Investigating the Effect of an Anti-Inflammatory Drug in Determining NURR1 Expression and Thus Exploring the Progression of Parkinson's Disease

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Received June 21, 2023
Accepted October 12, 2023

Summary

Nonsteroidal anti-inflammatory drugs are the most widely used drugs for Parkinson's disease (PD), of which ibuprofen shows positive effects in suppressing symptoms; however, the associated risk needs to be addressed in different pathological stages. Initially, we developed an initial and advanced stage of the Parkinson disease mouse model by intraperitoneal injection of MPTP (20 mg/kg; 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine) for 10 and 20 days, respectively. Subsequently, ibuprofen treatment was administered for 2 months, and a pole test, rotarod test, histology, immunohistochemistry, and western blotting were performed to determine neuronal motor function. Histological analysis for 10 days after mice were injected with MPTP showed the onset of neurodegeneration and cell aggregation, indicating the initial stages of Parkinson's disease. Advanced Parkinson's disease was marked by Lewy body formation after another 10 days of MPTP injection. Neurodegeneration reverted after ibuprofen therapy in initial Parkinson's disease but not in advanced Parkinson's disease. The pole and rotarod tests confirmed that motor activity in the initial Parkinson disease with ibuprofen treatment recovered ($p<0.01$). However, no improvement was observed in the ibuprofen-treated mice with advanced disease mice. Interestingly, ibuprofen treatment resulted in a significant improvement ($p<0.01$) in NURR1 (Nuclear receptor-related 1) expression in mice with early PD, but no substantial improvement was observed in its expression in mice with advanced PD. Our findings indicate that NURR1 exerts anti-inflammatory and neuroprotective effects. Overall, NURR1 contributed to the effects of ibuprofen on PD at different pathological stages.

Key words

Parkinson disease • MPTP • NURR1 • Ibuprofen • NSAIDs

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Introduction

The mechanisms involved in neuronal cell death in most diseases associated with neurodegeneration, including amyotrophic lateral sclerosis, Huntington's disease, Alzheimer's disease, and Parkinson's disease, are not fully understood [1]. However, common pathways related to oxidative stress, mitochondrial energy impairment, secondary glutamate excitotoxicity, inflammatory mechanisms, and a similar understanding of pathogenic processes contribute to the development of novel neuroprotective therapy [2,3]. Parkinson's disease (PD) is a type of neurodegenerative disorder that is well known by other names, such as tremors, paralysis, tremor, slow motion, cognitive disorder, abnormal gait, sleep disorders, and sensory and autonomic disorders [4,5,6]. Currently, there is no specific treatment available for PD [7] but the symptoms related to it and disease progression are delayed using anti-inflammatory drugs [8].

Nuclear receptor-related 1 (NURR1), a member of the nerve growth factor-induced nuclear receptors 1 and 2, is a neuroprotective transcription β subfamily of orphan factors found primarily in dopaminergic neurons. Parkinson's disease (PD) is characterized by progressive

degeneration of neuronal nuclear receptor-related 1 of the midbrain (NURR1), which is critical for its development and survival [9]. Several recent studies [9,10] have indicated that NURR1 and its transcriptional targets in the midbrain may play an important role in PD pathogenesis. NURR1 and its transcriptional targets are downregulated in dopaminergic neurons in this region, which overexpress the disease-causing protein α-synuclein. Clinical and experimental evidence suggests that disruption of NURR1 function contributes to abnormalities in dopaminergic neurons in the early stages of Parkinson's disease [11]. NURR1 expression is believed to be significantly affected by a protein called α-synuclein (α-SYN), which is one of the most defining characteristics of PD. The results indicated that there was a reduction in NURR1 and its downstream gene expression due to the overexpression of α-SYN [11].

In previous studies, the orphan nuclear receptor NURR1 (also known as NR4A2) has been implicated in the differentiation, survival, and development of dopamine neurons in the middle brain. Furthermore, it has been reported that NURR1 is associated with Parkinson's disease (PD). As a result, NURR1 may represent a potential therapeutic target for Parkinson's disease [11].

Further, the NURR1 gene plays a vital role in neural toxicity mediated by α-SYN overexpression as a downstream molecular target [12]. Decressac *et al.* [11] found that increased levels of α-SYN reduces performance of dopaminergic neurons to GDNF (glial cell-derived neurotrophic factor) through decreasing NURR1 activity [11]. In the midbrain DA neurons, Lin *et al.* [13] found mutant α-SYN conditionally expressed degraded NURR1, which led to progressive neurodegeneration [11]. In the past, no studies have described the molecular mechanisms involved in ibuprofen's inhibitory effect on α-SYN mediated NURR1 transcription until now.

It is reported that α-SYN modulates NURR1 levels and downstream genes. The NF-κB, was identified as the highest scoring NURR1 transcription factor using a JASPER database as α-SYN modulated through the NURR1 promoter region. The results indicated that α-SYN affected NURR1 expression by affecting NF-κB binding to NURR1. The results showed NF-κB-related mechanisms that regulate NURR1 expression [9].

As a result of studies conducted *in vitro* and *in vivo*, emerging evidence points to the potential benefits of NURR1 activating compounds, as well as NURR1 gene therapy, for the enhancement of DA neurotransmission and for the protection of DA neurons from damage caused by environmental toxins or microglia-mediated inflammation. Modulators that inhibit NURR1 or regulate its activity, such as the retinoid X receptor, cyclic AMP-responsive element-binding proteins, neurotrophic factors derived from glial cell lines, and Wnt/β-catenin pathway, have also been found to improve NURR1-based therapies in Parkinson's disease [14]. Pharmaceutical compounds that activate NURR1 expression can protect against dopaminergic neuronal injury and improve neurotransmission [14].

