Pharmacological Blocker of NF-κb and Mitochondrial Ros Restrict NLRP3 Inflammasome Activation and Rescue Dopaminergic Neurons in Vitro and in Vivo Parkinson’s Disease

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Research Article

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Abstract

Several activators of NLRP3 inflammasome have been described; however, the central mechanisms of NLRP3 inflammasome activation in brain microglia, especially at the activating step through free radical generation, still require further clarification. Hence the present study aimed to investigate the role of free radicals in activating NLRP3 inflammasome driven neurodegeneration and elucidated the neuroprotective role of perillyl alcohol (PA) in vitro and in vivo models of Parkinson's disease. Initial priming of microglial cells with lipopolysaccharide (LPS) following treatment with hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) induces NF-κB translocation to nucleus with robust generation of free radicals that act as Signal 2 in augmenting NLRP3 inflammasome assembly and its downstream targets. PA treatment suppresses nuclear translocation of NF-κB and maintains cellular redox homeostasis in microglia that limits NLRP3 inflammasome activation along with processing active caspase-1, IL-1β and IL-18. To further correlates the in vitro study with in vivo MPTP model, treatment with PA also inhibits the nuclear translocation of NF-κB and downregulates the NLRP3 inflammasome activation. PA administration upregulates various antioxidant enzymes levels and restored the level of dopamine and other neurotransmitters in the striatum of the mice brain with improved behavioural activities. Additionally, treatment with Mito-TEMPO (a mitochondrial ROS inhibitor) was also seen to inhibit NLRP3 inflammasome and rescue dopaminergic neuron loss in the mice brain. Therefore, we conclude that NLRP3 inflammasome activation requires a signal from damaged mitochondria for its activation. Further pharmacological scavenging of free radicals restricts microglia activation and simultaneously supports neuronal survival via targeting NLRP3 inflammasome pathway in Parkinson's disease.

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by degeneration of dopaminergic neurons within the substantia nigra par compacta (SNpc) causing depletion of dopaminergic transmission within the striatum that impact motor coordination [1]. The cardinal features of the disease include symptoms like tremor at rest, rigidity, muscle stiffness, slowness of movement (bradykinesia) and poverty of movement (hypokinesia) [2]. The prime neuropathological hallmarks of the disease include intracellular inclusions containing aggregates of α-synuclein and formation of Lewy bodies [3]. Mounting evidence states the role of oxidative stress and microglia mediated neuroinflammation as a nearly pathological changes in the progression of neurodegeneration [4]. However, underlying mechanism still requires further investigation and novel potential therapeutics are needed to be explored in halting microglia mediated neurodegeneration.

Microglia are immune cells of brain which principally function as a surveillance system in regulating several signals to maintain brain homeostasis. Neuronal survival directly depends upon the microglia cells function, which includes biochemical homeostasis, neuronal integrity and neuronal remodelling within brain [5]. Microglia can be activated by a variety of stimuli including pathogen-associated molecular pattern (PAMPS) like lipopolysaccharide (LPS) and endogenous damage-associated molecular pattern (DAMPs) which varies from ATP, mitochondrial DNA, potassium efflux, urate crystal and reactive
oxygen species (ROS) [6]. Nucleotide-binding oligomerization domain (Nod)-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome is a widely studied in context to microglia activation and plays an essential role in initiating inflammatory cascade in PD brain [7]. Active IL-1β and IL-18 production remains the foremost inflammatory cytokines generates from microglia and plays a crucial role in both acute and chronic neuroinflammation through amplifying innate and adaptive immunity [8]. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) mediated NLRP3 inflammasome regulation and production of active IL-1β and IL-18 are tightly controlled both at transcriptional and post-translational levels [9]. Generally, a two-step mechanism of NLRP3 activation and generation of active IL-1β and IL-18 is widely studied and accepted. Priming signal (Signal 1) acts via TLR-4 receptors and causes NF-κB translocation to the nucleus and produces biologically inactive intracellular precursors of both pro-IL-1β and pro-IL-18 [10]. The activation signal (Signal 2) involves inflammasome oligomerization in response to endogenous signals from malfunctioned mitochondria and elicit its action by activating the caspase-1 enzyme which then cleaves pro-IL-1β and pro-IL-18 to its matured forms responsible for neuroinflammation and pyroptosis within brain microglia [11, 12].

Mitochondrial dysfunction remains as one of the prominent features in several neurodegenerative disorders and is associated with generation of several endogenous insults which causes neuroinflammation within the brain regions [13–15]. Similarly, to cross talk, the role of mitochondria in maintaining the glial homeostasis is well documented in PD [16, 17, 15]. NLRP3 inflammasomes can sense any redox changes within its environments allowing its activation through a variety of stimuli including generated reactive oxygen species (ROS). Several studies have proven that many NLRP3 stimulators cause mitochondrial dysfunction but still there is a lacuna for understanding the role of NLRP3 activators in mitochondrial damage. Recently some studies have shown the role of mitochondrial ROS (mtROS) which act as one of the major endogenous DAMPs (Signal 2) in activating NLRP3 inflammasomes complex within the glia [18, 19]. However, the central mechanisms of NLRP3 inflammasomes activation in microglia through free radicals generation is unclear and require further explanation.

Perillyl alcohol (PA), a monoterpenic is found in essential oils of plants such as peppermint, sage, mints, cherries, citrus fruits and lemongrass [20]. PA has been reported to exhibit anti-inflammatory [21], antioxidant, anticancer [22] and neuroprotective effects [23]. PA is shown to intensify the memory formation in scopolamine treated rats by restoring dopaminergic neurons and acetyl-cholinesterase activity in brain [24]. PA was found promoting neurogenesis in Neuro-2a cells by inhibiting ubiquinone (CoQ) synthesis [25]. Line of evidences has also suggested its beneficial effects in Alzheimer’s disease owing its antioxidant and anti-inflammatory activity [26]. Further, PA was also seen to inhibit neuronal death in unilateral 6-hydroxydopamine (6-OHDA) toxicity by promoting mitochondrial biogenesis [27]. However, the effect of PA in context to microglial mediated neuroinflammation in PD largely remained unexplored. Therefore, the current study is aimed to understand the role of mitochondrial dysfunction and NLRP3 inflammasome activation utilizing complex I inhibitor (MPTP) and exogenous ROS generation (hydrogen peroxide) that mediates inflammation-driven neurodegeneration. Furthermore, the study also involves
screening the neuroprotective activity of PA and to understand how targeting mtROS generation resilience inflammasome mediated neurodegeneration in PD mice model.

