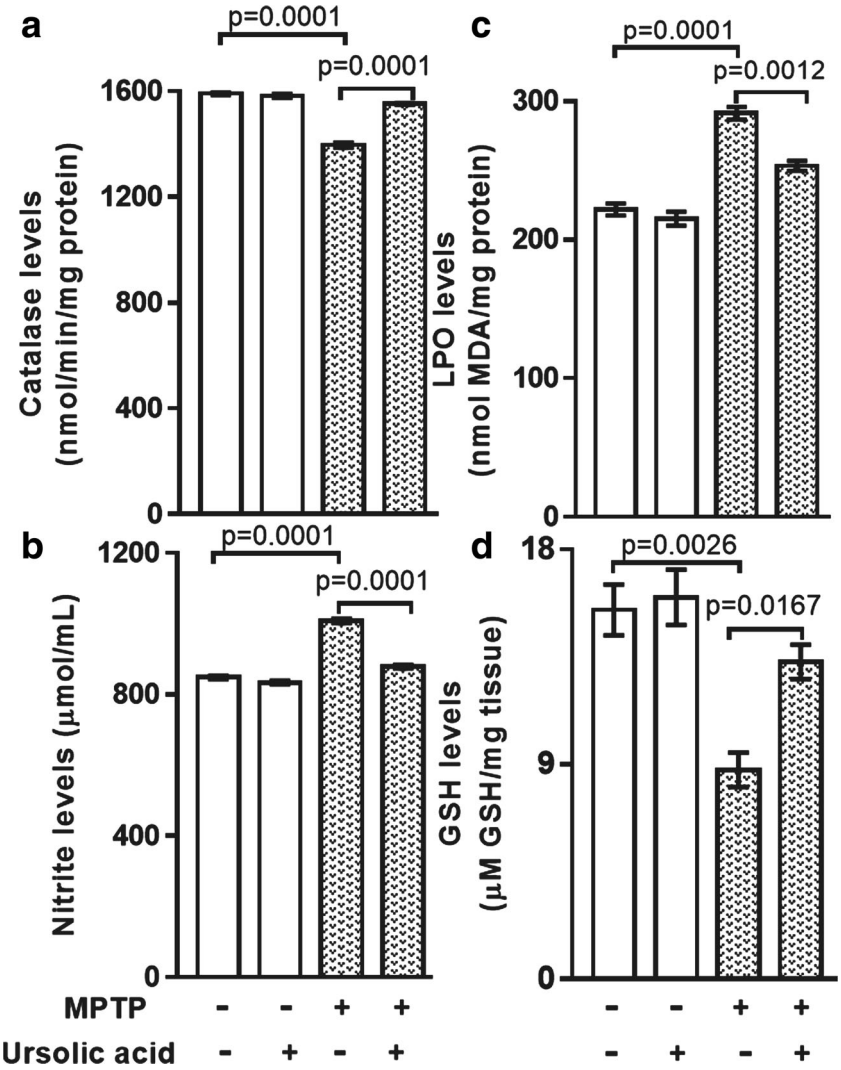

Fig. 2 Estimation of CAT, nitrite, MDA, and GSH in the nigrostriatal region of mice. MPTP-injected mice showed significant decrease in CAT activity ($p = 0.0001$) and GSH level (0.0026) and increase in the nitrite ($p = 0.0001$) and MDA levels (p = 0.0001) as compared with the control group. While UA treatment in the MPTP group significantly increase the level of catalase ($p = 0.0001$) (**a**), decrease in the level of nitrite ($p = 0.0001$) (**b**) and MDA ($p = 0.0012$) (**c**), and increase in the level of GSH ($p = 0.0167$) (**d**). ns, non-significant; MDA, malondialdehyde; GSH, glutathione; CAT, catalase; SEM, standard error of mean.

we have shown the antioxidative property of UA by its ability in scavenging noticeable amount of ROS generated by MPTP as evident by the level of catalase and GSH. MDA level is a distinguished oxidative stress marker of lipid peroxidation in Parkinsonian mice (Rai et al. 2016, 2017; Yadav et al. 2014). In our study too, the level of MDA was enhanced in MPTP-induced Parkinsonian mouse which was significantly reduced by the UA, further exhibiting its antioxidative activity.