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in clinical settings for the treatment of swelling, pain, muscle stiffness, and inflammation [15]. In addition, NSAIDs also attract patients with Parkinson's disease because they have a neuroprotective role in animal models of Parkinson's disease [16]. NSAIDs [17] are potent drugs for reducing the incidence of Alzheimer's disease [18]. In the case of Parkinson's disease, NSAIDs have the potential to substantially reduce the disease condition, but the risk and safety aspects of using NSAIDs are questionable [19]. Ibuprofen falls under NSAIDs, and its routine intake could help prevent or delay the onset of Parkinson's disease among its users [19], but similar therapeutic effects were not observed for other NSAIDs such as aspirin or acetaminophen [20].

In several studies, ibuprofen blocks PGE2 release, reduces inflammation, and enhances healing as a non-steroidal anti-inflammatory drug [21]. Despite the fact that all NSAIDs inhibit COX-2, only ibuprofen protects dopaminergic neurons and prevent oxidative damage in PD [22]. In the present study, using a Parkinson's disease mouse model, the effect of ibuprofen on NURR1 expression was analysed to assess the risk factors associated with NSAIDs.

Materials and Methods

Inducing Parkinson's disease condition in mice

Two-month-old male C57BL/6 mice (n=30, weighing 30-40 g) strains of mice that were acclimatized to the laboratory environment and used to develop Parkinson's disease. Normally, mice were kept in a cage at a temperature of approximately 22±1 °C with an alternative 12-hour light and dark cycle. All animals that were subjected to the present experimental procedure were approved according to the guidelines of the

Institutional Ethics Committee affiliated with Research Review and Ethics Board (RREB) of The Second Affiliated Hospital of Xi'an Medical University, Shaanxi, China (approval number: XMU-DM-45/3/A-22).

The mice were randomly divided into three groups of six mice each. Each group of mice was maintained separately and monitored twice daily. The first group of mice (n=6) was intraperitoneally injected with saline solution for 20 days and served as a control. The second group of mice (n=12) was injected intraperitoneally, with a neurotoxic chemical, namely, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; 20 mg/kg, Sigma Aldrich, USA) for 10 consecutive days and the remaining 10 days with saline following the standard protocol [23,24], which was regarded as the initial stage of PD (Initial PD or IPD group). The third group consisted of mice (n=12) that received MPTP for 20 days after the injection protocol and were considered an advanced stage of PD (Advanced PD or APD group). All mice were kept undisturbed for one week. To begin treatment, each of the six mice in the second and third groups was treated with sodium ibuprofen (50 mg/kg) intraperitoneally dissolved in saline [25] for a continuous 2 months and considered group IV (IPDI) and group V (APDI), respectively (Fig. 1).

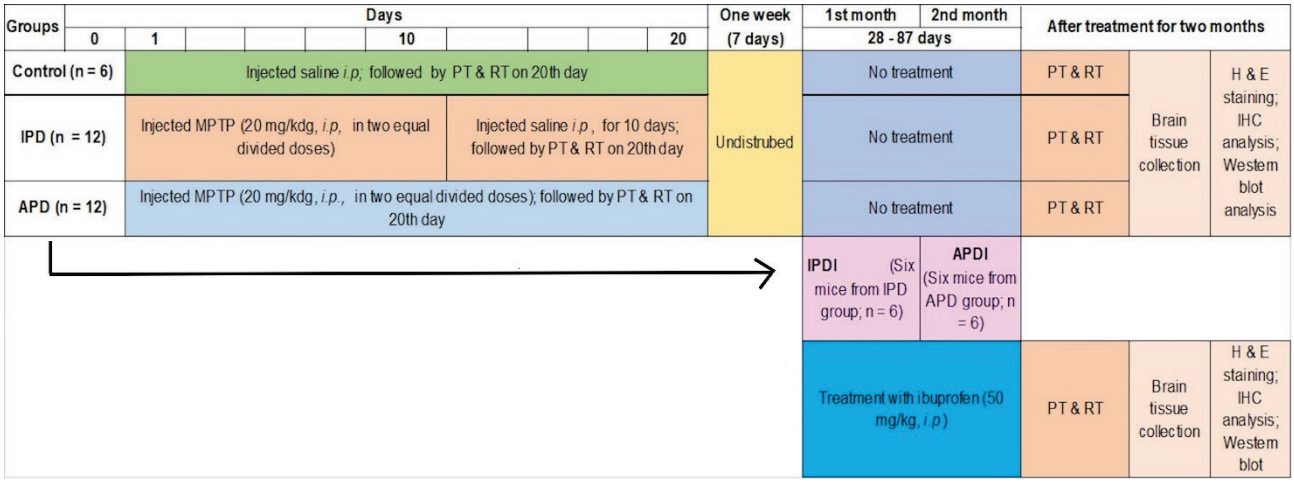

Fig. 1. Schematic representation of experimental protocol. IPD = initial Parkinson's disease; APD = advanced Parkinson's disease; IPDI = initial Parkinson's disease treated with ibuprofen; APDI = advanced Parkinson's disease treated with ibuprofen. PT = Pole test; RT = Rotarod test; MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; IHC = immunohistochemistry.

Administration of MPTP

MPTP hydrochloride (20 mg/kg, Sigma Aldrich, USA) was diluted in 0.9 % sodium chloride and then administered intraperitoneally at intervals of approximately one hour for a total dose of 20 mg/kg in two doses of 10 mg/kg each at an average of one hour.

Administration of ibuprofen

Immediately before injection, ibuprofen (Sigma Aldrich, USA) was dissolved in sodium chloride (0.9 % NaCl). The drug was administered daily for two months at a dose of 50 mg/kg after MPTP treatment. Based on preliminary studies on NURR1 expression in the early and advanced stages of PD, the doses of ibuprofen tested were 30, 50, and 80 mg/kg i.p. after MPTP treatment. When 50 mg/kg of i.p. was administered for two months in the early stages of Parkinson's disease, significant expression of NURR1 was observed compared to other doses. Therefore, we selected a dose of 50 mg/kg to be administered intraperitoneally for treatment [25,26,27].

Pole test

It is an experimental task that helps to evaluate complex neuronal dysfunction [28]. Before subjecting the mice to experimental analysis, they were trained in a vertical wooden pool with a rough surface for at least 3 days. The apparatus was kept inside the home cage and the animals were placed on top of the wooden pole. On the test day, each mouse received five trials at 20-minute interval and one of the best performances was recorded.

