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Toxicology *Original Article*

Neuroprotection of Kolaviron by Regulation of Nuclear Factor Erythroid 2-related Factor 2 in 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine Mice Model of Parkinson Disease

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ABSTRACT

Objectives: Parkinson's disease (PD) is the most prevalent movement disorder. Available therapies are palliative with no effect on disease progression. We have previously demonstrated that kolaviron (KV), a natural anti-inflammatory and antioxidant agent, suppressed behavioral defect, redo-inflammation, and nigrostriatal pathology in rotenone PD model. The present study investigates the neuroprotective effect of KV focusing on DJ-1/nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway.

Material and Methods: All-trans retinoic acid (ATRA, 10 mg/kg/day) was used to inhibit Nrf2. PD was established with four doses of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (20 mg/kg) at 2 h interval. MPTP mice were pre-treated with either KV (200 mg/kg/day), ATRA or both for 7 days before MPTP. Mice were evaluated for locomotor defects and indices of oxidative stress, neuroinflammation and neurotransmission as well as pathological tyrosine hydroxylase expression PD were evaluated in the striatum.

Results: ATRA alone in mice did not exhibit neurobehavioral defect but caused striatal toxicity, mild nigrostriatal pathology, significant nitrosative stress, and Nrf2 cascade inhibition. KV+ATRA mice were slow in movement with frequent short-lived interruptions and oxidative striatal pathology. ATRA aggravated MPTP-associated locomotor incompetence and could not prevent nigrostriatal toxicity with evident vacuolated striosome and pyknotic/degenerating dopaminergic neurons. MPTP induced acute locomotor, exploratory, and motor incompetence, which was prevented by KV treatment. In addition, KV treatment restored MPTP-mediated depletion of endogenous antioxidant, striatal nitrosative stress, and oxidative damage with elevated DJ-1 level, potentiated Nrf2/NAD(P)H; quinone oxidoreductase-1 cytoprotective capacity, reduced Kelch-like ECH-associated protein 1 expression, and limited striatal pathology. However, ATRA treatment attenuated all the protective effects of KV on MPTP-challenged mice. Meanwhile, other ATRA-combinations elicited significant DJ-1 and Nrf2 induction but are associated striatal toxicity/pathology.

Conclusion: This suggests that KV may be conferring protection through a yet-undetermined DJ-1 downstream cytoprotective effect dependent on the KV-mediated attenuation of oxidative environment.

Keywords: Kolaviron, Parkinson disease, Nuclear factor erythroid 2-related factor 2, Behavioral defect, Neurodegeneration, DJ-1

INTRODUCTION

Parkinson's disease (PD) is a gradual and progressive multifactorial nigrostriatal dopaminergic denervation which pathologically manifests in aged population above 65 as a motor syndrome.[1,2] Clinically, PD is diagnosed as impairment of classical motor features such as resting tremor, postural deformities, bradykinesia, hypokinesia, gait imbalance, cogwheel rigidity, and muscle stiffness.[2] Meanwhile, about 60% of the dopaminergic neurons have been degenerated before the apparent manifestation of the neuropathological motor deficits in PD patients.[3] According to an estimate, about 1 in 1000 is affected with PD worldwide.[2] Okubadejo's group has reported that PD accounts for 20 of 1360 neurological cases in Nigeria,[4] an environment where PD medicines are limited and pricey.[5] Despite the increase in the prevalence and incidence of this neurological disorder, there is currently no disease modifying therapy for the condition even as the precise molecular mechanisms underlying etiology of dopaminergic neuronal loss remains clouded.[6] However, apart from the combined effect of the interplay among genetic factor, environmental factor and age, consistent laboratory-based evidences have shown that mitochondrial dysfunction, chronic neuroinflammation, oxidative stress, and their cross talks are linked to PD onset and progression. The substantial nigra region is particularly susceptible to oxidative stress due to the low level of antioxidant glutathione (GSH), moderate activities of superoxide dismutase (SOD), and GSH peroxidase thereby rendering it a vulnerable hotspot to oxido-inflammatory modification.[7]

Meanwhile, the body has an endogenous cytoprotective system relevant for the maintenance of mitochondrial integrity and attenuating/mitigating oxidative stress and its damage. This system is critically regulated by the nuclear factor erythroid 2-related factor 2 (Nrf2), the master regulation of cellular redox status.[8] In response to cellular or neuronal insults, Nrf2 becomes activated, translocate to the nucleus, bind the antioxidant response element (ARE) of targeted genes and transactivate the expression of a battery of cytoprotective players. Examples include reduced GSH, SOD, catalase (CAT), NAD(P)H; quinone oxidoreductase-1 (NQO1), and glutathione-s-transferase (GST).[9] Nevertheless, the fate of the master regulator Nrf2 is constitutively determined by Kelch-like ECH-associated protein 1 (Keap1) and Park-7/DJ-1.[10,11] Keap1 prevents Nrf2 nuclear mobilization as it tags Nrf2 for ubiquitin-proteasomal degradation, while DJ-I stabilizes Nrf2 in the cytoplasm through its journey to the nucleus.[12] The involvement of Nrf2 in PD has been extensively established.[13] Considering the role of Nrf2 in mitochondrial function and cellular redox maintenance, pharmacological activation of Nrf2 in the brain is likely to preserve neuronal health. Thus, therapeutic agents that activate Nrf2-dependent cytoprotective genes would be a promising lead for the treatment of PD.

Preliminary findings from our laboratory have earlier demonstrated the neuroprotective relevance of Kolaviron (KV), a biflavonoid compound with natural antioxidant and anti-inflammatory potentials derived from the seed of *Garcinia kola*, in experimental models of PD.[14] Review of the literature also indicates the neuroprotective role of KV in the toxicity induced by vanadium in rats[15] and lipopolysaccharide associated microglia neuroinflammation in BV2 cells.[16] Farombi and Owoeye (2011) suggested KV as a compound with capacity to cross the blood–brain barrier. However, the molecular mechanisms underlining the neuropharmacological effects of KV in PD have not been fully unraveled. Based on the fact that, Nrf2 is a key interception junction in the crosstalk of several antioxidant and anti-inflammatory pathways, the present study therefore hypothesized that KV might elicit its neuroprotective effect in PD through Nrf2 activation. Here, we reported the identification of KV as a potent Nrf2 activator, using all-trans retinoic acid (ATRA) as Nrf2 inhibitor, thereby providing protection against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-mediated dopaminergic neuronal damage in the nigrostriatal pathway. The mechanism of activation involves sustained elevation of PARK-7/DJ-1 with concomitant reduced expression of Keap1.