2. Material And Methods

2.1 Chemicals and reagents

MPTP-HCl (Cat no: M0896), hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) (Cat no: 386790), Lipopolysaccharide (LPS) from \textit{Escherichia coli} serotype 026:B6 (Cat no: L827), Perillyl alcohol (Cat no: 218391), Mito-TEMPO (Cat no:SML0737), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Cat no: M2128), ATP estimation kit (Cat no: MAK190), mitochondrial isolation kit (Cat no: MITOISO1), radioimmunoprecipitation assay (RIPA) buffer (Cat no: R0278), protease inhibitor cocktail (Cat no: P8340), phosphatase inhibitor cocktail (Cat no: P2850), Cresyl violet acetate (Cat no: C5042), Nor-epinephrine bitartrate salt (Cat no: N5785), Dopamine hydrochloride (Cat no: H8502), Serotonin hydrochloride (Cat no: H9523) and 5-Hydroxyindole-3-acetic acid (Cat no: H8876) were procured from Sigma Aldrich (St. Louis, MO, USA). Cytokine analysis kits were used to estimate IL-1\textbeta (Cat no: BMS6002), Amplex™ Red Hydrogen Peroxide/Peroxidase Assay Kit (Cat no: A22188), tissue protein extraction reagent (T-TER) (Cat no: 78510), tetraethyl benzimidazolyl carbocyanine iodide (JC-1) dye (Cat no: T3168), MitoTracker™ Deep Red FM (excitation/emission: 644/665) (Cat no: M22426) were procured from Thermofisher scientific. Total protein was estimated using a Bicinchoninic acid assay (BCA) Protein Assay kit (Cat no: 71285-3) from Novagen, EMD, Millipore, MA, USA. TNF-α estimation ELISA kit (Cat no: KMC3011) was obtained from Novex life technologies. NLRP3 monoclonal antibody (Cat no: MA5-23919), Iba-1 polyclonal antibody (Cat no: PA5-27436), Alexa Flour™ 488 goat anti-rabbit IgG (H+L, Cat no: A11034), goat anti-rat IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 555 (Cat no: A21434) and Alexa Flour™ 594 goat anti-mouse IgG (H+L) (Cat no: A11032) were obtained from Invitrogen. β-actin (Cat no: 4967S), tyrosine hydroxylase (TH, Cat no: 2792S), phospho-NF-κB p65 (Cat no: 3033), anti-rabbit IgG (Cat no: 7074S), anti-mouse IgG (Cat no: 7076S), anti-rat IgG (Cat no: 7077), normal goat serum (Cat no: 5425) were obtained from cell signaling technology. Anti-caspase-1(p20) (Cat no: AG-20B-0042) was obtained from Adipogen. Mouse IL-1β (Cat no: AF-401-NA) was obtained from R&D system. IL-18 rabbit pAb (Cat no: A1115) was procured from ABclonal, USA.

2.2 Cell culture, cell viability, and treatment

N9 mouse microglial cells (passage no-16) were obtained as gift sample from Dr. Anirban Basu (National Brain Research Centre, Manesar, India). Briefly, N9 microglial cells were cultured in complete RPMI-1640 medium containing 5mM glucose, 2mM glutamine, 10% FBS, and 1% streptomycin/penicillin in a humidified CO\textsubscript{2} incubator (5% CO\textsubscript{2} and 37°C). N9 microglial cells were seeded in a sterile 96 well plate at a density of 1x 10\textsuperscript{4} cells per well. The cytotoxicity of the test compounds PA, LPS, H\textsubscript{2}O\textsubscript{2} and combination of LPS and H\textsubscript{2}O\textsubscript{2} were assessed by taking different concentrations.
**In vitro** microglial neuroinflammation model was established by priming LPS and H₂O₂ at different concentration. For LPS/H₂O₂ priming treatment, cells were serum-starved for 2 h and priming was done with LPS at a concentration of 1µg/ml for next 4 h. Further, the media was washed with sterile PBS to remove any trace of LPS, and then H₂O₂ (100 µM) was further added for 2 h. The cell viability was assessed through standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and absorbance were recorded at a wavelength of 570nm. Cell viability was expressed as a percentage of the value in the control cells. Subsequently, neuroinflammatory protein markers was assessed by immunoblotting and ELISA assay.

### 2.3 Evaluation of mitochondrial membrane potential

Mitochondrial membrane potential (Δψm) was evaluated by utilizing specific mitochondrial fluorescent probe JC-1 dye. Under normal conditions, Δψm JC-1 forms an aggregate with high red fluorescence intensity. Loss in the Δψm is indicated with decrease red fluorescence and increase green fluorescence as the dye shift from aggregate to monomeric form. Thus, the ratio of red/green serves as an indicator of loss of Δψm.

### 2.4 Evaluation of mitochondrial superoxide generation

Mitochondrial ROS was measured using MitoSOX™ Red FM. After priming the cells with LPS/H₂O₂ and treatment with test compound, cells were stained with 5µM MitoSOX™ Red for 10min at 37°C. Cells were washed with warm PBS buffer and observed under a confocal microscope (Leica TCS SP-8) with absorption/emission maxima: ~510/580 nm.

### 2.5 Detection of extracellular hydrogen peroxide using Amplex red assay

N9 microglia cells (2x10^5 cells per well) were cultured in 6 well plates and kept overnight for proper attachment. The first experiment involves LPS (1 µg/ml) priming for 4 hrs followed by H₂O₂ (100µM) treatment for different time points (0, 0.5, 1, 1.5, 2 hr). Further second experiment involve successive drug treatments (PA100, PA200, MITO100) in LPS primed N9 cells H₂O₂ treated microglia to detect the H₂O₂ release by a standard Amplex red assay kit. The generated fluorescent product (resorufin) was measured using microplate reader (PROMEGA: GloMax® Discover) (excitation: 540 ± 20nm, emission: 590 ± 20nm). The extracellular H₂O₂ releases from different treatment groups were determined using a standard H₂O₂ curve (0–20µM) as per manufacturer’s protocol. The liberated H₂O₂ was expressed as picomoles/minute (pmoles/minute) [28, 29].

### 2.6 Immunocytochemistry (ICC)

N9 cells were plated on poly D-lysine coated coverslips in a 6 well culture plate at a density of 2x10^6 cells per well. After priming the cells with LPS/H₂O₂ and treatment with test compound, cells were washed with PBS, fixed with 4% paraformaldehyde and then permeabilized with 0.2% Triton X-100. Cells were blocked
with 5% normal goat serum (NGS), washed and incubated overnight with primary antibodies: NLRP3 (1:250 dilution), and pNF-κB 65 (1:800 dilution) at 4°C. Next, cells were washed with PBS and then incubated with secondary antibodies namely Alexa Flour™ 488 goat anti-rabbit IgG(H + L) and Alexa Flour™ 594 goat anti-mouse IgG(H + L). Subsequently, nuclei were stained with Vectashield mounting medium for fluorescence with 4',6-diamidino-2-phenylindole (DAPI) (Cat no. H-1200, Vector Laboratories, Burlingame, CA). Negative control slides were prepared by the exclusion of the primary antibody. Slides were kept in a cool place until observed under oil emersion at 40x magnifications with a confocal microscope.

2.7 Animal studies

Male C57BL/6 mice (10-weeks-old, weighing between 22g to 25g) were obtained from Palamur Biosciences Private Limited, Hyderabad, India, and were acclimatized for two weeks before initiation of the study.

The study and protocol procedures were approved by the Institutional Animal Ethics Committee (IAEC) (Approval No- NIPS/NIPER/18–032) of the Nemcare Group of Institutions-Mirza, Kamrup (CPCSEA No.1996/PO/Re/S/17/CPCSEA).