Rotarod test

The motor performance and endurance ability of the mice were evaluated using simple rotarod tests as previously described [29]. Before initiating the experiment on the experimental day (20^{th} day), the mice were

trained in the accelerated rotarod at 6, 12, and 15 rpm for three consecutive days before the experimental day. At the end of the test, the time taken by the mouse to maintain grip on the rolling rod was recorded. Five trials were administered to the mice, and the best performance was considered. Before each trial experiment, a 15 min rest was provided between the experiments.

Brain tissue processing

Chloral hydrate anesthesia (350 mg/kg) was used to sacrifice the mice after treatment. A 0.05 M phosphate buffer saline solution was perfused transcardially into the brain, followed by the infusion of cold 4 % paraformaldehyde in a 0.1 M phosphate buffer solution. Brains were quickly removed and placed in a tray of ice along the sagittal plane.

Brain dissection in the substantia nigra (dopaminergic midbrain)

The telencephalon was removed using a scalpel which was used to cut along the medial edge of each telencephalon zone (telencephalic vesicles can be used to explant the corpus striatum). The meningeal sheath was then removed. A dorsoventral cut was made at the rostral end of the mesencephalic flexure. A second incision was made caudal to the mesencephalic flexure in the dorsoventral plane. A microdissection knife was used to cut along the midline of the dorsal part in a rostrocaudal direction to expose the ventral midbrain tissue underneath the dorsal midline. Please be careful not to damage dopaminergic neurons located in the ventral midbrain tissue. A rostrocaudal cut was made lateral and parallel to the ventral midline to remove the dorsal midbrain tissues. Explants were prepared using a microdissection knife to ensure that the remaining ventral midbrain tissue was divided into pieces. Explants were placed in 0.1× Minimum Essential Medium Eagle (pH 4, sterile) containing 5 % FBS until further processing was performed [30].

Dissection of the striatum

The telencephalic regions were dissected during the midbrain dissection. Forceps were used to remove the thalamus from the striatum by cutting it between the thalamus and striatum. A mediolateral cut should be made rostral to the striatum to remove the rostral structures, such as the olfactory bulb. This step was repeated for tissues located caudal to the striatum. Coronal views of the striatum were obtained by placing the remaining slices in the appropriate position. The striatum was easy to identify because it had a slightly lower density (more transparent) than other parts of the tissue. The striatum was excised, and the explants were separated from the striatum using a microdissection knife. There was a slight difference in color at the midline, which contained several migrating neurons from the lateral and medial ganglionic eminences. Darker tissue was avoided. Explants were placed in 0.1× Minimum Essential Medium Eagle (pH 4, sterile) containing 5 % FBS until further processing was performed [30].

To postfix the tissue explants (dopaminergic midbrain) at 4 °C overnight, the tissue samples were placed in 0.1 M phosphate buffer containing 4 % paraformaldehyde. Subsequently, the tissues were dipped in 30 % sucrose solution in 0.05 M PBS to ensure cryoprotection. A freezing microtome (Leica Instruments, Germany) was used to cut 30 µm-thick coronal sections in series. The slices were stored in cryoprotectant at 4 °C until further use for immunohistochemistry.

Western blot analyses were performed on the mice as soon as they were killed by cervical dislocation. Brains were removed as quickly as possible and washed with ice-cold PBS. We dissected the ventral midbrains using a rapid dissection technique, cooled them to -80 °C, and stored them for additional experiments. Nine animals were processed for H&E staining, 12 animals for immunohistochemical analysis, and the remaining nine animals were used for western blot analysis.

Hematoxylin and Eosin staining (H&E)

A series of brain sections was fixed in neutral formalin buffer for 24 h under ice-cold conditions, dehydrated with isopropyl alcohol, and cleared with xylene. Several tissue sections were processed and embedded in paraffin, and 10-µm sections were mounted on slides. The deparaffinized sections were stained with hematoxylin for 2 min, transferred to a 1 % hydrochloric acid alcohol differentiation solution, stained with eosin for 3 s, and examined under a light microscope (Olympus, Japan).

Immunohistochemistry (IHC) analysis

The immunohistochemistry (IHC) analysis of NURR1 was carried out as previously described [24]. The sections were dewaxed and rehydrated with 3 % hydrogen peroxide. To retrieve the protein antigens from the sections, they were treated with 0.1 % trypsin solution 0.1 % for 15 min at 70 °C. Immediately after retrieval,

slides were cooled in distilled water for five minutes. PBS was used to wash the slides between each step of the procedure. Endogenous peroxidase activity in the sections was quenched by treatment with 3.0 % hydrogen peroxide. Following the blocking of nonspecific sites with 4 % BSA solution, the sections were incubated with mouse anti-NURR1 (1:500, rabbit, Santa Cruz Biotechnology, USA) for 4 h at 4 °C. After blocking at room temperature, the sections were washed three to four times with Tris-buffered saline with Tween-20 (TBST). After washing, the secondary antibody was incubated at 37 °C for one hour with horseradish peroxidase (HRP). Subsequently, the slides were washed with 1× TBST to remove nonspecific antibody binding and then stained with 3,3′-diaminobenzidine (DAB) solution to obtain a brown color (NURR1). Photographs were taken at higher magnification with an optical light microscope (Olympus, Japan) equipped with a 20× objective lens.

Western blot

After washing the samples with ice-cold 1× PBS, the tissue was homogenized using RIPA lysis buffer (pH 7.8) and protease inhibitors and then subjected to total protein extraction. Each sample was analyzed using bicinchoninic acid (BCA) to determine its protein concentration. The homogenate was centrifuged at 3000 rpm at 4 °C for 15 min. The supernatant was decanted, and each lane was loaded with an equal concentration (40 µg) of protein samples and run at 50 V on a 12 % SDS-PAGE gel for one hour. Once bromophenol blue reached the lower half of the separating gel, the proteins resolved in the separating gel were deposited onto PVDF membranes for one hour at 150 mA. Non-specific sites were blocked in the membrane using 4 % skim milk in 1× TBST, and the target protein, NURR1 or α-synuclein, was probed using an antibody against NURR1 (1:300 dilution) or anti-α-synuclein (1:800 dilution) in the PVDF membrane. Following the washing step, the blots were incubated for 40 min with an HRP-conjugated secondary antibody (1:3000 dilution) and then washed again with PBS. A chemiluminescence reaction was used to develop blots using chemiluminescent reagents. A scan of the images of non-saturated immunoblot films was used to examine the band densitometry analysis of the membrane. The intensity of the pixels of the bands obtained in each experiment was normalized using β-actin as an internal control.