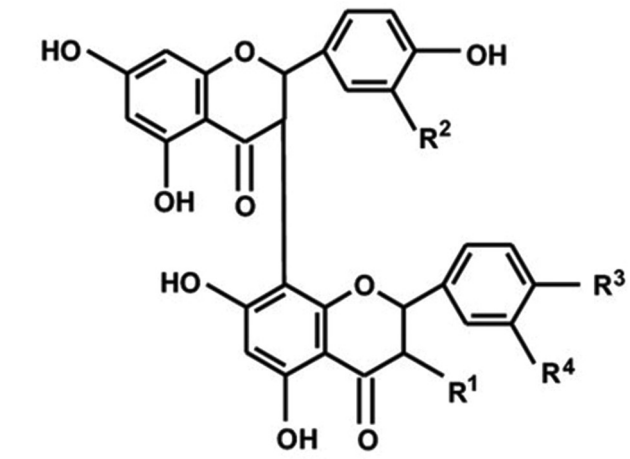
MATERIAL AND METHODS

Chemicals and reagents

We purchased MPTP and N-propyl gallate from AK Scientific (California, USA) while ATRA was obtained from Santa Cruz Biotechnology (CA, USA). Anti-Nrf2, anti-Keap1, anti-NQO1, anti-tyrosine hydroxylase (TH), and rabbit polyclonal primary antibodies were acquired from Elabscience (Texas, USA). Mouse reactive PARK-7/DJ-1 enzyme linked immunosorbent assay (ELISA) kit was bought from Elabscience (Texas, USA) and Cusabio Biotecnology LLC (Wuhan, China), respectively. The secondary antibody – anti-rabbit NL493 – was a product of R&D Systems (Minnesota, USA). Except for Coomassie brilliant blue G-250, which was obtained from Bio-basic Inc. (Canada), the remaining reagents and chemicals were of good analytical qualities produced by Sigma-Aldrich (St Louis, USA) and British Drug House (Dorset UK).

KV

KV [Figure 1] was extracted from *Garcinia Kola* seeds, which were purchased from Bodija market, Ibadan. KV was extracted according to the method of Iwu *et al*.[18] which was modified by Farombi *et al*.[19] The seeds were dehusked and ground at a local mill to obtain the granulated form. The *Garcinia kola* granules were then soaked in hexane solution



	R1	R2	R3	R4
GB1	OH	H	OH	H
GB2	OH	H	OH	OH
Kolaflavanone	OH	H	OCH_3	OH

Figure 1: Structure of Kolaviron.

using Soxhlet extractor for defatting. The defatted residue was then extracted in methanol solution using Soxhlet extractor. KV was obtained by a twin purification dilution process and extraction using chloroform to yield a yellowish gold extract. KV purification and identity were authenticated as *Garcinia* biflavanones GB1, GB2, and kolaflavanone were identified in the golden yellow extract through thin-layer chromatography with a solvent mixture of methanol and chloroform, ratio 1:4 (v/v). Identified compounds were further confirmed by comparison of 1H nuclear magnetic resonance spectra data with their standards. Isolated KV had purity of 96%.

Animal and treatments

A hundred and twenty male BALB/c mice (20–24 g; *n*-15/group) were used for the experiment. They were procured from the Central Animal House, College of Medicine, University of Ibadan. The animals were fed with mice pellets purchased from Ladokun Feeds Nigeria Limited and were given clean supply of water *ad libitum*. Experimental protocols, which was in line with the guideline of the Care and Use of Laboratory Animals (produced by the National Academy of Science and National Institute of Health, USA) was approved by the University of Ibadan Animal Care and Use Research Ethics. Freshly prepared solutions of MPTP and ATRA in normal saline and corn oil, respectively, were administered. KV was first prepared as a stock solution in DMSO before it was diluted to 200 mg/kg with corn oil. MPTP and ATRA were administered intraperitoneally (i.p.) while KV was treated orally. The animals were randomly divided into eight groups, 15 animals in each cage, as follows [Figure 2]:

Group 1 (control)

Mice were orally administered Corn oil-DMSO solution oil for 7 days. On the 8th day, they were injected with normal saline (i.p) 4 times every 2 h.

Group 2 (KV)

Mice were orally administered 200 mg/kg body weight of KV for 7 days and were given four injections of normal saline on the 8th day. The dose has been used in a previous study.[14]

Group 3 (MPTP)

Mice were administered four doses of 20 mg/kg body weight of MPTP (i.p) on the 8th day at 2 h interval as previously described.[20] Before this, the mice were treated with corn oil-DMSO solution orally for a week.

Group 4 (ATRA)

Mice were administered 10 mg/kg body weight of ATRA (i.p) for 7 days. The dose was selected from previous reports[21,22] where ATRA was used as Nrf2 inhibitor.

Group 5 (KV + ATRA)

Mice were co-treated with 200 mg/kg of body weight of KV and 10 mg/kg body weight of ATRA for 7 days and were given four injections of normal saline at 2 h interval on the 8th day. Throughout the administration period, KV was administered at 8.00 while ATRA was administered at 15:00.

Group 6 (MPTP + ATRA)

Mice were treated with 10 mg/kg body weight of ATRA for 7 days and were given four injections of 20 mg/kg body weight of MPTP on the 8th day at 2 h interval.

Group 7 (KV + MPTP)

Mice were administered 200 mg/kg body weight of KV from day 1 to 7 after which they were injected with four doses 20 mg/kg body weight of MPTP on the 8th day at 2 h interval.

Group 8 (KV + MPTP + ATRA)

Mice were co-administered with 200 mg/kg body weight of KV and 10 mg/kg body weight of ATRA for 7 days, as described above. On the 8th day, four doses of MPTP (20 mg/kg) were administered at 2 h interval.

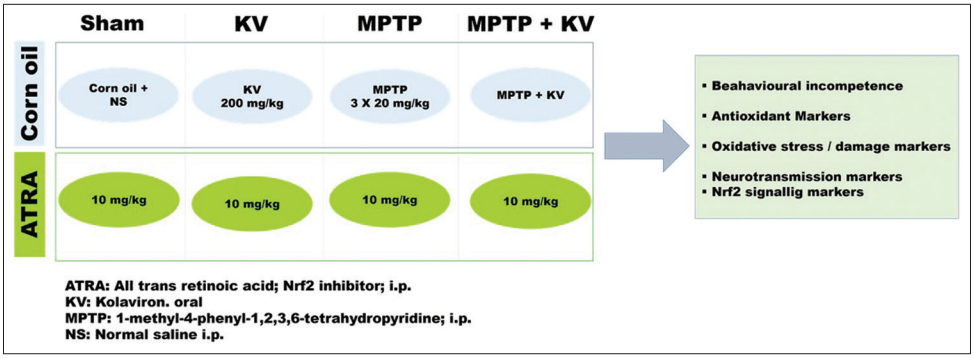


Figure 2: Diagrammatic representation of the experimental design. The treatment groups were broadly categorized into two: those that received all-trans retinoic acid (ATRA) (4, 5, 6 and 8) or not (1, 2, 3 and 7). ATRA, 10 mg/kg, i.p. was used to inhibit nuclear factor erythroid 2-related factor 2. Parkinson's disease was established with four doses of1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (20 mg/kg, i.p) at 2 h interval. MPTP mice were pre-treated with either kolaviron (200 mg/kg/day, p.o), ATRA or both for 7 days before MPTP.

Broadly, the treatment groups were categorized into two [Figure 2]; those that received ATRA (4, 5, 6, and 8) or not (1, 2, 3, and 7).

Behavioral assessments

Pole-climbing test

The climbing time and pattern, a suggestive marker of motor coordination, of the experimental animals down a pole was assessed on day 9 using a pole that is 30 cm long with diameter of 1 cm. Mice (n = 10) were placed on top of the pole and the time taken for each mouse to traverse from top to bottom of the pole was recorded. The pattern of climbing was also noted during the period.

Open field test

On day 10, the open field test was carried out as reported in Farombi *et al.* (2019). A square domain made of white wood with dimensions, 56 cm length, 56 cm breadth, and a height of 20cm was used. The apparatus' floor is evenly divided into 16 squares with its central and peripheral parts defined. At the beginning of each session, each mouse was placed at the center of the arena and its movement was recorded for 6 min with the use of an overhead camcorder (DNE webcam, Porto Alegre, Brazil). Analysis of the video was done with the use of ANY-maze video tracking software (Steolting CO, USA) to evaluate behavioral indices such as muscle stiffness (immobile latency, and freezing latency), bradykinesia (track plots, distance covered, mean speed, and time immobile), and exploration (meandering, and rotations).