Animals were kept in individually ventilated Cages (IVC) (Tecniplast, UK) in a standard environment condition (22 ± 2°C; humidity 65 ± 5%; 12-hour light/dark cycle).

All the experiments were conducted as per the guidelines laid by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India.

All the animals were accessed to food and water ad libitum.

2.8 Experimental design and drug treatment

Mice were randomly divided into five groups consisting of 15 animals in each and the treatment was performed as mentioned below:

Group 1 (Normal control): Animals were administered orally with corn oil (5ml/kg body weight) for 14 consecutive days and normal saline (1ml/100g body weight) was administered intraperitoneally (i.p.) for 5 consecutive days starting from day 1 to day 5.

Group 2 (MPTP-25mg/kg): Animals were administered i.p. with MPTP-HCl (dissolved in sterile normal saline, 25mg/kg body weight) for 5 consecutive days starting from day 1 to day 5. Corn oil (5ml/kg body weight) as a drug vehicle was administered orally for 14 consecutive days.

Group 3 (MPTP + PA-100mg/kg): Mice were administered i.p. with MPTP-HCl(dissolved in sterile normal saline, 25mg/kg body weight) for 5 consecutive days starting from initial day 1 to day 5 and a suspension of PA (100mg/kg body weight) in Corn oil (5ml/kg body weight) was administered orally for 14 consecutive days.
Group 4 (MPTP + PA-200mg/kg): Animals were administered i.p. with MPTP-HCl (dissolved in sterile normal saline, 25mg/kg body weight per day) for 5 consecutive days starting from initial day 1 to day 5 and a suspension of PA (200mg/kg body weight) in Corn oil (5ml/kg body weight) was administered orally for 14 consecutive days.

Group 5 (MPTP + Mito-TEMPO-1mg/kg): Animals were administered i.p. with MPTP-HCl (dissolved in sterile normal saline, 25mg/kg body weight) for 5 consecutive days starting from initial day 1 to day 5 and Mito-TEMPO (1mg/kg body weight) dissolved in normal saline was administered i.p. for 14 consecutive days.

The dose and duration of treatment of PA and Mito-TEMPO were selected based on previously published report [30]. The dose and duration of MPTP to induce experimental Parkinson-like phenomenon in mice were selected based on our pilot study and earlier reports [31]. MPTP-HCl solution preparations, dosing of animals, and handling of bedding material were done in accordance with the safety guidelines outlined by Przedborski et al [32]. The behavioral parameters were recorded on 0th day, 7th, and 14th day. On the 15th day, animals were euthanized using a high dose of isoflurane, and the brains was immediately collected and stored at -80°C for further analysis (Fig. 1).

2.9 Behavioral tests

2.9.1 Catalepsy test

Catalepsy (bar test) test was performed to assess the behavioral abnormalities in rodents. In this method, front limbs of the mice were suspended on a wooden bar 5 cm high. Length of time in seconds were recorded by the control and treated animals to move the limb back to the surface [33].

2.9.2 Grip strength test

Grip strength of the forelimbs was measured using a digital grip strength meter (Ugo Basile, Gemonio (VA) Italy) [34]. Mice were allowed to grab an iron grid with its forelimbs and were gently pulled back to record its grip strength. The grip strength was expressed in gf (gram x force).

2.9.3 Rota-rod test

Rota-rod experiment was performed to assess the motor coordination and grip performance of all experimental animals. Briefly, mice were placed on the apex of the rota-rod treadmill (Ugo Basile, Gemonio (VA) Italy), and the latency to fall (in seconds) were reordered automatically by individual sensors. Before start of experiment, baseline training was given to all experimental mice. Data was collected on the 0th, 7th, and 14th day in acceleration mode (4–45 rpm) over 5 min.

2.9.4 Pole test
Pole test is used to measure the degree of bradykinesia, basal ganglia related motor disturbances in experimental animals as described earlier [35]. The mice were allowed to run a vertical pole 50cm length and the time taken to reach the ground was recorded in seconds.

2.9.5 Walking track analysis

Both the limbs of trained mice were dipped in black ink and were allowed to walk on a white paper (4.5 cm wide, 42 cm long) towards a dark cage. Before start of experiment, mice were subjected to training trials to acclimatize the environment. Stride length was measured as the distance between successive paw prints [34]. Data were presented as the average of five stride lengths for three different animals from each group.

2.9.6 Open field test

An open field test (OFT) is used to evaluate spontaneous locomotor activity. The apparatus consists of a wooden box (50×50×38 cm). Mice were placed into the centre square and behavioral activities were videotaped for 5 min. The parameters used for analysis includes the total distance travelled; average speed, and immobility time for a five-minute period. All the behavior analysis was done by Any-maze video tracking software version-6.1(Stoelting Co, USA).

2.10 UHPLC analysis of neurotransmitters

Briefly, the animals were sacrificed and the striatum region was isolated and homogenized in 0.1M perchloric acid (PCA) followed by centrifugation at 13000 rpm at 4°C for 10 min. The supernatant was collected and filtered through a 0.22 µm filter and 15µl volume was injected in a thermo scientific-UHPLC-ultimate 3000 with an electrochemical detector (ECD) using an isocratic elution system. The column used was hyper cell gold-150×4.6mm, flow rate-0.8ml/min, run time-45min, ECD-1 potential-550mv, ECD-2 potential-300mv. A linearity standard curve (1000ng/ml, 500ng/ml, 250ng/ml, 100ng/ml, 50ng/ml, 25ng/ml 5ng/ml) was plotted by using freshly prepared standards of nor-epinephrine (NE), dopamine (DA), serotonin (5-HT) and 5-hydroxy indole acetic acid (5-HIAA). The mobile phase was prepared by using 75 mM sodium dihydrogen phosphate, 1.7 mM 1-octanesulfonic acid, 100 µl/l triethylamine, 25 µM EDTA, 10 % acetonitrile and pH of mobile phase was adjusted to 3 with phosphoric acid. The calibration curve was generated with the Chromeleon-7 software and relative amines concentrations were obtained as an integrated peak area for the individual analyte.

2.11 Western blotting

Substantia nigra par compacta (SNpc) region of the midbrain was excised out and tissues were lysed with RIPA lysis buffer containing protease and phosphatase inhibitors cocktail. The total protein was estimated using the BCA protein assay kit. Approximately30 µg of protein was loaded in each lane and separated on a10%, 12%, and 14% SDS polyacrylamide gel. Protein was transferred on a nitrocellulose membrane (Bio-Rad), blocked with 5% bovine serum albumin (BSA), and incubated in primary antibody overnight at 4°C. Primary antibodies: NLRP3 (1:1000), caspase-1 (1:1000), IL-1β (1:1000), IL-18 (1:1000), P-NF-κB p65 (1:1000), Iba-1 (1:1000) and tyrosine Hydroxylase (1:1000) were used. After washing,
membranes incubated with appropriate anti-rabbit HRP conjugated secondary antibody (1:1000) for 2h at room temperature. β-actin was used as internal loading control for each blot. Membranes were washed and visualized in Fusion Fx chemiluminescence (Vilber Lourmat) system using ECL reagent (Cat no. 1705061 Bio-Rad). Densitometric analysis was performed using Image J analysis software (National Institutes of Health, Bethesda, Maryland, USA).