Statistical analysis

There were four values taken from each animal. The mean of these four values was used as the individual result, and the mean of the six rats was used as the group result. This was employed for quantitative H&E staining and immunohistochemistry analyses. Graph Pad Prism 9.5 was used to analyze the data. Normality of the data was determined using a Shapiro-Wilk test and Kolmogorov-Smirnov test. Data was found to be non-normally distributed ($p<0.05$). A Kruskal-Wallis test followed by Dunn's multiple comparison test (non-parametric test) was applied to non-normally distributed data and multiple groups. A Mann-Whitney test was used to compare the two groups. Data are represented as the mean ± standard deviation and were considered significant when $p<0.05$.

Results

Developing a Parkinson disease mouse model using MPTP

MPTP is a key neurotoxic chemical that helps to develop a Parkinson disease model and in the present study MPTP in the dose range of 20 mg/kg was used to develop the initial and advanced stages of Parkinson disease. For the development of the initial form of Parkinson's disease, MPTP (20 mg/kg) was administered continuously for 10 days, and for the development of the advanced stage of Parkinson's disease, MPTP (20 mg/kg) in the same dose range was continued for 20 days. Mice in the initial and advanced stages of Parkinson's disease following ibuprofen treatment were subjected to histopathological analysis.

Figure 2a shows normal neuronal cells with prominent nuclei, cytoplasm, and striatum architectural formation in control brain tissue. It was also observed that the cell bodies were larger along with the supporting cells or satellite cells. Nissl bodies were also observed in the cytoplasm. We found oligodendrocytes (small glial cells) near the cell bodies with no signs of degeneration. Figure 2a shows that neurons have basophilic nuclei located peripherally with a granular eosinophilic cytoplasm. In addition, they had lightly stained round to oval granular nuclei located centrally. A large number of neuronal nuclei were arranged in clusters and granulated. There was no discernible difference between the cell membranes.

Figure 2b represents the initial stage of Parkinson's, where the substantia nigra portion forms a loose structure with degeneration of the tissue