In this study, we have shown that MPTP intoxication produces neuroinflammation-induced reactive nitrogen species (RNS) overproduction, while UA reduces this RNS accumulation. It is well-established that inflammation is the downstream event of oxidative stress (Rai et al. 2017). This might be suggested that UA uses its anti-inflammatory activities along with antioxidative activity to inhibit these oxidative and inflammatory loads. In this study, 21 days after MPTP administration, increased amount of nitrite, suggestive of neuroinflammation, was observed as reported by our previous study (Rai et al. 2016). Whereas, this increased level of nitrite was attenuated on UA administration, indicating its anti-neuroinflammatory activity.

Upon MPTP challenge, a significant loss of DA neurons was seen through immunofluorescence analysis of SN brain sections in mice. The findings from this study also showed that MPTP had a deleterious effect on SN DA neurons which is consistent with the previous studies (Yadav et al. 2014; Kim et al. 2015a, b). In the present study, we have observed that in the SNpc, MPTP intoxication considerably reduces the expression of TH-positive DA neurons. The DA neuronal loss in SNpc is one of the pathological hallmark of PD (Rai et al. 2016, 2017; Yadav et al. 2014). However, UA administration in MPTP-intoxicated mice has significantly protected the neurons from degeneration, suggesting its beneficial property in rescuing from neurodegeneration (Rai et al. 2016). The dose-dependent increase in the bioavailability of UA has been well studied in brain tissue of mice. Moreover, bioavalability of UA is seen to be increased in various tissues like brain, heart, liver, kidney, colon, and bladder in a dose-dependent manner