2.12. Cresyl Violet staining of SNpc tissue (Nissl-stained positive neurons)

Brain tissues were embedded in Optimal cutting temperature (OCT) medium and sectioned at 20 µm thickness using a cryostat. Sections were mounted on Poly d-lysine coated slides and dried for several hours at room temperature. Slides were stained with cresyl violet stain for 6 to 8 mins in a 60°C preheated water bath. Slides were allowed to cool and washed with running tap water to drain excess stain. Slides were then rehydrated with a series of alcohol and cleared in xylene. Sections were mounted with DPx mounting media and observed under a light microscope (Evos FL Auto 2). Nissl-stained positive neurons were quantified using ImageJ (Fiji version) software as described by Paul R et al [36]. Low (40x) and high magnification images (400x) of the SNpc region were acquired by a bright field microscope.

2.13. Immunohistochemistry (IHC)

Animals were anesthetized with ketamine and xylazine combination at a dose of 100mg and 10mg/kg body weight respectively and perfused intracardially with 50 mL of PBS followed by 100 ml of 4% paraformaldehyde (PFA). After euthanasia, brain tissue was isolated and further fixed in 4% PFA for 24h at 4 ºC. Then brain tissue was immersed in 10%, 20%, and 30% sucrose solutions gradually for cryoprotection until the tissue sink at the bottom. Midbrain was excised out and embedded in an OCT medium and sections approximately 20 µm were cut with a cryostat throughout the midbrain and identified the SNpc region. Antigen retrieval step was done in the microwave oven and heating the sections at 95 ºC in 10mM trisodium citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by dipping sections in 3% hydrogen peroxide. Sections were washed with PBS and blocked with NGS for 1h at room temperature. Sections were incubated with primary antibody (tyrosine hydroxylase (1:200) and Iba1 (1:200) in a humidity chamber and kept at 4ºC overnight. Sections were washed and incubated with biotinylated universal antibody and avidin-biotin complex as per manufacture's protocol (Vecta stain Elite-ABC Universal kit, Vector Labs PK-6200). Sections were visualized with 3,3’-Diaminobenzidine(DAB) until brown color develops. Sections were further dehydrated with graded alcohol, cleared in 100% xylene and mounted in DPx mounting medium. Sections were observed under a bright field illumination using a trinocular microscope (Evos FL Auto 2). The total numbers of immunoreactive neurons and microglia in the entire extent of SNpc were stereologically counted from four mouse brains per group by observer blind to the experiment. Tyrosine hydroxylase and Iba-1 were quantified using ImageJ (Fiji version) software as described by Paul R et al [36]. Low (40x) and high magnification images (200x) of the SNpc region were acquired.
2.14. Isolation of mitochondria and ATP estimation

Mitochondria rich fractions was isolated from the SNpc region using a standard MITOISO1 kit (Sigma, St. Louis, MO) as per manufacture instructions. Protein content in the sample was estimated using the BCA protein estimation kit. The ATP content was measured in the mitochondrial pellet by a colorimetric method using a standard kit. The amount of ATP in the samples was expressed as nanogram per mg of tissue.

2.15 Estimations of pro-inflammatory cytokines (TNF-α and IL-1β)

SNpc homogenates were prepared using 50mM PBS, pH 7.4 containing protease inhibitor cocktail (1%). The concentrations of TNF-α and IL-1β were measured using respective mouse-specific TNF-α and IL-1β commercial ELISA kits. The respective concentrations were obtained by drawing a standard reference curve and expressed as picograms per mg of protein.

2.16 Biochemical Tests

2.16.1 Measurement of lipid peroxidation

Lipid peroxidation is the marker for the generated oxidative stress in the tissue. The malondialdehyde (MDA) is the formed end-product of lipid peroxidation. The obtained supernatant of the SNpc was processed separately for MDA level estimation. Briefly, 100μl supernatant was mixed with 200μl of 8.1% SDS, 20% acetic acid (pH 3.4) and thiobarbituric acid (0.8%). The resultant mixture in glass tubes kept for 1 hour in a water bath at 95°C followed by cooling it under running tap water. Finally, absorbance was measured at 532 nm on a multi-plate reader (SpectraMax i3x, Molecular devices). Thereafter, the results were compared among the groups and expressed as nmoles of MDA per mg of protein (Jangra et al., 2016b).

2.16.2 Measurement of reduced glutathione level

The reduced glutathione is the indicator of the glutathione-based antioxidant system in the tissue. Supernatant of the SNpc homogenate was mixed in equal ratio with 20% (w/v) trichloroacetic acid and centrifuged at 1000xg for 10 min at 4°C. The obtained supernatant was diluted with 0.3 M disodium hydrogen phosphate buffer and 250μl of 1mM freshly prepared DTNB [5,5-dithiobis (2-nitro benzoic acid) solution dissolved in 1% w/v sodium citrate and incubated for 20 min in dark. Final absorbance was taken at 412 nm on a multi-plate reader (SpectraMax i3x, Molecular devices). The results were calculated as nmoles of GSH/mg of protein [37, 38].

2.16.3 Determination of superoxide dismutase (SOD) and catalase (CAT) activity

The superoxide dismutase (SOD) and catalase (CAT) activity were quantified using the SOD and CAT assay kits, respectively. The assays were performed as per manufacturer’s protocol. Final absorbance of SOD and CAT activity was measured at 440 nm and 520 nm, respectively using a multi-plate reader.
SOD activity was represented as U/mg of protein while CAT activity was expressed as µmol of H$_2$O$_2$ consumed/min/mg of protein.

3. Data Analysis

All the results were indicated as the mean ± standard deviation (SD). For comparison between only control and treatment group, data were analysed by one-way analysis of variance (ANOVA) followed by Dunnett analysis. Two-way ANOVA was used for comparing multiple groups and was used for behavior studies. For comparing between different treatment groups, one-way ANOVA followed by Tukey’s post hoc test was applied. Statistical analysis was performed using Graph Pad Prism 8.01 software (GraphPad Software Inc., La Jolla, CA, USA). A value of $p < 0.05$ was considered statistically significant.