Evaluation of oxidative stress, neuroinflammation, and neurotransmission indices

On day 11, brain tissues of mice (n = 10) were excised, striatal sections were removed and homogenized in four volumes of phosphate buffer. These were later centrifuged at 14,000 rpm for 10 min at 4°C. Thereafter, the supernatant, post-mitochondrial fraction, was used for biochemical assays to assess indices of oxidative stress and damage, neuroinflammation and neurotransmission. The Bradford method[23] was used to determine the concentration of protein in the collected tissues. To evaluate the antioxidant defense system, the activities of CAT, SOD, and GST were determined following the protocols described by Clairborne,[24] Misra and Fridovich,[25] and Habig *et al.*,[26] respectively. The concentration of reduced GSH in each sample was measured through the method of Jollow *et al.*[27] The level of nitric oxide (NO) activity was determined according to the Griess method described by Crespo *et al.*[28] Malondialdehyde (MDA) levels were evaluated to determine the extent of oxidative damage according to the method of Varshney and Kale.[29] The neurotransmission marker, acetylcholinesterase, was evaluated using the method described in Owoeye *et al.*[30]

Assessment of DJ-1 in striatal homogenate

DJ-1 level was determined in the striatum with the use of ELISA kit according to the manufacturer's instructions.

Immunofluorescence

Five animals per group were perfused with 2% buffered solution for immunostaining. After perfusion, the brains were excised, post-fixed in 2% buffered formalin for 30 min and immersed in 25% sucrose solution until they were sectioned (30 µm thick) coronally to reveal the striatum and substantia nigra pars reticulata (SNr) using a cryostat. Thereafter, the sections, placed on slides, were incubated with blocking solution (3% BSA solution in PBS containing 0.05% Triton -X-100) for 30 min and stained with diluted (5 ug/ml in blocking buffer) Nrf2, Keap1, NQO1, primary antibodies for 90 min. After this, NL-493- Conjugated secondary antibody (1:200) was added to the sections in the absence of

light for 60 min and counterstaining was done with 1 μg/ml Hoechst solution. The slides were mounted with in-house N-propyl gallate-supplemented glycerol/PBS solution and viewed with SP-98-FL inverted fluorescent microscope (Brunel Microscope Limited). Acquired images were further processed in Fiji-imageJ software (NIH, USA) to remove background noise and quantify fluorescence signal. Exposure settings, light intensity, and ImageJ parameters were uniformly applied to all images.

Immunohistochemistry (IHC)

The perfused cryopreserved brain samples ($n = 5$) were used for IHC analysis. Expression of TH in the SNr and striatum was evaluated with a "2-step plus Poly-HRP Anti-Rabbit IgG detection system-with DAB solution" IHC kit (Elabscience, USA) according to the manufacturer's guideline. Briefly, 30 μm coronal sections exposing the SNr and striatum were cut with cryostat and placed on slides (Leica, Germany). Endogenous peroxidase activity was wedged through the incubation of the sections in 3% H_2O_2 for 10 min. After this, the slides were cleansed in PBS, treated with normal goat serum for 30 min and incubated with primary antibodies (1:500) for 90 min at room temperature. After this, a polymer auxiliary detection system was added to the sections for 20 min before incubation with a polyperoxidase anti-rabbit IgG for another 30 min. Immunoreaction was visualized with the addition of 3,3'-diaminobenzidine till a brown color appeared. Sections were counterstained with hematoxylin, dehydrated in graded (96–100%) alcohol and cleared in xylene before mounting. The slides were examined by a digital camera coupled to a microscope. Slides were visualized with light microscope (Leica DM 500, Germany). Acquired images were further processed with Fiji-imageJ (NIH, USA). Five micrographs per sample were viewed by two scientists who were blinded to the experimental groups.

Statistical analysis

Analysis of data was done with two way analysis of variance and presented as mean ± standard error of mean. Statistical significance was considered at $P < 0.05$. For immunostaining analysis, $n = 5$, and for biochemical analysis, $n = 10$.

RESULTS

KV attenuates locomotory and exploratory defects in MPTP mice, which were reversed by ATRA treatment

We have previously demonstrated that treatment with KV suppressed behavioral incompetence and redo-inflammation associated with Parkinsonism in rotenone intoxicated mice. However, even though rotenone and MPTP both inhibit complex 1, an important step implicated in the death of dopaminergic neurons in PD, the treatment regimen, time to disease development, and features of PD exhibited by treated animals are different.[31] More importantly, acute MPTP treatment has been shown to elicit both hyper or hypo locomotory behavior in mice, perhaps depending on the time of analysis.[32] Confirming the previous report, KV reversed the locomotor defect associated with repeated but acute MPTP (20 mg/kg) intoxication, when compared with MPTP and control mice [Figure 3a]. This was largely evident with distance covered and means speed reversed to the control level [Figure 3b and c]. On the average, it took MPTP + KV mice a similar length of time with control mice to freeze [60 s, Figure 3d] or become immobile [78 s] for the 1st time after being introduced into the open field apparatus whereas MPTP mice barely spent 5s and 22 s, respectively. Although freezing episodes were similar across the experimental groups [Figure 3e], KV reversed the number of times MPTP-treated mice experienced immobility to the control level [Figure 3f-g]. In the same vein, KV modulated the MPTP-mediated reduced efficiency to rotate and increased climbing down duration to a level comparable to the control.

To explore the involvement of Nrf2 in the neuroprotective role of KV following injury to dopaminergic neurons, we employed ATRA as a small molecule inhibitor of Nrf2. ATRA exhibits different activities at varying doses and treatment duration. For instance, at a low dose of 1 mg/kg for about 30 days, ATRA has been used as a cytoprotective and antioxidant agent.[33,34] At a higher dose between 7 and 10 mg/kg, ATRA induces *in utero* defects, nephrotoxicity and worsening of existing conditions.[35-37] Meanwhile, ATRA at 10 mg/kg for 14 days has been validated as Nrf2 inhibitor in C57BL/6 mice[21,35,38] thus we adopted this regimen for our study. However, using BALB/c mice, we observed in two independent pilot studies that 85% of mice treated with ATRA at 10 mg/kg died before 2 weeks with mortality onset on day 11 (data not shown). Thus, we limited ATRA treatment to 7 days after confirming that significant Nrf2 protein expression in the substantia nigra was inhibited. In this study, administration of ATRA at 10 mg/kg for 7 days did not elicit any behavioral anomaly in BALB/c mice except on the number of rotations which was reduced when compared with control [Figure 3h]. When administered with KV, ATRA reduced the distance travelled, mean speed, rotations, and time to climb down a pole [Figure 3i], with significant reduction in freezing episodes and enhanced immobile episodes without any effect on their latencies, when compared with KV-treated mice. ATRA aggravated the freezing parameters but reduced significantly the time taken to climb down a pole, when mice were pre-treated with ATRA before MPTP intoxication. However, all the benefits of KV on MPTP-related behavioral deficit were either inhibited or reversed by ATRA treatment when combined with KV and MPTP.