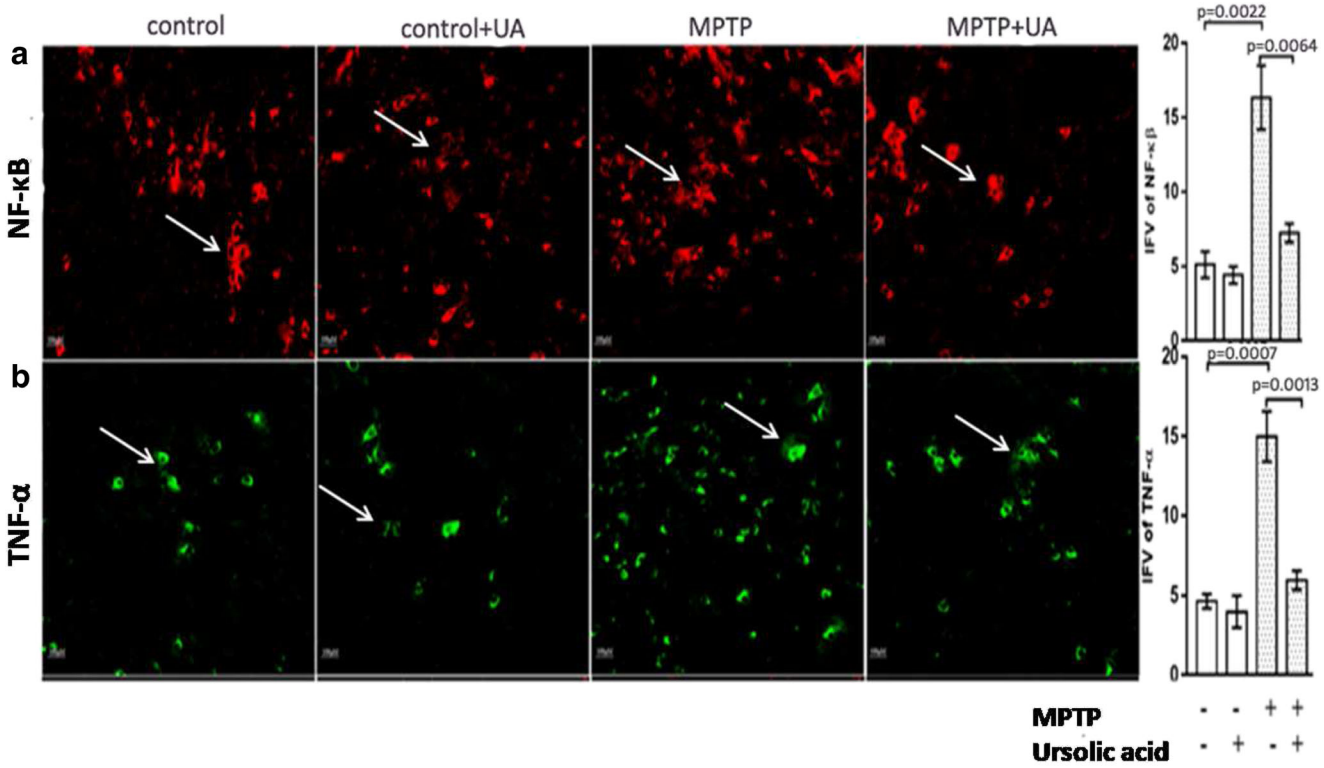

Fig. 3 Immunofluorescence expression of NF-κB and TNF-α in SNpc. With ×20 magnifications after staining. Immunofluorescence expression of NF-κB and TNF-α in SNpc of CONT, MPTP, and MPTP+UA mice by using Image J Software at ×20 magnification. The MPTP-intoxicated PD mice showed significantly enhanced expression level of NF-κB ($p = 0.0022$) (**a**) and TNF-α ($p = 0.0007$) (**b**) positive cells as compared with the control. On UA treatment, PD mice showed significantly alleviated expression level of NF-κB-positive ($p = 0.0064$) and TNF-α-positive (0.0013) cells as compared with MPTP mice. Values are expressed as integrated fluorescent value (IFV). ns, non-significant. TNF-α, tumor necrosis factor alpha; SNpc, substantia nigra pars compacta