4. Results

4.1. Combined LPS and H$_2$O$_2$ treatment causes mitochondrial damage and activates NLRP3 inflammasomes expression in N9 microglia

NLRP3 inflammasome complex is widely studied in correlation to microglial activation but the exact mechanism for NLRP3 inflammasome activation is still debatable. Our prime focus in this study was to establish a link between LPS and hydrogen peroxide (H$_2$O$_2$) to mimic the exposure of inflammation and oxidative stress as a double-hit model in NLRP3 inflammasome activation. To test the hypothesis whether exogenous H$_2$O$_2$ treatment could act as a potent free radical in activating NLRP3 inflammasome in LPS primed microglia, we initially primed the microglia cells with a fixed dose of LPS i.e. 1000ng/ml (Signal 1) for 4h and then treated with different concentrations of H$_2$O$_2$ ranging from 50µM to 200µM for next 2h (Fig. 2B). Further data from kinetic studies using the combination of LPS + H$_2$O$_2$ treatment at different time interval showed a robust release of H$_2$O$_2$ at time points from 1hr, 1.5 hr with highest release at 2hr incubation (Fig. 2C). Thus, from the above preliminary studies, exposure of a combination of LPS and H$_2$O$_2$ at a dose of 1000 ng/ml and 100 µM for 4 hr and 2hr respectively was sufficient to release ROS without causing significant cell death. Next sets of experiments involve in addressing the question whether the combination of LPS and H$_2$O$_2$ was sufficient to activate NLRP3 inflammasome complex and its downstream target. Immunoblot data suggested that selected concentrations of LPS and H$_2$O$_2$ were sufficient enough to activate NLRP3 inflammasome, together with increased protein expression of active caspase-1(p20), IL-1β and IL-18 (Fig. 2D, 2E, 2F, 2G and 2H). These results were further supported by cytokine analysis from the cell supernatant showing a significant increase in the IL-1β level in LPS primed H$_2$O$_2$ treated microglia compared to LPS primed microglia and H$_2$O$_2$ treatment alone (Fig. 2I). However, we fail to detect any active processing of caspase-1(p20) mature IL-1β or mature IL-18 form neither in
LPS alone primed microglia nor in H$_2$O$_2$ treatment alone (Fig. 2F, 2G and 2H). Thus, from our data, it can be assumed that H$_2$O$_2$ can itself act as a DAMP (Signal 2) in augmenting the NLRP3 inflammasome complex together with cleavage of caspase-1 and release of active IL-1$\beta$ and IL-18 in LPS primed microglia. Moreover, cytokine analysis of microglia supernatant treated with LPS and H$_2$O$_2$ and H$_2$O$_2$ alone showed a significant increase in TNF-\(\alpha\) level as compared to unprimed cells (Fig. 2J). This indicates that TNF-\(\alpha\) does not require a secondary signal in the form of DAMP for its activation.

4.2. PA ameliorates H$_2$O$_2$ induced ROS generation and destabilization of MMP in LPS primed microglia

ROS play a major role in the initiation and progression of neurodegenerative diseases including PD. Combined LPS and H$_2$O$_2$ treatment caused a significant increase in the ROS generation which can act as Signal 2 in activating microglia through NLRP3 inflammasomes oligomerization. To test the hypothesis whether H$_2$O$_2$ treatment in LPS primed microglia caused the mitochondrial membrane depolarization with increase ROS generation, and understand the antioxidant potential of PA and Mito-TEMPO, a series of experiments related to \(\Delta \psi_m\) and ROS generation were conducted. We found that combined LPS and H$_2$O$_2$ treatment destabilize the inner MMP (\(\Delta \psi_m\)) and produced an increase in the conversion of the monomeric form of JC-1 (green) (Fig. 3A). Further, MitoSOX (Fig. 3B) revealed that combined LPS and H$_2$O$_2$ treatment significantly augmented the generation of mitochondrial superoxide anion levels in microglia cells. The results were further supported by flow cytometry analysis where increased JC-1 monomer (Fig. 3C) and decrease JC-1 aggregates (Fig. 3D) were seen in LPS primed H$_2$O$_2$ treated groups. Treatments with PA and Mito-TEMPO was seen to rescue \(\Delta \psi_m\) damage and causes a decrease mitochondrial superoxide anion levels compared to untreated microglial cells. Further treatment with PA and Mito-TEMPO was also found to inhibit the release free H$_2$O$_2$ generation as seen from the Amplex red assay (Fig. 3E). From these findings, we believe that H$_2$O$_2$ treatment in LPS primed microglia augment the ROS generation which cause damage to mitochondrial membrane leading to metabolic crises within microglia.

4.3 PA suppresses the NF-\(\kappa\)B mediated NLRP3 activation in LPS primed H$_2$O$_2$ treated microglial cells

The mechanism of NLRP3 inflammasome activation is a two-step process that involves priming signal (Signal 1 by LPS) and activation signal (Signal 2 by H$_2$O$_2$ mediated ROS generation). LPS is widely known as an inflammatory cytokine inducer which acts through activation of the NF-\(\kappa\)B signaling. Further ROS produced from H$_2$O$_2$ and generated H$_2$O$_2$ itself are believed to be the main activators of NLRP3 inflammasome complex and its downstream targets in LPS primed microglia. Hence, controlling the NF-\(\kappa\)B signaling and the ROS generation could be a potential strategy in inhibiting microglia activation. The findings of our study demonstrated that combined LPS and H$_2$O$_2$ treatment was sufficient to activate NLRP3 inflammasomes and its downstream targets i.e. generation of active caspase-1, IL-1$\beta$ and IL-18
(Fig. 4A), whereas PA treatment attenuates the NLRP3 inflammasome activation in a dose-dependent manner (Fig. 4B, 4C, 4D, and 4E). Treatment with PA and Mito-TEMPO further inhibit the increase expression of Iba-1 (microglia activation marker) and decreases the extracellular release of IL-1β level in LPS primed H₂O₂ treated microglia (Fig. 4F, 4G). The results were further confirmed by immunocytochemistry experiments where PA inhibits the nuclear co-localization of P-NF-κBp65 (Fig. 4H) and inhibits the expression of NLRP3 inflammasome protein in microglia cells (Fig. 4I). In our study, treatment with Mito-TEMPO (a standard mitochondria superoxide scavenger) exhibited similar activity alike PA. Thus, results of our study demonstrated that PA by virtue of its antioxidant property scavenges free radicals and inhibits the NF-κB mediated NLRP3 inflammasome activation in microglia cells.

### 4.4. PA ameliorates behavioral anomalies and restored the dopamine level in SNpc in MPTP-administered mice

All mice were trained before the initiation of experiments, and mice failed to pass the baseline behavioural performance were excluded from the experimental study. Five doses of MPTP (25mg/kg body weight) were injected i.p. to produce PD-like symptoms in mice. MPTP treated mice showed a decrease in neuromuscular tone and poor grasping strength with a shorter force to pull the bar in grip strength meter test (Fig. 5A). Similarly, in the rota-rod test, the latency to fall of time in seconds was reduced in the MPTP treated mice on the 7th and 14th day of experiment when compared with the vehicle control group which represents severe motor incoordination and disease progression (Fig. 5B). Mice treated with MPTP showed a marked increase in the cataleptic response on Day 7 and Day 14 when compared with vehicle control group (Fig. 5C). Pole climbing and OFT were performed to assess the bradykinesia (slow movement) in the MPTP-induced mice. The time taken by the mice to descend from the pole was recorded and compared with vehicle control to assess the disease severity in the MPTP-administered mice (Fig. 5D). Total distance travelled, average speed and immobility time was recorded in the OFT to correlate the motor behaviour in vehicle control, MPTP group and treatment groups (Fig. 5F). Abnormal gait and the stride length were measured in mice on Day 14 to evaluate motor incoordination as well as walking pattern behavior (through foot print analysis) (Fig. 5G).

Treatment with PA at both the doses 100 and 200mg/kg body weight ameliorates the MPTP-induced motor deficits in the mice and showed beneficial effect in improving motor coordination as evidenced from experiments involving rota-rod, pole climbing, OFT and footprint analysis. Catalepsy and grip strength test showed that PA treatment at both the doses 100 and 200 mg/kg body weight dose-dependently improved neuromuscular tone when compared with MPTP-administered mice. Moreover, treatment with Mito-TEMPO also improved all behavioral anomalies compared to MPTP-administered mice.