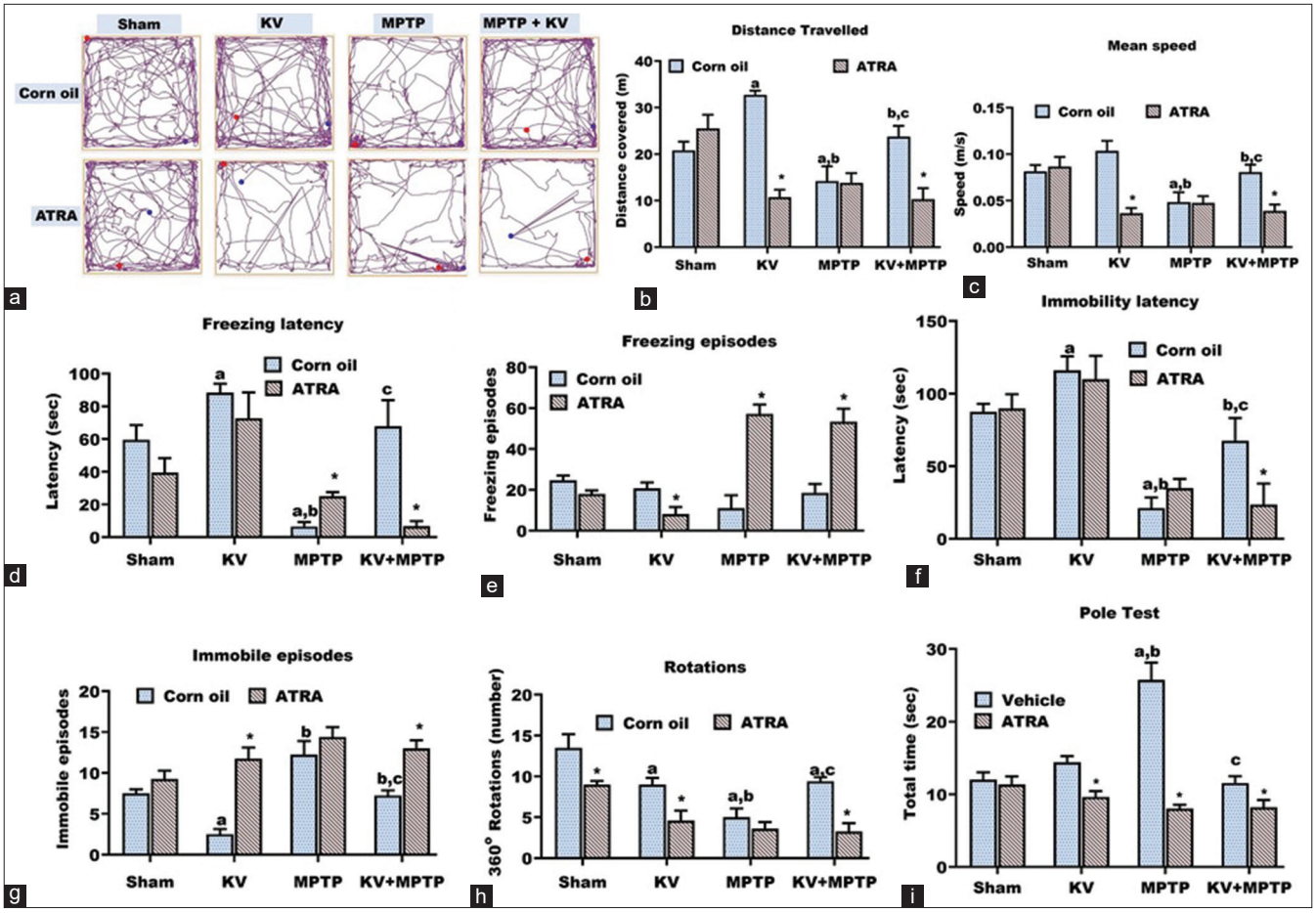


Figure 3: Kolaviron attenuates the locomotory and exploratory defects in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mice, which were reversed by All-trans retinoic acid (ATRA) treatment. Following treatment with ATRA, kolaviron (KV) and/or MPTP, mice (n = 10) were placed in an open field apparatus or at the tip of a pole for neurobehavioural assessment. For the open field, mice were video-recorded from the top and subsequently analyzed with ANY-maze video-tracking software to show the tracking plot (a) and data on distance travelled (b) mean speed (c) freezing latency (d) freezing episodes (e) immobility latency (f) immobile episodes (g) and rotations (h). The time to climb down the pole (i) was also monitored. The data represent mean ± SEM. $^{a}P < 0.05$ versus sham; $^{b}P < 0.05$ versus KV; $^{c}P < 0.05$ versus MPTP; $^{*}P < 0.05$ within group comparison.

Contrastingly, the freezing and immobility latencies, which were unaffected when KV was co-administered with ATRA, were markedly reduced in the mice that received the three treatments. While ATRA reduced the freezing episodes by 50% when administered with KV, it however elevated the episode by 174% when administered with KV and MPTP.

Taken together, this result indicated that four doses of MPTP induced acute locomotor, exploratory and motor incompetence, which was prevented with KV treatment. Mice that received both KV and ATRA were very slothful in movement with frequent short-lived interruptions. ATRA may aggravate MPTP-associated locomotor defect in mice, although with protective effect on motor coordination. Despite the individual and varying effect of ATRA when administered with either KV or MPTP, the reversal effect of ATRA on the protective role of KV in MPTP mice appears to be independent of its individual effect with either KV or MPTP.

Effect of ATRA and/or KV on striatal antioxidant status of MPTP-treated mice

The pivotal role of oxidative stress in the pathogenesis of PD is well established and we had previously documented the protective role of KV in this regard. In this study, acute MPTP injury elicited redox disturbance in the striatal tissue without affecting the H_2O_2 production [Figure 4]. Using biochemical evaluation of striatal redox status, we additionally confirmed the anti-Parkinsonian effect of KV in mice mediated by its antioxidant role. Specifically, KV significantly reduced the levels of striatal NO and MDA generation and activities of GST and CAT, relative to control, while increasing the level of reduced GSH. Although the SOD activity of MPTP mice