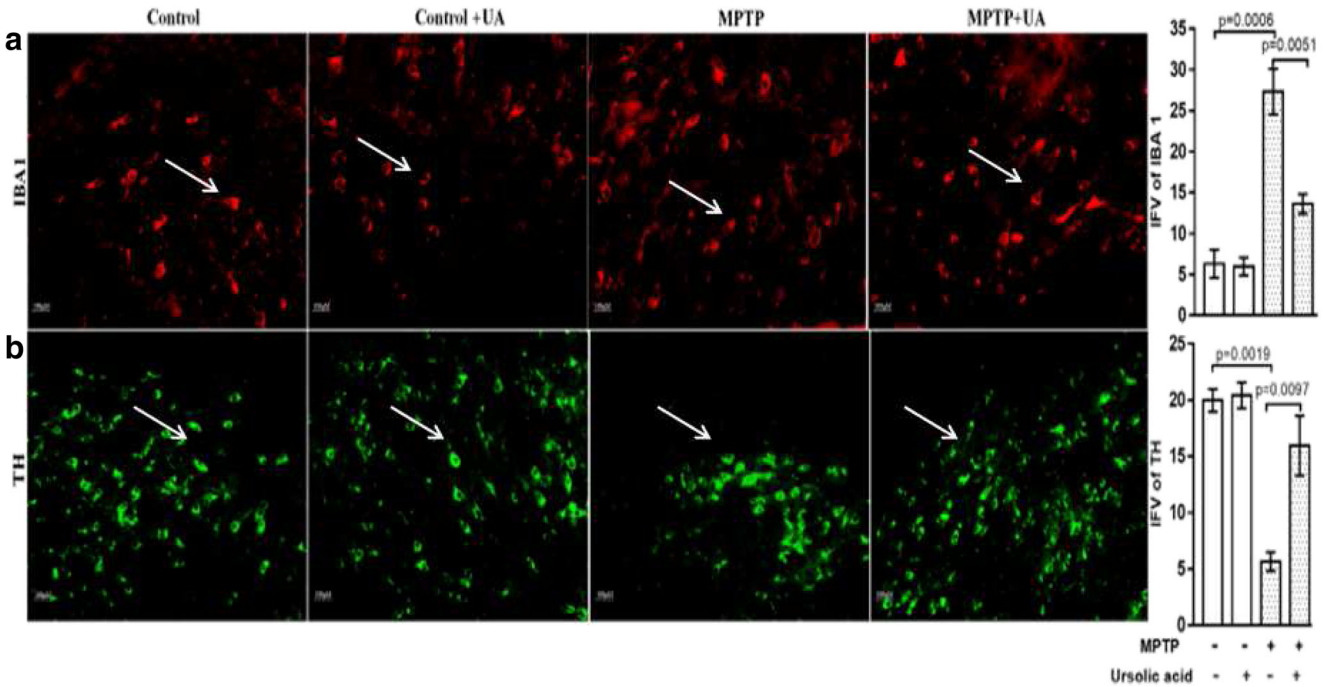

Fig. 4 Immunofluorescence expression of Iba1 and TH in SNpc. With ×20 magnifications after staining. IBA-positive microglial cells expression was increased in MPTP-treated mice ($p = 0.0006$) while UA treatment ($p = 0.0051$) in MPTP mice significantly reduced the Iba1 expression (**a**). TH-positive dopaminergic neuron expression was reduced in MPTP-treated mice ($p = 0.0019$) while UA treatment ($p = 0.0097$) in MPTP-treated mice significantly enhanced the expression of TH (**b**). Values are expressed as integrated fluorescent value (IFV). ns, non-significant; Iba1, ionized calcium-binding adaptor molecule 1; TH, tyrosine hydroxylase