### 4.5 PA restored the antioxidant enzyme activities and inhibited the NLRP3 inflammasomes activation in brain
MPTP, as a complex-I inhibitor of mitochondrial ETC, causes robust generation of ROS with a decrease in several antioxidant enzyme levels along with mitochondria bioenergetic crises. The generated superoxide and peroxide ions were capable of activating NLRP3 inflammasomes together with its downstream targets as seen from the immunoblot data (Fig. 6A, 6B, 6C, 6D, and 6E). Further MPTP causes NF-κB activation which remains one of the major signaling mechanisms for the generation of inflammatory cytokines together with NLRP3 expression (Fig. 6F). The upregulated expression in different proteins were sufficient to activate microglia as seen from immunoblot data of Iba-1 in the SNpc region of the brain (Fig. 6G) along with reduced tyrosine hydroxylase (TH) positive stained neurons in the SNpc region of mice brain (Fig. 6H). PA treatment caused a significant increase in GSH level, SOD and Catalase activity with lowered MDA levels were seen in the treatment groups thereby exhibiting its strong antioxidant property (Fig. 6K, 6L, 6M and 6N).

ATP levels were estimated in the mitochondrial fraction of the SNpc region of mice brain where we can correlate mitochondrial dysfunction with reduction in ATP levels in MPTP-administered group. PA treatment as well as Mito-TEMPO showed a dose dependent restoration in the ATP levels with improved mitochondrial functions (Fig. 6O).

4.6 PA attenuates the dopaminergic neuronal loss and rescues the altered neurotransmitter level in MPTP-administered mice

MPTP-administration produced a significant reduction in Nissl-positive neurons (Panel A, Fig. 7) and tyrosine hydroxylase positive stained dopaminergic neurons (Panel B, Fig. 7) in the SNpc region of the mice brain compared with vehicle control group. PA treatment dose-dependently restored the dopaminergic neuronal loss as evidenced from Nissl positive staining and TH antibody immunostaining. To understand the microglia activation, we further confirmed the protein expression of Iba-1 in SNpc by employing immunohistochemistry (IHC). The protein expression of Iba-1 was significantly decreased in SNpc region of MPTP induced mice when compared with vehicle control group. Treatment with both PA and Mito-TEMPO significantly reduced the protein expression of Iba-1, and prevented the microglial activation when compared with MPTP-administered mice (Panel C, Fig. 7). The above data was further supported by estimation of neurotransmitter levels in the striatum region of the mouse brain. The concentrations of NE, 5-HT, 5-HIAA and DA were estimated in the striatum region of the brain tissue. We observed a significant decrease in the concentrations of NE; 5-HT and DA in brain of MPTP-administered mice when compared to vehicle control group. Whereas, the concentration of 5-HIAA was significantly increased in brain tissue of MPTP-administered mice. Depleted neurotransmitter levels and loss of dopaminergic neurons can be correlated with behavioural data where there was decreased motor function in MPTP-administered mice (Fig. 7F). Treatment with both PA and Mito-TEMPO significantly restored the concentrations of NE, 5-HT and DA, and decreased concentration of 5-HIAA in brain tissue when compared to MPTP-administered mice. The aforementioned findings concluded the neuroprotective effects of PA were comparable to Mito-TEMPO and this ensures PA exhibit strong antioxidant and anti-
inflammatory properties. Thus, tightly controlling signals that govern NLRP3 inflammasome activation could be a potential treatment regime in controlling inflammation-induced neurodegeneration in PD.

5. Discussion

Microglia are the resident immune cells within the brain that plays a fundamental role in maintenance of neuronal survival by phagocytising and eliminating cytotoxic insults [39]. Substantial evidences concluded chronic and persistent microglia activation function as a key regulator in initiating neurodegeneration in several neurological diseases including PD [40–42, 15]. This statement was further proven by the post-mortem brain analysis of patients suffering from PD was found with activated microglia in SNpc region where dopaminergic neurons are mostly concentrated [43]. NLRP3 inflammasome activation is commonly associated with microglial activation and release inflammatory mediators that cause neuronal damage and further progresses process of neurodegeneration in PD brain [44–46]. NLRP3 inflammasome activation is triggered by a variety of stimuli including microbial invasion, misfolded protein aggregates, and other DAMPs including ATP, ROS generation that triggers inflammation induced cell death (pyroptosis) [47, 48]. However, the regulatory mechanism underpinning NLRP3 activation in microglia through free radical generation is still remains controversial and need great attention [49]. Hence the present study focused the role of NLRP3 inflammasomes in microglial activation by adding a ROS inducer, H$_2$O$_2$ after priming step of LPS induced activation in N9 microglial cells. The study further involves elucidating the underlying neuroprotective mechanisms of PA and Mito-TEMPO by employing both in vitro N9 microglial cells and in vivo MPTP-administered PD model in mice.

Generally NLRP3 inflammasome activation is a two-step signalling process and represents major players in immune cell activation [50]. Signal 1 or pattern recognition induced priming signal (mainly by LPS) through TLR-4 receptors, induces transcription of NLRP3, pro-IL-1β, and pro-IL-18 by NF-κB dependent pathway [51]. Signal 2 activation occurs by endogenous insults such as ATP, urate crystals, mitochondrial dysfunction, potassium efflux that activate NLRP3 inflammasome and drives active caspase-1 processing which in turn synthesis of active IL-1β, and IL-18[7, 52]. Earlier reports have depicted the role of LPS + ATP combination in activating the NLRP3 inflammasomes complex, but these studies do not imitate exact condition of mitochondria mediated ROS generation in NLRP3 inflammasome activation [53, 10, 54]. Hence, within this study we tried to establish a cellular model whether changing the redox microenvironment regulates NLRP3 inflammasome activation through oxidative stress in LPS primed microglia. In this study, H$_2$O$_2$ was selected as an exogenous stimulant for mimicking the condition of oxidative stress in LPS-primed microglia. Remarkably, H$_2$O$_2$-treatment at a dose of 100 µM causes a robust increase in NLRP3 activation along with cleavage of caspase-1 and release of mature IL-1β level and IL-18 level in LPS primed microglia which remains as potent pro-inflammatory cytokines for further exacerbating neuroinflammatory cascade following neurodegeneration. Similar studies in microglia and macrophages have shown the NLRP3 inflammasome activation with LPS priming following extracellular ATP treatment but not with LPS or ATP alone [55, 56]. Contrary to this a few studies states the inflammasome activation occurs only by LPS priming but not its downstream cascade proteins (IL-1β, IL-
Furthermore, studies also provide overview with H$_2$O$_2$ alone may capable to stimulate NLRP3 inflammasome in human placenta and human primary macrophages [59, 60]. This provides the overview that NLRP3 inflammasome activation depends on nature, dose and duration of different signals exposure. Our study has utilized the bidirectional approach (Signal 1: LPS; Signal 2: H$_2$O$_2$) of employing the exact cellular environment where two signals are necessary for NLRP3 inflammasome activation while H$_2$O$_2$ alone fails to activate the NLRP3 downstream targets.