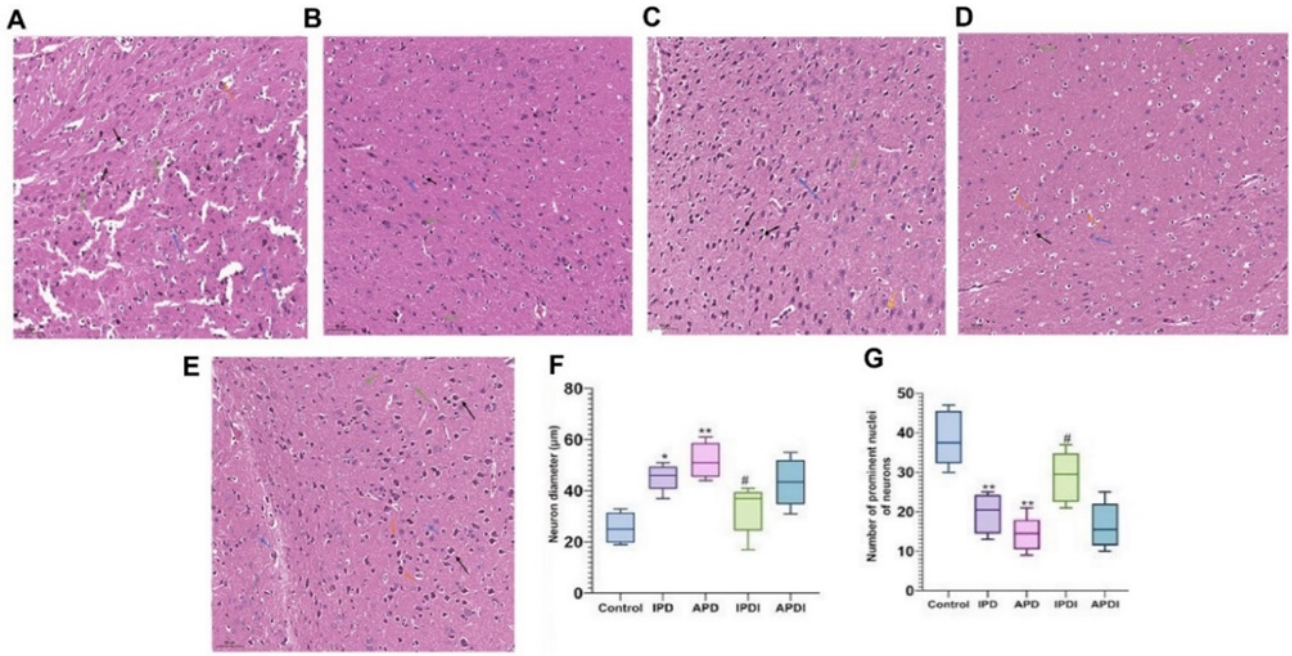

Fig. 2. (**A**) The histopathological observations show normal neuronal cells with prominent nuclei, cytoplasm, and striatum architectural formation in control brain tissue. It was also observed that the cell bodies were larger along with supporting cells or satellite cells. Nissl bodies were also observed in the cytoplasm. We found oligodendrocytes (small glial cells) near the cell bodies with no signs of degeneration. It shows neurons have basophilic nuclei located peripherally with granular eosinophilic cytoplasm. In addition, they have lightly stained round to oval granular nuclei located centrally. A large number of neuronal nuclei were arranged in clusters and granulated. There was no discernible difference between the cells' membranes (*black arrow indicates nucleolus of the neuron; red arrow indicates Nissl bodies; blue arrow indicates oligodendrocytes or glial cells; green arrow indicates basophilic nuclei*). (**B**) The histopathological observations represent the initial stage of Parkinson's disease condition, the portion of substantia nigra forms loosen structure with degeneration of tissue arrangement showing minimal cell aggregates. There were numerous pigmented multipolar neuronal cell bodies, axons, and dendrites in the substantia nigra. There were basophilic nuclei in the pigmented neuronal cytoplasm. A peripherally located, compact nucleolus was embedded in a thin, indistinct membrane that covered the granulated, round-oval nuclei. Moreover, oligodendrocytes with pericytoplasmic hollows and central nuclei were seen abundantly. Hyperplasia was present in these features to a mild to moderate degree *(blue arrow indicates oligodendrocytes or glial cells; black arrow indicates round-oval nuclei; green arrow indicates basophilic nuclei)*. (**C**) In the advanced stage, Lewy body structures along with more degenerative tissue conditions were observed. Pyknotic nuclei were seen with staining and degeneration of the neurons, as well as necrosis and neuronal degeneration with fewer neurons and cytoplasmic shrinkage, with deeply stained basophilic nuclei that were centrally located and occupied almost the entire cytoplasm in the middle. A large number of large synuclein aggregates form eosinophilic cytoplasmic inclusions, called Lewy bodies (LBs), found as fibrils of synuclein polymers deposited in nerve cells, astrocytes, and oligodendroglia. It was also noted that there was an abundance of oligodendrocytes with a central nucleus and pericytoplasmic chambers. There was evidence of moderate to severe hyperplasia with gliosis based on physical features. The pigmented neurons were significantly higher than those for control neurons. It was also found that the neuron diameter increased in a similar manner ($p>0.05$), indicating that the pigmentation in the neuron had increased as well. Dark brown cells are dopaminergic (DA) neurons contained neuromelanin *(black arrow indicates Lewy bodies; blue arrow indicates oligodendrocytes or glial cells; yellow arrow indicates round-oval nuclei; green arrow indicates basophilic nuclei)*. (**D**) The histopathological observations showed the initial Parkinson's disease mice treated with ibuprofen for continuous 2 months, demonstrating prominent reversion from histological abnormalities; especially tissue degeneration is recovered to original control tissue and the brain tissue showed less evidence of pyknotic nuclei, Lewy bodies, and neuronal degeneration as well as less inflammation of the brain tissue with distinct boundaries, and a significantly ($p<0.05$) more prominent nuclei of neurons, which indicates less damage to the brain tissue. H&E stain *(red arrow indicates pyknotic nuclei; blue arrow indicates oligodendrocytes or glial cells; black arrow indicates Lewy bodies; green arrow indicates basophilic nuclei)*. (**E**) Reversion of histopathologic condition is not attained for mice developed with advanced stages of Parkinson's disease following treatment with ibuprofen. Neuronal degeneration, necrosis, and pyknotic nuclei (arrow marks) were seen, along with shrinkage of the cytoplasm and deeply stained basophilic nuclei occupying most of the cytoplasm. Neurochemically, Lewy bodies (LBs) consist of lamellated eosinophilic cytoplasmic inclusions made of α-synuclein polymers, while α-synuclein neurites are deposited in neuronal processes and in astrocytes and oligodendroglial cells. There were also abundant oligodendrocytes with pericytoplasmic hollows and a central nucleus. Hyperplasia with gliosis appeared moderate to severe. There was a significant increase in pigmented neurons. Additionally, cell diameter increased ($p>0.05$) with increased pigmentation within the cell. Dark brown cells are dopaminergic (DA) neurons that contain neuromelanin in the substantia nigra pars compacta. H&E stain *(black arrow indicates Lewy bodies; blue arrow indicates oligodendrocytes or glial cells; red arrow indicates dark brown cells; green arrow indicates basophilic nuclei)*. Scale bar – 50 µm. (**F**) Increased neuron diameter (µm) indicating pigmentation of neurons. * $p<0.05$ & ** $p<0.01$ vs. control group; # $p<0.05$ vs. IPD. (**G**) Number of prominent nuclei in neurons indicating less damage to brain tissue. ** $p<0.01$ vs. control group; # $p<0.05$ vs. IPD (n=9).

arrangement showing minimal cell aggregates. Numerous pigmented multipolar neuronal cell bodies, axons, and dendrites were observed in the substantia nigra. Eosinophilia was observed in the pigmented neuronal

cytoplasm. A compact, peripherally located nucleolus is embedded in a thin, indistinct membrane that covers the granulated round oval nuclei. Furthermore, abundant oligodendrocytes with hollow pericytoplasmic and central nuclei were also observed. Hyperplasia was present in these mice to a mild-to-moderate degree.In the advanced stage, Lewy body structures were observed along with more degenerative tissue conditions (Fig. 2c). Pyknotic nuclei were observed with staining and degeneration of neurons, as well as necrosis and neuronal degeneration with fewer neurons and cytoplasmic shrinkage, with deeply stained basophilic nuclei that were centrally located and occupied almost the entire cytoplasm in the middle. Synuclein accumulation is a major pathological feature of Parkinson's disease. A large number of large α-synuclein aggregates form eosinophilic cytoplasmic inclusions called Lewy bodies (LBs), which are found in the nervous system throughout and outside the brain as fibrils of α-synuclein polymers deposited in nerve cells, astrocytes, and oligodendroglia. There is evidence that synuclein accumulation alters mitochondrial, lysosomal, and endoplasmic reticulum function. In addition, it interferes with microtubular transport. In addition, oligodendrocytes with a central nucleus and pericytoplasmic chambers were abundant. There was evidence of moderate to severe hyperplasia with gliosis based on physical characteristics. The cell counts of pigmented neurons were considerably higher than those of unpigmented neurons. The diameter of the neurons also increased (p=0.001, Table 1), indicating that pigmentation in the neurons was increased relative to control group. Dark brown cells are dopaminergic neurons (DA) because they contain neuromelanin. It is evident that dopaminergic cells were lost in the compacta substantia nigra of Parkinson's disease.