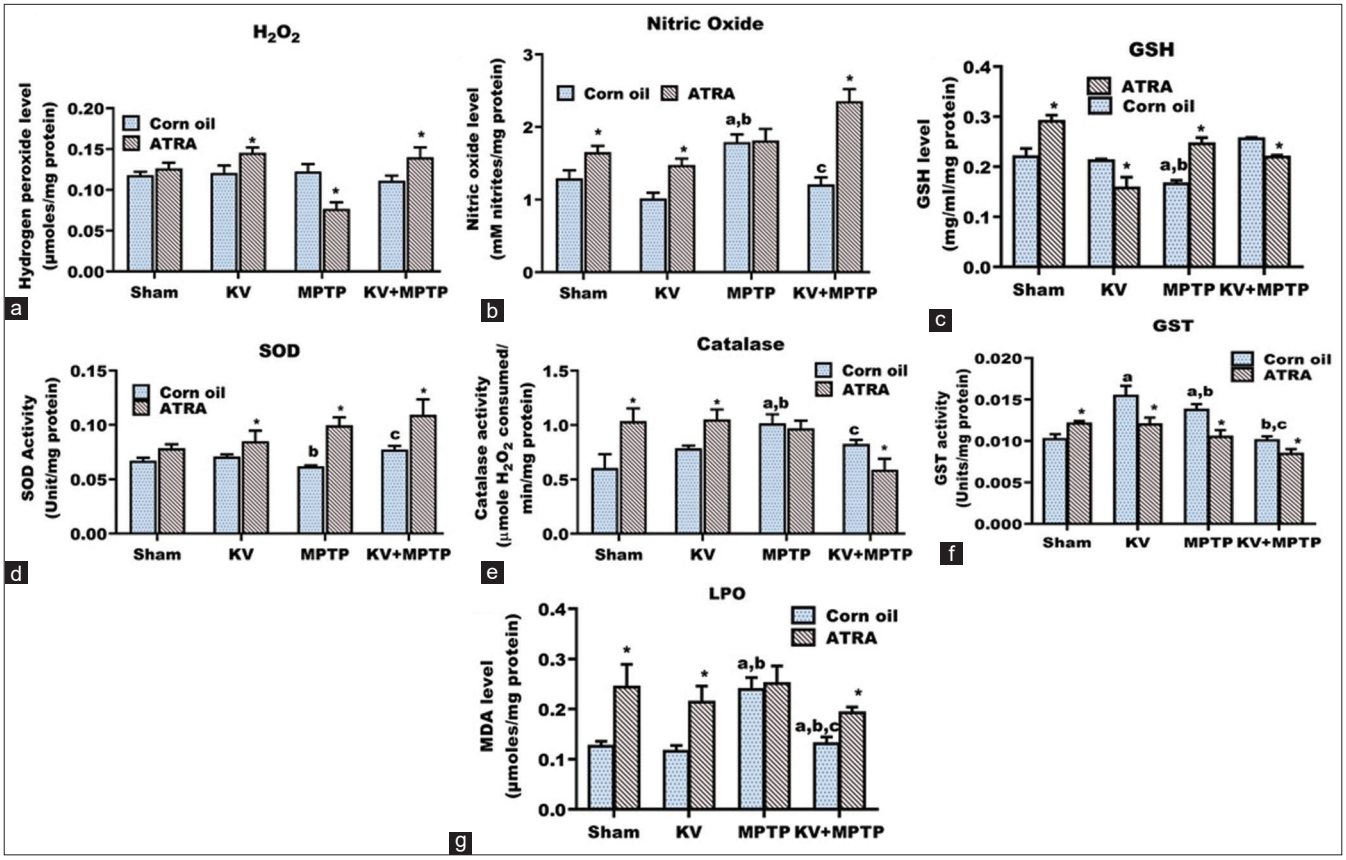


Figure 4: Effect of All-trans retinoic acid and/or kolaviron (KV) on the striatal antioxidant status of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice. Following treatment, striatal section was removed from the brain, and biochemical assays were used to quantitate hydrogen peroxide level (a) nitric oxide level (b) reduced glutathione level (c) superoxide dismutase activity (d) catalase activity (e) glutathione-S-transferase activity (f) and lipid peroxidative products level in the striatal homogenate. The data represent mean ± SEM. [a]$P < 0.05$ versus sham; [b]$P < 0.05$ versus KV; [c]$P < 0.05$ versus MPTP; *$P < 0.05$ within group comparison.

did not change, when compared with control, KV treatment induced the activity by 34% notwithstanding, relative to MPTP-treated group.

In contrast to the result obtained with the behavioral test, ATRA treatment alone caused significant nitric oxide generation which culminated in marked oxidative damage, significant depletion of reduced GSH and induction of enzymic antioxidant system CAT and GST without affecting superoxide dismutation and its product, H_2O_2 [Figure 4]. While KV did not have any effect on the evaluated indices – compared with the control – except GST activity, which was induced by 47%, KV+ATRA treatment markedly increased generation of both H_2O_2 and NO reactive species, depleted both GSH and GST – components of xenobiotic conjugation system – with elevated level of the oxidative product MDA and enhanced activities of SOD and CAT, relative to the KV group. When administered with MPTP, ATRA was unable to rescue the elevated MPTP-induced H_2O_2 and MDA levels, even though GSH level was boosted by 41%, H_2O_2 level was reduced by 39% and SOD activity was increased by 61% possibly because CAT activity was not affected, and GST activity was reduced by 24%. However, ATRA attenuated all the modulatory effect of KV in MPTP mice. Notably, suggesting potentiation of striatal toxicity, ATRA+KV+MPTP treatments significantly elevated NO level, relative to KV+MPTP, ATRA+MPTP and ATRA+KV, by 95%, 29%, and 59%; and markedly reduced CAT and GST activities by 32%, 39% and 44%, and 13%, 19.5%, and 29.5%.

Taken together, this result indicated that ATRA alone caused nitrosative stress and damage in mice. In the presence of KV, oxidative stress contributed to the ATRA-mediated striatal damage and redox imbalance. ATRA treatment could not prevent striatal toxicity when coadministered with MPTP. However, KV prevented MPTP-related striatal oxidative damage, and this effect was attenuated by ATRA treatment.

ATRA treatment prevents the modulatory activity of KV on MPTP-mediated dopaminergic neuronal loss

In this study, we seek to establish if the protection of dopaminergic neurons and terminals by KV involves Nrf2 signaling, using ATRA as an Nrf2 inhibitor. Following MPTP intoxication and treatment with KV and/or ATRA, striatum and SNr were stained for TH immunoreactivity and the results are presented in Figures 5 and 6. As expected, we noted a profound depletion of about 50% TH immune complex in the striatal terminals [Figures 5a and b] and SNr [Figures 6a and b] of MPTP-treated group, which was restored by KV pre-treatment to 81% and 83% of the control level, respectively. Of note, the restoration of dopamine depletion was abrogated by ATRA-treatment. Interestingly, ATRA alone depleted moderately TH optical density in both striatum and SNr by 16% and 20%, respectively. When KV was administered with ATRA, TH immunoreactivity in the striatum and SNr reduced by 36% and 34%, respectively. Meanwhile, administration of ATRA with the neurotoxin increased the immunoreactivity of TH from 50% to 80% and 72% in the striatum and SNr, respectively, relative to the MPTP group. In addition, we observed vacuolated striosome and pyknotic/degenerating neurons in ATRA treated groups.

Nrf2 accounts for the neuroprotective effect of KV in MPTP mice

The transcription factor Nrf2 is a key regulator of several cytoprotective genes including those encoding for antioxidant enzymes. NQO1 is considered as one of the Nrf2/ARE downstream proteins with antioxidant and cytoprotective functions. To ascertain the involvement of Nrf2/DJ-1 signaling pathway in the neuroprotective activity of KV against the dopaminergic neurotoxicity induced by MPTP, we inhibited endogenous Nrf2 in mice with 10 mg/kg ATRA. Mice were then treated with KV followed by intraperitoneal MPTP administration. We monitored the expression of Nrf2, its key regulator Keap1 and effector NQO1 in the substantial nigra using immunofluorescence and evaluated the quantity of secreted/expressed DJ-1 in the striatal homogenate with ELISA. The levels of DJ-1 in the striatum of mice treated with the vehicle, KV or MPTP were comparable [Figure 5c] while KV treatment before the acute MPTP injury significantly elevated DJ-1 level by 51%. Of note, the induction was abrogated when KV was combined with ATRA before MPTP injury. Meanwhile, ATRA or its combination with KV or MPTP increased DJ-1 production by 27%, 60%, and 46%, relative to control, KV and MPTP groups.