Oxidative stress mediated NLRP3 inflammasome activation has been recognised as the main perpetrator for inflammation in several neurological disorders [61, 13]. Mitochondria are highly sensitive organelle which can respond even to mild redox imbalance via generating H$_2$O$_2$ which is a potent inducer of oxidative related cellular damage [62, 63]. Our study showed that treatment with LPS and H$_2$O$_2$ combination showed a robust production of both cytosolic and mitochondrial superoxide anion levels and H$_2$O$_2$ release in microglial cells due to mitochondrial membrane depolarisation. Thus, inhibiting or scavenging both cytosolic and mitochondrial ROS (mainly superoxide: O$_2$•− and H$_2$O$_2$) could be a potential target in controlling NLRP3 inflammasome activation. The analysis of specific ROS is determinant factor in induction of oxidative stress and relevant downstream inflammatory signaling. Therefore, their evaluation is utmost necessary at a particular time point with certain specific fluorescent probes which are highly stable against specified generated ROS (such as MitoSOX, Amplex red) [64, 65]. Results from MitoSOX and Amplex red assay showed that LPS primed H$_2$O$_2$ treatment in microglia triggers generation of O$_2$•− and H$_2$O$_2$ which might act as signal 2 in activating NLRP3 inflammasome. These in vitro findings were further correlated in MPTP mice model where there was increased generation of free radicals with depleted endogenous antioxidant (reduced GSH) and reduced antioxidant enzymes (SOD and catalase) activities. This may be the exact reason for NLRP3 inflammasome activation both in vitro and in vivo models. Treatment with PA was seen inhibiting both cytosolic and mitochondrial: O$_2$•− anions levels thereby rescuing the loss of mitochondrial membrane potential when compared with LPS primed H$_2$O$_2$ treated microglia. These results were further supported in animal model where PA treatment upregulates SOD and catalase activity with restoration of reduced GSH level. Further treatment with Mito-TEMPO (a mitochondria-targeted antioxidant) was also seen inhibiting ROS generation and maintains mitochondrial membrane integrity. These findings ensure that both PA and Mito-TEMPO act as a direct and potent inhibitor of ROS by scavenging generated free radicals and have potential antioxidant property. To further clarify the role of generated ROS in activating NLRP3 inflammasomes in microglia we conducted a series of experiments. Treatments with PA was seen with suppressing NF-κB nuclear translocation and further inhibit activation of NLRP3 inflammasome complex which in turn inhibit release of IL-1β and IL-18 in LPS primed H$_2$O$_2$ exposed microglia in a dose dependent manner. Similarly, treatment with Mito-TEMPO was also found inhibiting NLRP3 inflammasome complex and its downstream targets. Hence, we conclude that mtROS plays a major role in activating NLRP3 inflammasome complex and compounds which alleviate ROS originated oxidative stress not only maintain mitochondrial integrity but also inhibiting NLRP3 inflammasome activation.
Data obtained from *in vitro* experiments were well correlated with *in vivo* studies; where administering MPTP, to activate NLRP3 inflammasome in the SNpc region of mouse brain which was well corroborated to earlier reports [7, 66]. MPTP being a complex-I inhibitor of mitochondrial electron transport chain and possibly via conversion to its active metabolite MPP⁺ in the microglia results in robust production of mtROS and accelerate NLRP3 activation *in vivo* [67, 12]. Further like *in vitro* model LPS priming it not possible in animal models as MPTP itself can act as priming signal. Our results showed that MPTP-administered mice exhibited severe motor deficits as seen from grip strength, rota-rod, catalepsy test and pole climbing. The results were further supported from open field test and measuring the distance between two successive paws where severe bradykinesia with reduction in gait velocity were noticed in MPTP-administered mice. Treatment with both PA and Mito-TEMPO mitigated several behavioral abnormalities including motor-impairment, tremor and shuffled gait after MPTP-administration.

To further elucidate the neuroprotective mechanism of PA and Mito-TEMPO *in vivo* models, SNpc region of mice brains were excised out from MPTP-challenged, PA and Mito-TEMPO treated mice. The major mechanism through which MPTP exerts its toxicity is through induction of oxidative stress and mitochondrial damage via generation of ROS such as O₂•⁻ and H₂O₂. The generated ROS hampers the anti-oxidant defence mechanism and therefore compromised anti-oxidants levels along with depleted ATP level that exacerbate bioenergetic crises. Further the generated free radicals were found activating brain microglia through NLRP3 inflammasome complex oligomerization which triggers cleavage of caspase-1 with generation of active IL-1β and IL-18 in MPTP-administered mice. These results further indicate that NLRP3 driven microglia activation may show significant involvement in progression of neurodegeneration with altered neurotransmitter levels evaluated in the striatum region together with decreased tyrosine hydroxylase expression level in the SNpc region of MPTP-intoxicated mice brain. Treatment with PA and mtROS scavenger (Mito-TEMPO) was seen protecting the cellular antioxidant (reduced GSH) reserves and their activity (SOD and catalase) defence systems and ATP levels when compared with MPTP-administered mice. Overall treatment with PA and Mito-TEMPO has shown inhibition of microglia activation via regulating NLRP3 inflammasome activation and its downstream targets which remains one of major main executor for inflammation induces neuronal cell death in PD (Fig. 8).

**6. Conclusion**

Thus, from both *in vitro* and *in vivo* experiments we can conclude that NLRP3 inflammasomes activation remains as major pathway for microglia induction in both mouse microglia (N9 cells) and in SNpc brain region of MPTP-administered mice. Both PA and Mito-TEMPO treatment was found exhibiting potential neuroprotective effects. Hence, alleviates motor defects, neuroinflammation and neuronal damage associated with PD by maintaining cellular redox homeostasis, limit microglia activation and simultaneously support neuronal survival via targeting NLRP3 inflammasome pathway.

**Abbreviations**
Declarations

Author's contributions

V.G.M. provided overall guidance from designing the experiment, model establishment, manuscript writing. SA contributed to the in vitro and in vivo experimental design, model establishment, histology, Immunohistochemistry and manuscript writing. SR and MK contributed for manuscript writing, western blot estimation, biochemical assays, and mice behavior analysis. BDS: data interpretation and carried revision of final manuscript.

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Compliance with ethical standards

All animal experimental procedures were done in accordance with the ethical principles as per the guidelines laid by the CPCSEA, Ministry of Environment and Forest, Government of India with approval no- NIPS/NIPER/18-032.

Consent to participate

Not applicable
Consent for Publication

All the authors give their consent for the publication

Conflicts of interest

The authors also declare no conflicts of interest.

Data Availability

The datasets that support the findings of this study are available from the corresponding author upon reasonable request.