However, Figure 2d shows the initial Parkinson disease mice treated with ibuprofen for 2 months, continuously demonstrating prominent reversion of histological abnormalities. Tissue degeneration was recovered to the original control tissue, and brain tissue showed less evidence of pyknotic nuclei, Lewy bodies, and neuronal degeneration, as well as less inflammation of brain tissue with distinct boundaries, and significantly (p=0.002) more prominent neuronal nuclei, which indicates less damage to brain tissue compared to IPD group (Fig. 2f, g, Table 2) (black – prominent nuclei).

Table 1. Increased neuron diameter (μm) indicating pigmentation of neurons.

Groups	**Number of animals**	**Mean**	**Median**	**S.D.**	**S.E.**	**Lower 95 % CI**	**Upper 95 % CI**	**p-value**
Control	6	25.5	25	5.788	2.363	19.43	31.57	-
IPD	6	45.17	46	5.076	2.072	39.84	50.49	0.023*
APD	6	51.83	51	6.735	2.75	44.76	58.9	0.001**
IPDI	6	33	37	9.23	3.768	23.31	42.69	0.025$^{\#}$
APDI	6	43.33	43.5	8.959	3.658	33.93	52.74	0.131

IPD – initial Parkinson's disease; APD – advanced Parkinson's disease; IPDI – initial Parkinson's disease treated with ibuprofen; APDI – advanced Parkinson's disease treated with ibuprofen. * $p<0.05$ & ** $p<0.01$ vs. control group; $^{\#}$ $p<0.05$ vs. IPD.

Table 2. Number of prominent nuclei in neurons indicating less damage to brain tissue.

Groups	**Number of animals**	**Mean**	**Median**	**S.D.**	**S.E.**	**Lower 95 % CI**	**Upper 95 % CI**	**p-value**
Control	6	38.33	37.5	6.683	2.728	31.32	45.35	-
IPD	6	19.67	20.5	4.885	1.994	14.54	24.79	0.003**
APD	6	14.5	14.5	4.37	1.784	9.914	19.09	0.002**
IPDI	6	29.00	29.50	6.542	2.671	22.13	35.87	0.035$^{\#}$
APDI	6	16.5	15.5	5.822	2.377	10.39	22.61	0.458

IPD – initial Parkinson's disease; APD – advanced Parkinson's disease; IPDI – initial Parkinson's disease treated with ibuprofen; APDI – advanced Parkinson's disease treated with ibuprofen. ** $p<0.01$ vs. control group; $^{\#}$ $p<0.05$ vs. IPD.

However, such a reversion of the histopathological condition was not achieved in mice with advanced stages of Parkinson's disease following ibuprofen treatment (Fig. 2e). Neuronal degeneration, necrosis, and pyknotic nuclei were observed along with shrinkage of the cytoplasm and deeply stained basophilic nuclei occupying most of the cytoplasm. Parkinson's is caused by the accumulation of α-synuclein. Neurochemically, Lewy bodies (LB) consist of lamellated eosinophilic cytoplasmic inclusions made of α-synuclein polymers, while α-synuclein neurites are deposited in neuronal processes, astrocytes, and oligodendroglial cells. The accumulation of α-synuclein affects the mitochondria, lysosomes, and endoplasmic reticulum. There were also abundant oligodendrocytes with hollow pericytoplasmic and central nuclei. Gliosis hyperplasia appeared moderate to severe. A significant increase in the number of pigmented neurons was observed. In addition, the diameter of the cells was increased with no significant differences compared to APD group ($p=0.131$), indicating increased pigmentation remained within the cells. Dark brown cells are dopaminergic neurons (DA) that contain neuromelanin. Loss of dopaminergic cells is evident in the substantia nigra pars compacta of mice with Parkinson's disease.

The pole and rotarod tests confirmed the impairment of motor function

Neurological dysfunction following MPTP treatment was analyzed by performing Pole and Rotarod tests. Following the trials, the mice were placed above the wooden pole, and the time taken to descend to the floor was calculated. As a control, the mice did not find any difficulty reaching the floor and completing the distance in 13.38±4.49 s. In the initial stages of Parkinson's disease, mice take more time to orient themselves downward and reach the floor after doubling the time taken by control mice (26.61±5.34 s). In the advanced stage of Parkinson's disease, mice take more time to turn and descend to the floor (52.24±3.74 s). Following ibuprofen treatment, initial Parkinson's disease mice spent 15.49±4.49 s reaching the floor, but this improvement were not observed in advanced stage Parkinson's disease mice (55.21±5.92 s; Fig. 3, Table 3).

To evaluate motor coordination among different mice with Parkinson's, a rotarod test was performed. Control mice showed more resistance and endured the rotarod for a longer time (214±9.32 s). However, mice that developed to an initial stage (168±19.36 s) and an advanced stage (119±9.92 s) of Parkinson's disease showed an inability to withstand the rotarod. Treatment with ibuprofen showed an improvement in motor coordination activity in initially Parkinson-developed mice (204±17.9 s) but did not show an improvement in mice with advanced stage Parkinson's disease (112±12.3 s; Fig. 4, Table 4).