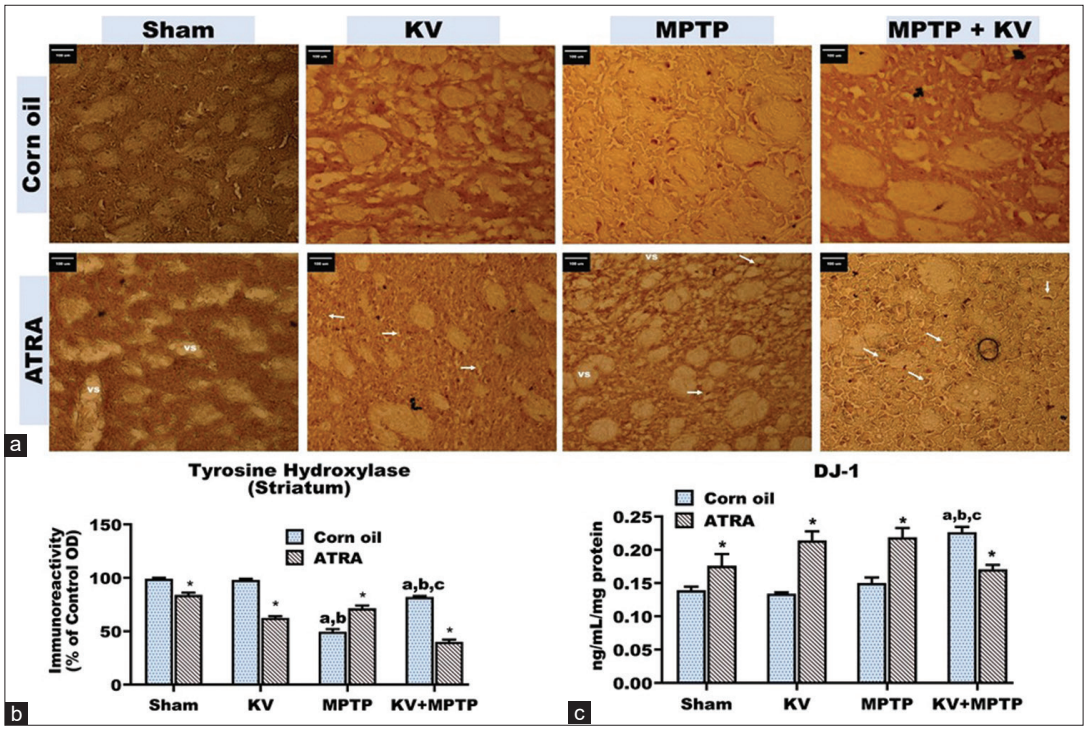


Figure 5: All-trans retinoic acid (ATRA) treatment prevents the modulatory activity of kolaviron in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mice. Immunohistochemistry was used to evaluate the expression of tyrosine hydroxylase in the striatum in the mice after treatment with ATRA, kolaviron (KV) and/or MPTP. Representative images from all groups are presented (a). The optical density of the images was analyzed with ImageJ and presented as mean ± SEM (b). DJ-1 level was also reversed to near (c) $^{a}P < 0.05$ versus sham; $^{b}P < 0.05$ versus KV; $^{c}P < 0.05$ versus MPTP; $^{*}P < 0.05$ within group comparison.

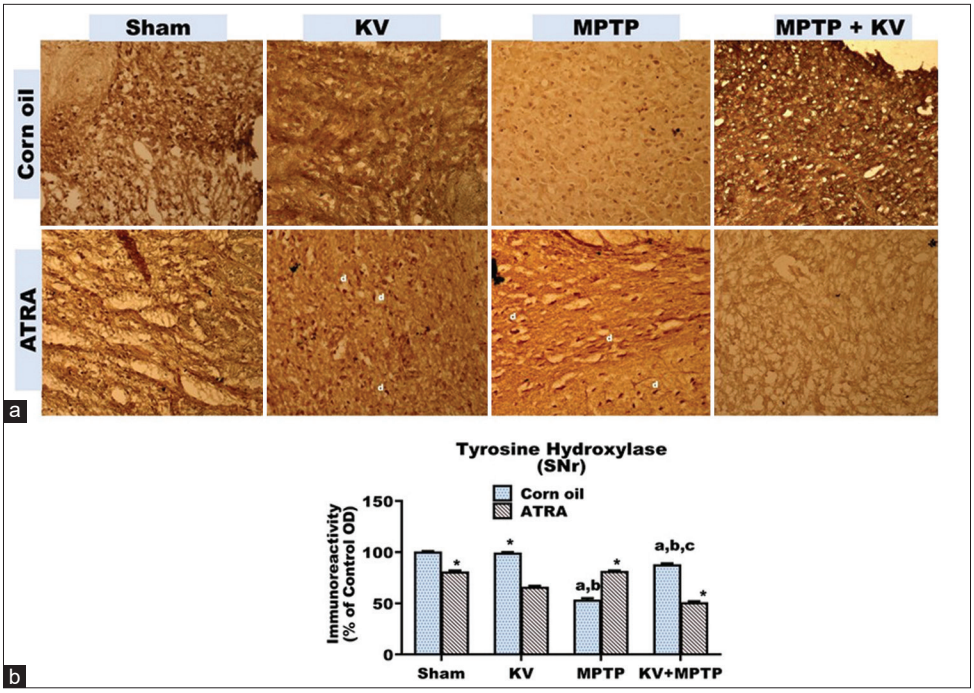


Figure 6: All-trans retinoic acid (ATRA) treatment prevents the suppresses the protective activity of kolaviron on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-mediated loss of dopaminergic neurons. Immunohistochemistry was used to evaluate the expression of tyrosine hydroxylase in the substantia nigra pars reticulata in the mice after treatment with ATRA, kolaviron (KV) and/or MPTP. Representative images from all groups are presented (a). The optical density of the images was analyzed with ImageJ and presented as mean ± SEM (b). $^{a}P < 0.05$ versus sham; $^{b}P < 0.05$ versus KV; $^{c}P < 0.05$ versus MPTP; $^{*}P < 0.05$ within group comparison.

Immunofluorescence staining showed that the expression of Nrf2 [Figure 7] and NQO1 [Figure 8] of KV-treated mice was similar to the constitutive level in control mice while the immunoreactivity of Keap1 was increased by 21%. Confirming that ATRA is a suitable Nrf2 inhibitor, the level of Nrf2, NQO1, and Keap1 was similar to the control level throughout the 7-day treatment period. Immune complex of Nrf2, NQO1, and Keap1 [Figure 9] was induced by MPTP intoxication by 63%, 26%, and 38%, respectively, above the control level. While the pre-treatment of MPTP mice with KV potentiated the Nrf2 and NQO1 expression levels by 8% and 14%, respectively, when compared with MPTP, ATRA treatment, relative to MPTP+KV group, abolished the potentiated response by 31% and 20%, respectively, to near basal level. Meanwhile, when treated with MPTP, ATRA did not affect NQO1 but significantly reduced Nrf2 by 4% and sustained Keap1 upregulation by 11%. However, in ATRA and KV mice, Nrf2 and NQO1 were increased by 46% and 19% while Keap1 was reduced by 20%, relative to KV group.

Considering the above result, it appears that ATRA uses different mechanism to regulate the protein level of DJ-1 relative to NQO1, Nrf2, and Keap1 expression. Induction of DJ-1, which is associated with the neuroprotective effect of KV, appeared to be a correlate of ATRA-mediated striatal toxicity. Mice used Nrf2 signaling adaptively in response to MPTP but without any effect on DJ-1 response. In response to MPTP, KV potentiated Nrf2 signaling cascade with additional induction of DJ-1 response. Co-administration of ATRA with MPTP mildly inhibits Nrf2 response.