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Figures
Figure 1

Time line of the study

Figure 2
H2O2 treatment induces NLRP3 inflammasome activation in LPS primed N9 microglial cells. (A) Structure of Perillyl alcohol. (B) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was done to check cell viability using different concentrations and combination of LPS and H2O2 for 4h and 2h respectively (n=6). (C) Kinetics of H2O2 release (0hr, 0.5hr, 1hr, 1.5 hr and 2hr) in LPS primed microglia treated with H2O2. (D) Western blot showing protein expression of NLRP3, cleavage of pro-caspase-1, pro-IL-1β and pro-IL-18 to its active caspase-1 p20, active IL-1β and active IL-18 form respectively (n=4). (E, F, G and H) Graphical depiction of western blotting and densitometric analysis of NLRP3, active caspase-1, IL-1β and IL-18 were done by Image-J (n=4) (I and J) Cytokine analysis of IL-1β and TNF-α levels in the supernatant of LPS primed microglia treated with H2O2 for 4h and 2h respectively by standard ELISA kit (n=6). Data's were expressed as mean±SD. Statistical significance was determined by one-way ANOVA followed by Dunnett analysis where all the groups were compared with normal control group with statistical significance defined as *p < 0.05, **p < 0.01, ***p < 0.001 respectively.

**Figure 3**

H2O2 causes mitochondrial dysfunction and increases ROS generation-Potential protective role of PA and mito-TEMPO. (A) JC-1 staining showing pictures of different treatment groups obtained from fluorescence microscope at 200x magnification with graph showing ratio of red/green intensities (n=6). (B) MitoSox staining showing pictures of different treated groups from confocal laser scanning microscope at 63x magnification with graph showing mean fluorescence intensities (n=6). (C and D)
Representative plot of JC-1 orange vs. green emission from flow cytometry on mitochondrial membrane potential as monomers and aggregate form (n=6). (E) Effects of PA and mito-TEMPO on LPS primed microglia treated with H2O2. Total H2O2 production in the supernatant was measured by Amplex Red Assay. Data’s were expressed as mean±SD. Statistical significance was determined by one-way ANOVA followed by Tukey’s post hoc analysis where #p < 0.05, ##p < 0.01, ###p < 0.001 represents control vs. LPS+H2O2 group and *p < 0.05, **p < 0.01, ***p < 0.001 represents LPS+H2O2 vs. different treatment group.

Figure 4

PA and mito-TEMPO suppresses NF-κB mediated NLRP3 expression in LPS primed H2O2 treated microglial cells. (A) Western blot showing protein expression of NLRP3, cleavage of pro-caspase-1, pro-IL-1β and pro-IL-18 to its active form caspase-1 p20, active IL-1β and active IL-18 form and Iba-1 expression in different treatment groups (n=4). (B, C, D, E and F) Graphical depiction of western blotting analysis and densitometric analysis of NLRP3, active caspase-1, IL-1β, IL-18 and Iba-1 were done by Image-J (n=4). (G) Cytokine analysis of IL-1β levels in the supernatant of LPS primed microglia treated with H2O2 for 12h and 4h respectively (n=6). (H and I) ICC photographs showing the intensity of NLRP3 and colocalization of NF-κB and in LPS-primed H2O2 treated microglia at 63x (n=4). (J and K) Graphical representation showing intensities and colocalization of NLRP3 and NF-κBp65 in different treated group (n=4). Data’s
were expressed as mean±SD. Statistical significance was determined by one-way ANOVA followed by Tukey’s post hoc analysis where #p < 0.05, ##p < 0.01, ###p < 0.001 represents control vs. LPS+H2O2 group and *p < 0.05, **p < 0.01, ***p < 0.001 represents LPS+H2O2 vs. different treatment group.

**Figure 5**

Perillyl alcohol ameliorates motor deficits and dopamine reduction in MPTP-treated mice. (A) Grip strength test. (B) Rota rod test. (C) Catalepsy test. (D) Pole climbing test. (E) Body weight. (F) Open field test with graph showing distance travelled (m), immobility time (sec) and speed acquired (m/s). (G) Walking track analysis with graph representing distance (cm) between two successive paws. All behavioral data were conducted using (n=8) mice per group. Data’s were expressed as mean±SD. Statistical significance was determined by two-way ANOVA followed by Tukey’s post hoc analysis where #p < 0.05, ##p < 0.01, ###p < 0.001 represents control vs. MPTP group, *p < 0.05, **p < 0.01, ***p < 0.001 represents MPTP vs. different treatment group with day 0 and day 7 and ^p < 0.05, ^^p < 0.01, ^^^p < 0.001 represents MPTP vs. different treatment group with day 0 and day 14 respectively. a$(p < 0.05) a$

$(p < 0.01)a$

$(p < 0.001)$ represents inter-comparison between different groups on day 0 with day 7 while b$(p < 0.05) b$

$(p < 0.01)b$
(p < 0.001) inter-comparison between different groups on day 7 with day 14.

Figure 6

PA enhanced the activity of anti-oxidant enzymes and in turn inhibits microglia and NLRP3 inflammasomes activation in mice brain. (A) Western blot showing protein expression of NLRP3, cleavage of pro-caspase-1, pro-IL-1β and pro-IL-18 to its active form caspase-1 p20, active IL-1β and IL-18 form in the SNpc region of mouse brain (n=4). (B, C, D and E) Graphical depiction of western blotting and densitometric analysis of NLRP3, active caspase-1, IL-1β and IL-18 were done by Image-J (n=4). (F) Western blot showing protein expression of P-NF-κBp65 in the SNpc region of mouse brain (n=4). (G) Western blot showing protein expression of Iba-1 and TH (n=4) in the SNpc region of mouse brain. (H, I and J) Graphical depiction of western blotting and densitometric analysis of PNF-κB p65, Iba-1 and TH were done by Image-J (n=4). (K, L, M and N) Anti-oxidant levels of various enzymes levels in SNpc region of mouse brain (n=6). (O) ATP level in the mitochondrial fraction of SNpc region of mouse brain (n=6). Data's were expressed as mean±SD. Statistical significance was determined by one-way ANOVA followed
PA attenuates dopaminergic neuronal loss and rescue altered neurotransmitter level in MPTP-treated mice. (A) Nissl-positive neurons stained in the SNpc region of mouse brain (magnification of 200x). (B) IHC staining of positive TH neurons in the SNpc region of mouse brain (magnification of 200x). (C) IHC staining of positive Iba-1 microglia in the SNpc region of mouse brain (magnification of 400x). (D and E) Graph representing Nissl positive and TH positive neurons in SNpc region of mouse brain (n=4). (F) Neurotransmitters mainly nor-epinephrine, dopamine 5-HIAA and 5-HT levels in the striatum region of mouse brain using HPLC-ECD method. Data's were expressed as mean±SD. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc analysis where #p < 0.05, ##p < 0.01, ###p < 0.001 represents control vs. MPTP group and *p < 0.05, **p < 0.01, ***p < 0.001 represents MPTP vs. different treatment group.
Figure 8

Representing signaling cascades involved in microglia activation through NLRP3 inflammasome activation. Generally a two-step process is involved in NLRP3 inflammasome activation through lipopolysaccharide (LPS) (priming/Signal 1) and reactive oxygen species (ROS) (activation/ Signal 2). The activated microglia releases neurotoxic factors which ultimately causes neuroinflammation and neuronal loss. Perillyl alcohol inhibits NF-κB mediated NLRP3 inflammasomes activation and subsequently rescues dopaminergic neuronal damage.