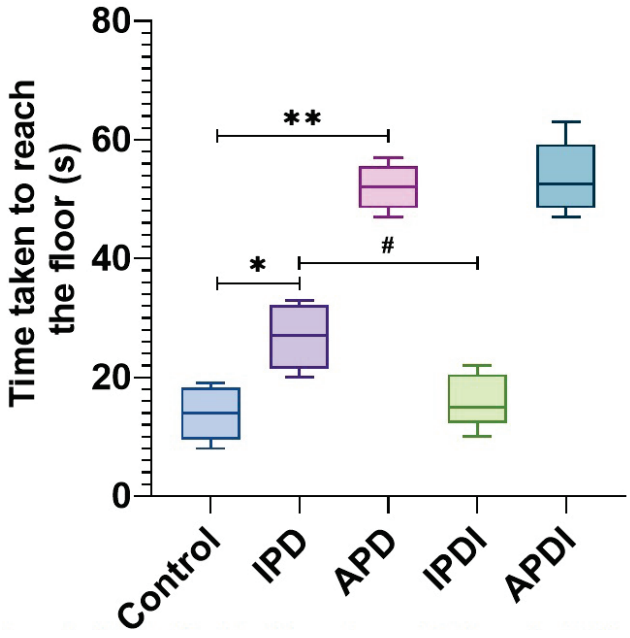

Fig. 3. Pole test for determining motor coordination and instability: Control, initial and advanced stage Parkinson's disease mice and ibuprofen-treated mice are subjected to pole test. The time taken by mice to attain the floor are documented in seconds. The experimental data are represented in mean ± SD. * $p<0.05$, ** $p<0.01$ was considered as statistically significant when compared to control. # $p<0.01$ when compared to initial stage of Parkinson's disease (IPD).

Table 3. Pole test to assess impairment of motor function in mice (s).

Groups	Number of animals	Mean	Median	S.D.	S.E.	Lower 95 % CI	Upper 95 % CI	p-value
Control	6	13.38	14	4.491	1.42	9.121	18.55	-
IPD	6	26.61	27	5.345	2.38	21.22	32.44	0.013*
APD	6	52.24	52	3.742	3.17	48.07	55.93	0.004**
IPDI	6	15.49	15	4.491	1.13	11.12	20.55	0.002#
APDI	6	55.21	52.5	5.922	4.11	47.45	59.88	0.357

IPD – initial Parkinson's disease; APD – advanced Parkinson's disease; IPDI – initial Parkinson's disease treated with ibuprofen; APDI – advanced Parkinson's disease treated with ibuprofen. * p<0.05 & ** p<0.01 vs. control group; # p<0.01 vs. IPD.

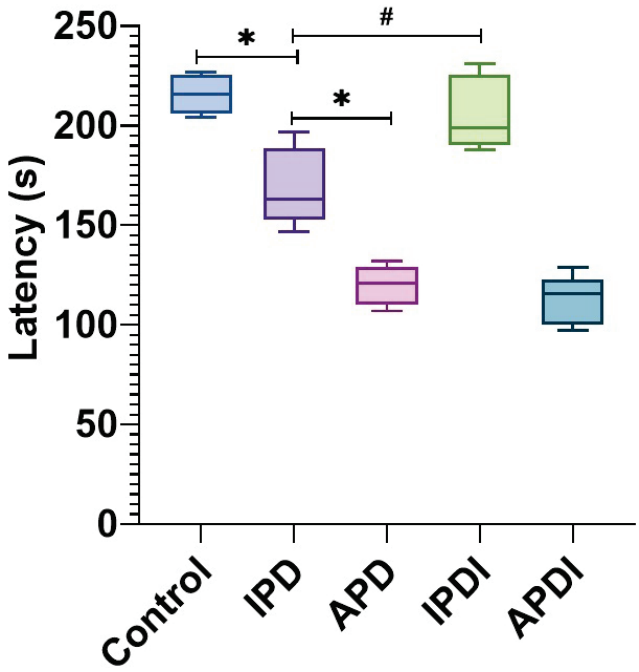

Fig. 4. Analyzing motor coordination using experimental setup of the Rotarod test: The withstand ability of mice in rotarod is compared to determine its motor skill. The graphs are plotted against time in seconds (Y axis) to different mice groups (X axis). The obtained results are represented in mean ± SD. * p<0.05, ** p<0.01 was considered as statistically significant when compared to control. # p<0.01 when compared to initial stage of Parkinson's disease (IPD).

Table 4. Rotarod latency test to impairment of motor function in mice (s).

Groups	Number of animals	Mean	Median	S.D.	S.E.	Lower 95 % CI	Upper 95 % CI	p-value
Control	6	214	216	9.326	2.48	206	225.6	-
IPD	6	168	163	19.36	7.903	148.2	188.8	0.022*
APD	6	119	121	9.92	4.05	109.6	130.4	0.004**
IPDI	6	204	199	17.9	7.306	186.6	224.1	0.010#
APDI	6	112	115.5	12.3	5.023	100.3	126.1	0.372

IPD – initial Parkinson's disease; APD – advanced Parkinson's disease; IPDI – initial Parkinson's disease treated with ibuprofen; APDI – advanced Parkinson's disease treated with ibuprofen. * p<0.05 & ** p<0.01 vs. control group; # p<0.01 vs. IPD.

NURR1 expression and ibuprofen treatment

The expression of NURR1 in a brain is more complex and more variable under different pathological conditions [31]. In recent studies of NURR1, the underlying mechanisms have been associated with brain inflammation. In the present study, NURR1 showed a high degree of expression in the control brain tissue from the substantia nigra. Images of NURR1 immunoreactivity showed that NURR1 products were found in the neuronal nucleus of the substantia nigra. The substantia nigra of normal rats contained many NURR1-positive cells (210.7±19.48, Fig. 5a) in the substantia nigra. However, NURR1 expression was gradually downregulated in immunohistochemical sections of mice with Parkinson's disease (Fig. 5b) and mice with Parkinson's disease at an advanced stage (Fig. 5c), demonstrating significantly fewer NURR1-positive cells in the substantia nigra suffering from early onset (105.0±17.42) and late-onset Parkinson's disease (61.0±19.8, Table 5), respectively.

Upon ibuprofen treatment, NURR1 showed upregulated expression in the initial Parkinson disease mice (Fig. 5d), resulting in a substantial increase (p=0.035) in NURR1-positive cells. Surprisingly, NURR1 expression was slightly higher than that in control tissues. However, their expression was insignificantly lower (p=0.420) even after ibuprofen treatment in mice with advanced stage Parkinson's disease (Fig. 5e), with a considerably lower number of NURR1-positive cells (117.7±8.52 vs. 189.8±18.94; Fig. 5f, Table 5).