DISCUSSION

Several animal and clinical evidence have demonstrated the biological relevance of KV in suppressing conditions associated with inflammation and redox imbalance.[17] In the past decade, emerging data are confirming that neuronal injury can also be prevented or repaired by KV.[39-42] Recently, we provided a proof of concept that KV could offer a potent neuroprotective advantage for the prevention and management of PD.[14] In the study, we showed that KV suppressed sensorimotor imbalance, loss of dopaminergic neurons in the SNc and striatal pathology in rotenone mice through a mechanism related to its antioxidant and anti-inflammatory properties.[14] However, given the intermediary role of Nrf2 in regulating the endogenous cytoprotective antioxidant capacity, and a previous report indicating that attenuation of LPS-induced neuroinflammation in microglial BV2 and neuronal HT22 co-culture system by KV is dependent on Nrf2/ARE, we hypothesized that the demonstrated neuroprotective ability of KV may involve Nrf2 signaling. In this study, using ATRA as an Nrf2 inhibitor,

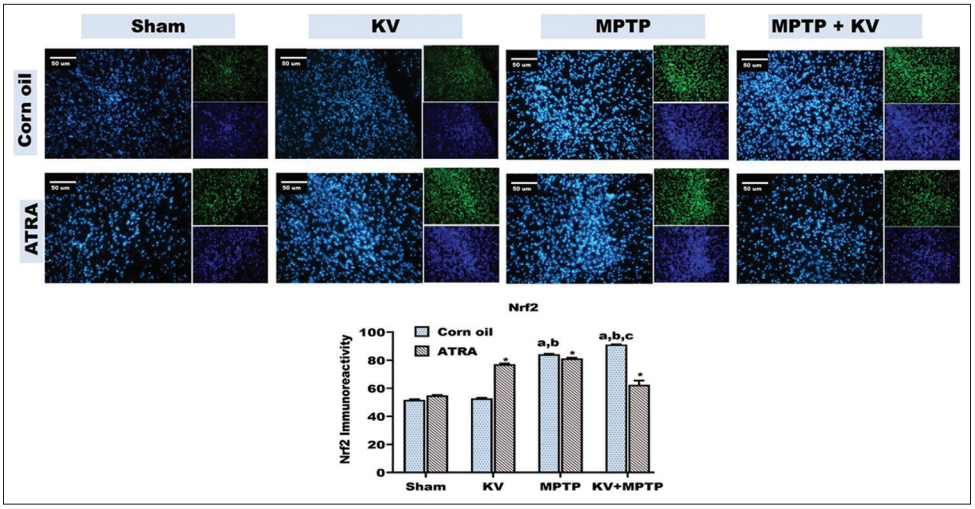


Figure 7: Kolaviron treatment potentiated the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced nuclear factor erythroid 2-related factor 2 (Nrf2) upregulation but All-trans retinoic acid (ATRA) treatment reverses the modulatory activity of kolaviron (KV). Brains from mice treated with ATRA, KV and/or MPTP were collected and sectioned to reveal the substantia nigra and processed for immunofluorescence staining. Representative images (n = 5) showing Nrf2 immunoreactivity and the optical density were presented. [a]$P < 0.05$ versus sham; [b]$P < 0.05$ versus KV; [c]$P < 0.05$ versus MPTP; *$P < 0.05$ within group comparison.

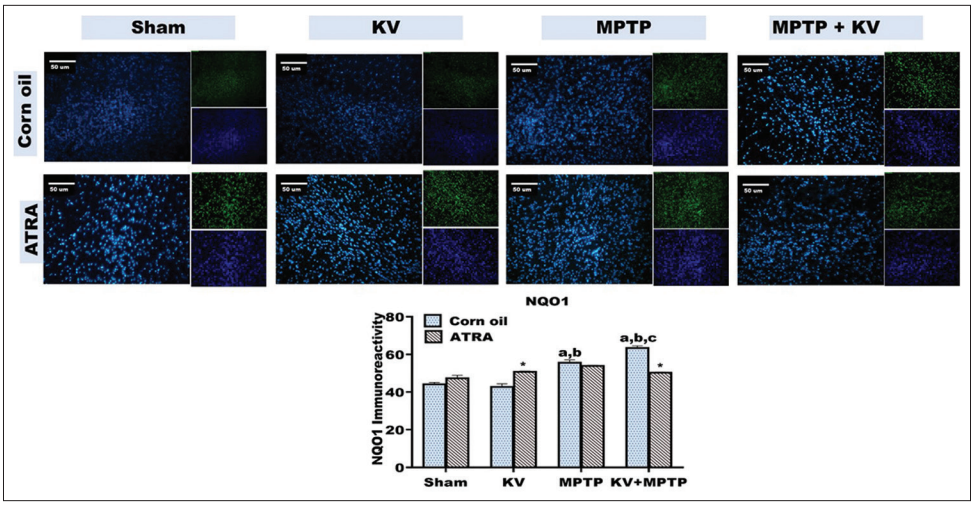


Figure 8: Kolaviron treatment potentiated the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced NQO1 upregulation but All-trans retinoic acid (ATRA) treatment reverses the modulatory activity of kolaviron (KV). Brains from mice treated with ATRA, KV and/or MPTP were collected and sectioned to reveal the substantia nigra and processed for immunofluorescence staining. Representative images (n = 5) showing NQO1 immunoreactivity and the optical density were presented. [a]$P < 0.05$ versus sham; [b]$P < 0.05$ versus KV; [c]$P < 0.05$ versus MPTP; *$P < 0.05$ within group comparison.

we demonstrated that the neuroprotective activities of KV against Parkinsonism-like symptoms in MPTP mice are reversible when Nrf2 is inhibited. Specifically, we showed that inhibition of Nrf2 abolished the KV-mediated improvement of locomotor defect, mitigation of striatal pathology and oxidative damage, suppression of nigral dopaminergic neuronal loss as well as enhancement of Nrf2 cytoprotective ability. This is the first *in vivo* study showing the involvement of Nrf2 in the neuroprotective effect of KV.

ATRA mediates its biological effect through the heterodimeric or homodimeric forms of two nuclear receptors RARs and RXRs.[43] Binding of ATRA to its nuclear receptors followed by the subsequent binding of the complex to the retinoid acid response elements (RARE) mediates the canonical biological response of ATRA, even though a fraction of ATRA's response involves epigenetic modification.[43] While the transcriptional induction in response to ATRA involves

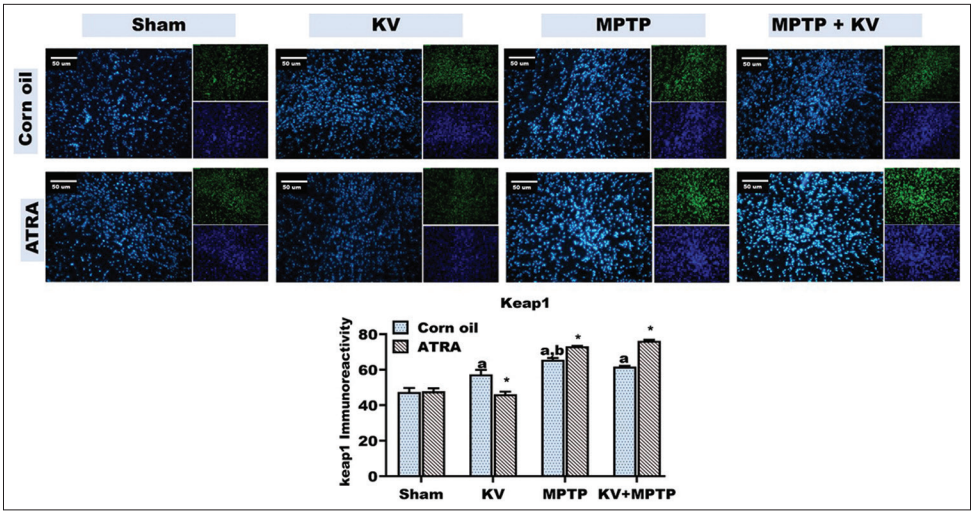


Figure 9: Kolaviron treatment downregulated the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-mediated Keap1 induction but All-trans retinoic acid (ATRA) treatment reverses the modulatory activity of kolaviron (KV). Brains from mice treated with ATRA, KV and/or MPTP were collected and sectioned to reveal the substantia nigra and processed for immunofluorescence staining. Representative images ($n = 5$) showing Keap1 immunoreactivity and the optical density were presented. [a]$P < 0.05$ versus sham; [b]$P < 0.05$ versus KV; [c]$P < 0.05$ versus MPTP; *$P < 0.05$ within group comparison.