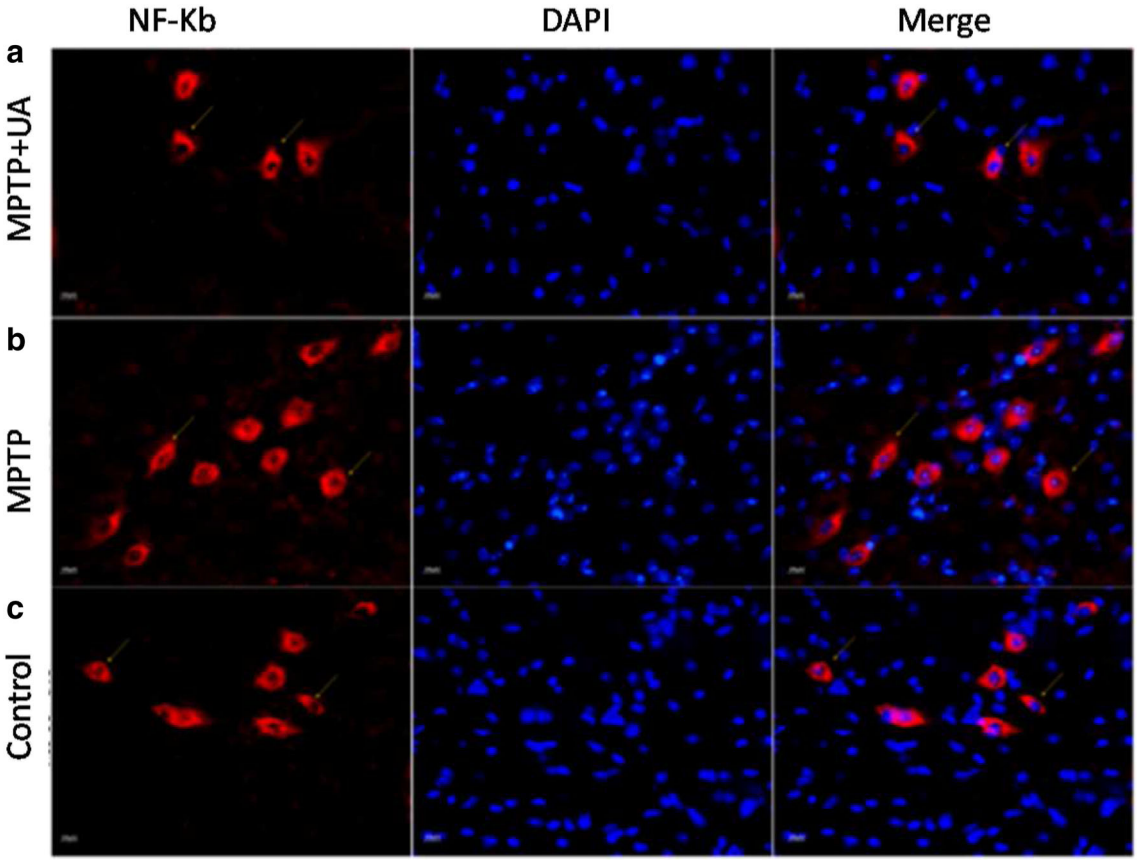

Fig. 5 Effect of UA on the nuclear translocation of NF-κB in SNpc. With ×40 magnifications after staining. SN nuclear translocation of NF-κB-positive cells was increased in the MPTP-treated group (**b**) as compared with the control group (**a**), while UA treatment inhibits this nuclear translocation of NF-κB (**c**)

(Yin et al. 2012). In our previous study too, we have shown that UA improves the immunoreactivity of TH and biochemical anomalies in a dose-dependent manner (Rai et al. 2016).

Neuroinflammation is one of the important factors responsible for the pathogenesis of PD (Niranjan 2014). Various inflammatory pathways and molecules were examined to study the

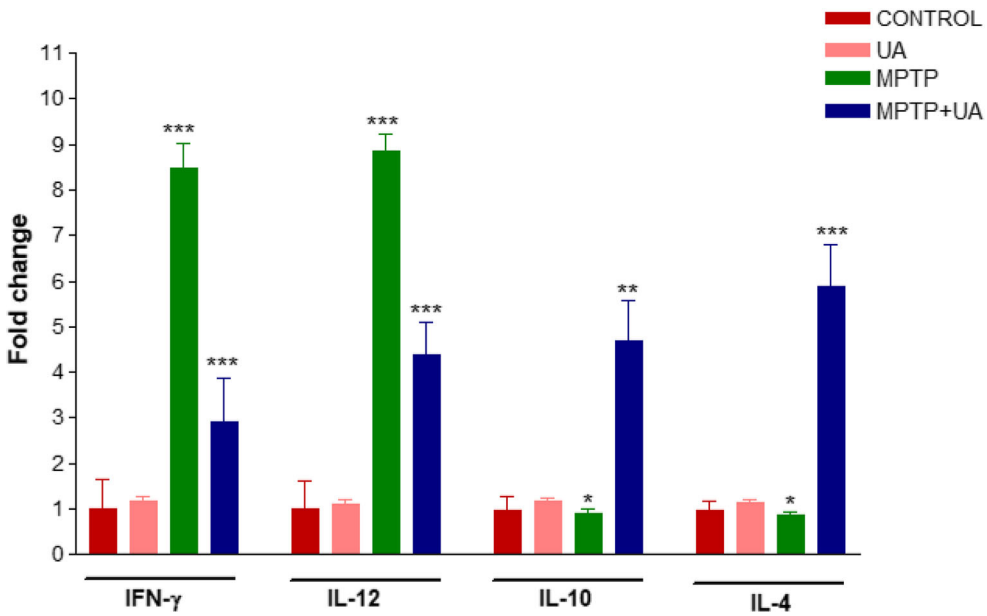

Fig. 6 Fold change in gene expression of pro- and anti-inflammatory cytokines in MPTP-intoxicated and UA-treated mice, in comparison with the healthy control. Expression of IFN-γ and IL-12 was found significantly upregulated in MPTP mice, whereas it was found to be attenuated in UA-treated mice. Expression of IL-10 and IL-4 was downregulated in MPTP mice and upregulated in UA-treated mice. Data were normalized with endogenous control (GAPDH) and value is expressed as mean ± SEM ($n = 5$) (*$p < 0.5$, **$p < 0.01$, ***$p < 0.001$)

potential effects of UA. Neuroinflammation is mainly mediated by microglia in the PD brain. Proinflammatory cytokines are seen to be released on exposure of MPTP in animal models of PD, causing neurodegeneration (Peterson and Flood 2012; Członkowska et al. 1996). Microglial activation was assessed by estimating the level of ionized calcium-binding adaptor molecule 1 (Iba1), i.e.; a calcium-binding protein specific in the microglia/macrophage (Ohsawa et al. 2004; Ito et al. 2001). In accordance with previous reports, MPTP challenge has also activated microglia, as evidenced by increased Iba1 expression due to ROS and neuroinflammation in the mouse model of PD (Thakur and Nehru 2013; Litteljohn et al. 2011). In this study, UA has considerably reduced the expression of Iba1 indicating its beneficial role in inhibiting the microglial activation in the mouse model of PD. Thus, UA can be considered the potential therapeutic agent in providing protection against neuroinflammation-induced neurodegeneration.