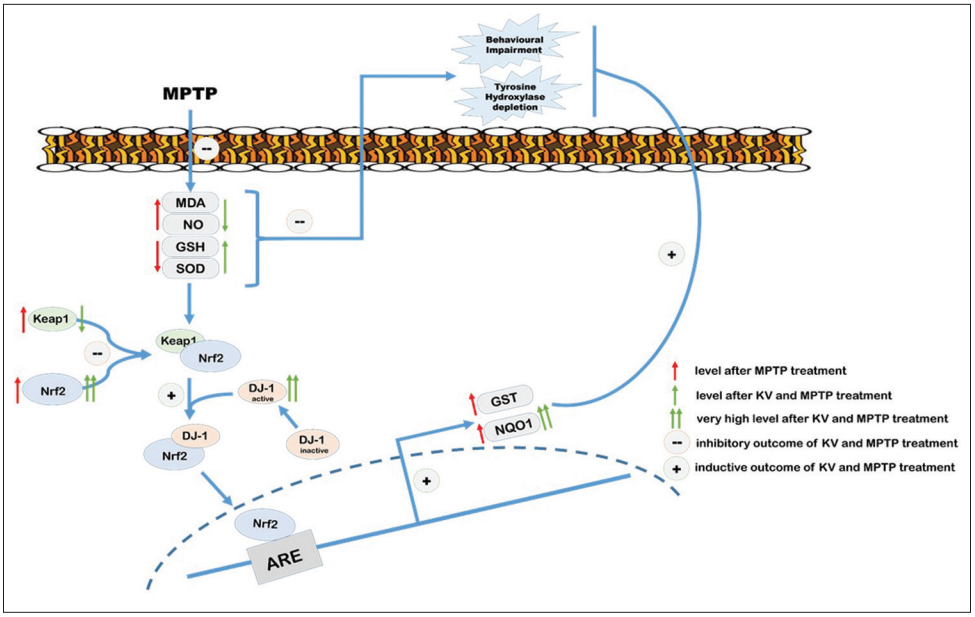


Figure 10: A scheme showing the effect of kolaviron on oxidative stress, tyrosine hydroxylase depletion and behavioural impairment in mice treated with MPTP. MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; ARE: Antioxidant response element; DJ-1, PARK7; GSH: Reduced glutathione; GST: Glutathione-S-transferase; Keap1: Kelch-like ECH-associated protein 1; MDA: Malondialdehyde, a lipid peroxidative product; NO: Nitric oxide; NQO1: NAD(P)H; quinone oxidoreductase-1; SOD: Superoxide dismutase.

upregulation of endogenous antioxidant system,[44] the presence of oxidative stress or states mitigates DNA binding of ATRA-mediated RAR/RXR heterodimers leading to ATRA resistance.[45] In addition, at higher and frequent ATRA dosing, IRF1 nuclear mobilization and response element binding are triggered culminating in the induction of RARE-independent gene expression including the apoptotic-facilitating ones.[46,47] Taken together, ATRA has the dual capacity as a pro- and anti-survival agent, depending on the doses. In this study, even though ATRA alone did not elicit pronounced behavioral defect, the treatment caused significant oxidative damage mediated by nitrosative stress and vacuolated striosome despite increased adaptive antioxidant response and minimal effect on Nrf2 expression. Thus, it appears that mechanisms controlling the toxic properties and

cytoprotective response of ATRA, which coexisted in this study, are independently regulated.

Further, we observed that the balance between the two opposing properties of ATRA may be influenced by the persisting environmental milieu. For instance, when ATRA-treated mice were administered MPTP, the toxicity of MPTP was exacerbated and prominent degenerating dopaminergic neurons were evident even though tyrosine TH expression was minimally enhanced, possibly because of the oxidative environment created by MPTP.[45] However, when ATRA was co-treated with KV for 7 days, our findings indicated that ATRA possibly antagonized KV. The antagonistic effect was associated with the upregulation of Nrf2 cascade. While ATRA has been reported to antagonize[36] or aggravate[48] the effect of toxic compounds, the mechanism responsible for antagonizing the non-toxic effect of KV is sketchy but related to oxidative stress.

However, ATRA showed a distinct property when it was administered with KV and MPTP. It was evident that neither did ATRA potentiated the toxic response with KV nor exacerbated the aggravated response with MPTP. In the presence of ATRA, the protective effect of KV on MPTP-mediated nigrostriatal injury and behavioral incompetence was suppressed in a fashion that encompassed what was excluded by the independent toxicity with either KV or MPTP. Notably, this observation correlated well with the suppression of DJ-1 level and not only with the inhibition of Nrf2 signaling cascade. This confirms an earlier report that susceptibility of neuronal cell to oxidative stress and PD-inducing 6-hydroxyl dopamine, another neurotoxin which inhibits complex 1 of the electron transport chain, is dependent on the presence and level of the multifunctional DJ-1.[49] Since other ATRA-treated groups, including ATRA alone group, with minimal Nrf2 signaling before MPTP challenge, also experience induction of DJ-1 but with associated toxicity, this indicates that KV must be activating a specific yet-undetermined downstream cytoprotective function of DJ-1/Nrf2. However, this function could be attributed to the reducing environment created by KV against MPTP challenge as the NO level in the ATRA, ATRA+KV, and ATRA+MPTP were increased by 37%, 22%, and 57%, respectively, relative to KV+MPTP. For DJ-1 to perform any of its multifunctional activity, it must be activated through the oxidation of its methionine and cysteine residues to the active DJ-1-SO_2H. Further oxidation results in the inactive DJ-1-SO_3H and loss of activity.[12] Like the UCP0054278, a modulator of oxidized DJ-1 which protected dopaminergic neurodegeneration in both 6-OHDA and rotenone model of PD,[50,51] KV may be stabilizing and sustaining the active DJ-1-SO_2H by direct binding or preventing its transformation to the inactive state by warding off binding ROS and other peroxidative agents. However, this warrants further investigation.

CONCLUSION

This report explores the involvement of Nrf2 to extend the evidence that KV may be beneficial in the management of PD. We have demonstrated that the neuroprotective activity of KV in MPTP model of PD is mediated through potentiation of Nrf2 and upregulation of DJ-1 [Figure 10]. Thus, this study suggests that KV can be explored further as a pharmacological Nrf2 and/or DJ-1 modulator in the management of oxidative stress-mediated movement disorders. However, further study is warranted to elucidate the specific relationship between KV and Nrf2 or DJ-1.

Declaration of patient consent

Patient's consent not required as there are no patients in this study.

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Conflicts of interest

There are no conflicts of interest.

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