Proinflammatory cytokines causes the activation of NF-κB signaling cascade by the phosphorylation and degradation of its inhibitor IκB (Litteljohn et al. 2011). In this study, UA has caused the inhibition of activation and ultimately translocation of NF-κB, as reported in previous studies too (Yadav et al. 2014). The transcription of proinflammatory genes such as TNF-α takes place when NF-κB (p65) is translocated into the nucleus where it binds to its target sites in DNA (Yadav et al. 2014; Shen et al. 2010). TNF-α expression was considerably reduced by UA treatment which further strengthened its anti-inflammatory activity. These overall processes ultimately lead to neuroinflammation, ultimately causing the progressive degeneration of DA neurons, which was subsequently reduced by UA treatment.

Activation of microglia enhances neurotoxicity by releasing inflammatory and immunomodulatory cytokines (IL-1β, IFN-γ, TNF-α, IL-6, IL-8, IL-12, IL-15, and IL-10), as a result causes neuronal degeneration. In addition to microglia, astroglia also plays a crucial role in inflammatory process by releasing various cytokines (Block and Hong 2007; Teismann and Schulz 2004). Studies suggested that, under pathological conditions, the expression of inflammatory cytokines such as IL-1β, TNF-α, and other cytokines were increased by astrocytes. In addition, release of TNF-α, IFN-γ, and IL-1β from microglia can also activate astrocytes (Saijo et al. 2009; Barcia et al. 2011). Therefore, IL-1β, IL-12, TNF-α, and IFN-γ are the proinflammatory cytokines whereas IL-10 and IL-4 shows the anti-inflammatory activity in neurodegenerative diseases like PD. Arimoto et al. showed in his study that IL-10 and IL-4 can protect inflammation-induced DA neurons degeneration in SN (Arimoto et al. 2007; Spittau 2017). Hence, IL-10 and IL-4 shows the neuroprotective activity in Parkinsonian mice.

It is evident that in MPTP-intoxicated mice, the mRNA levels of proinflammatory cytokines IFN-γ and IL-12 are increased while it is considerably restored in the UA-treated group. On the other hand, in the MPTP group, the mRNA level of anti-inflammatory cytokine IL-10 and IL-4 was negatively regulated whereas it was restored in the UA-treated group.

Thus, from the results found in this study and from the evidence presented by previous reports, UA seems to be a potential anti-inflammatory agent against degeneration of DA neurons in PD. Its efficacy and safe use is further strengthened by the absence of any adverse effects on brain tissue. Hence, UA can act as potential therapeutic drug targeted for providing protection from neuroinflammation-induced neurodegeneration.

Conclusion

In summary, our study demonstrates that UA blocks MPTP-induced dopaminergic neurodegeneration by NF-κB pathway. The anti-inflammatory activity of UA has mainly attributed to its neuroprotective activity. Although the molecular mechanism behind the neuroprotective action of UA is still to be investigated, our study has tried to contribute to the fact that UA can be used as potential drug in managing the symptoms of PD. Therefore, the potential of UA in rescuing the dopaminergic neurons from neurodegeneration could suggest its role for clinical intervention in PD. Moreover, further research work is needed to explore its downstream regulatory pathways which are involved in the anti-inflammatory activity of UA in the case of PD.

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Compliance with Ethical Standards

The experimental protocol used for the animals and the test that were carried out in this study was approved and permitted by the Animal Ethical Committee of Banaras Hindu University, Varanasi, India.

Conflicts of Interest The authors declare that they have no conflict of interest.

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