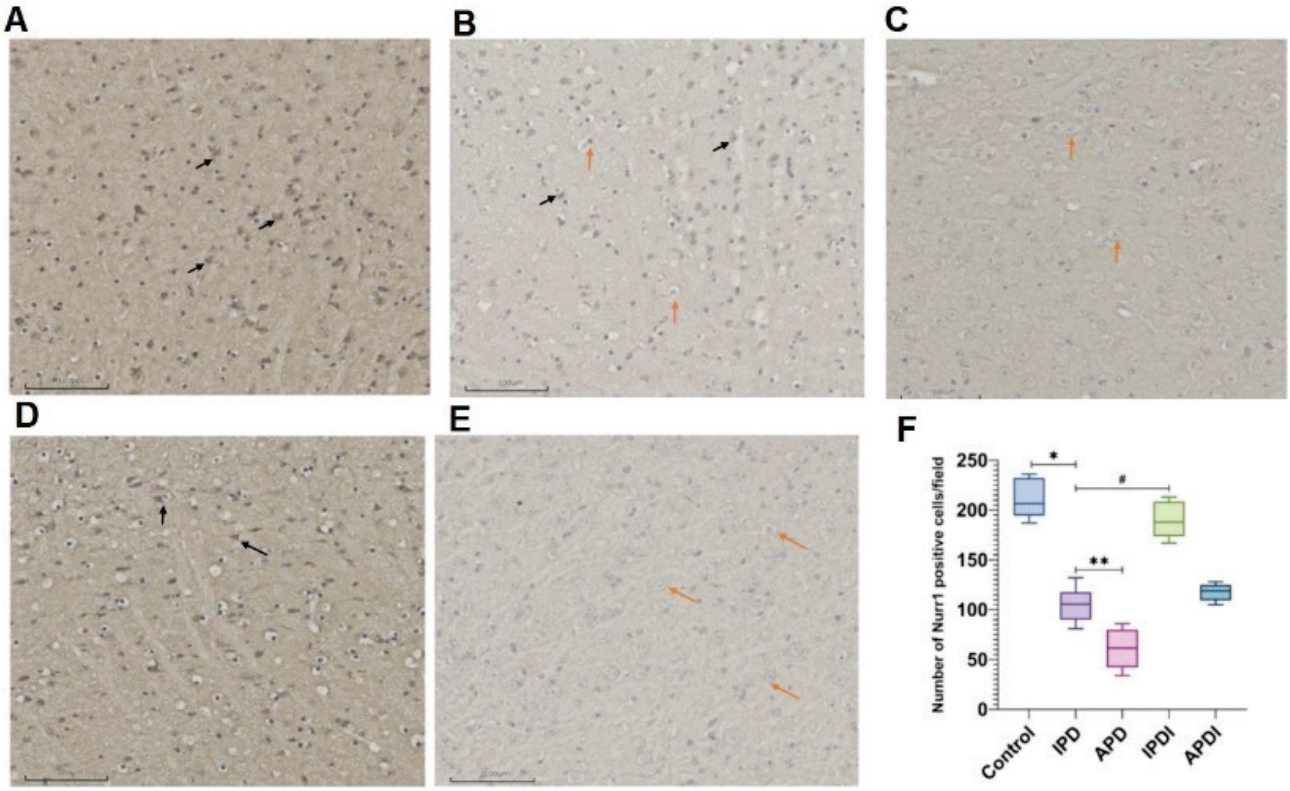

Fig. 5. (**A**) Immunohistochemistry studies showed with high degree of expression pattern of NURR1 in the control brain tissue of substantia nigra. NURR1 immunoreactivity displayed that the NURR1 products were found in the neuronal nucleus of substantia nigra containing many NURR1-positive cells in the substantia nigra ($p<0.01$). *(black arrow indicates NURR1-positive cells)* (**B**) Immunohistochemistry showed that NURR1 expression was gradually downregulated in immune-histological sections of mice in the initial stage Parkinson's disease with significantly ($p<0.05$) lower NURR1-positive cells in the substantia nigra. *(black arrow indicates NURR1-positive cells; red arrow indicates oligodendrocytes or glial cells)* (**C**) Immunohistochemistry showed that NURR1 expression was increasingly downregulated in immune-histological sections of mice in the advanced stage Parkinson's disease with significantly ($p<0.01$) lower NURR1-positive cells in the substantia nigra. *(red arrow indicates oligodendrocytes or glial cells)* (**D**) Immunohistological studies showed up-regulation NURR1 expression in initial Parkinson's disease mice upon treatment with ibuprofen resulted in the substantial increase ($p<0.05$) of Nurr1-positive cells. Surprisingly the expression of NURR1 was significantly high ($p<0.05$) when compared with the control tissue. *(black arrow indicates NURR1-positive cells)* (**E**) Immunohistological studies showed the expression of NURR1 was significantly lower ($p<0.05$) even after treatment with ibuprofen in advanced stage Parkinson's disease developed mice with considerably low number of NURR1-positive cells ($p<0.01$). *(red arrow indicates oligodendrocytes or glial cells).* Scale bar – 100 μm. (n=12). (**F**) Number of NURR1 positive cells per field. * $p<0.05$ & ** $p<0.01$ vs. control group; # $p<0.05$ vs. IPD.

Table 5. Number of NURR1 positive cells in mice.

Groups	Number of animals	Mean	Median	S.D.	S.E.	Lower 95 % CI	Upper 95 % CI	p-value
Control	6	210.7	206.5	19.48	7.953	190.2	231.1	-
IPD	6	105	105.5	17.42	7.113	86.71	123.3	0.035*
APD	6	61	61.5	19.8	8.083	40.22	81.78	0.001**
IPDI	6	189.8	188	18.94	7.731	170	209.7	0.037#
APDI	6	117.7	119	8.524	3.48	108.7	126.6	0.765

IPD – initial Parkinson's disease; APD – advanced Parkinson's disease; IPDI – initial Parkinson's disease treated with ibuprofen; APDI – advanced Parkinson's disease treated with ibuprofen. * p<0.05 & ** p<0.01 vs. control group; # p<0.05 vs. IPD.

The experimental data generated by immunohistochemistry were subjected to western blot analysis (Fig. 5). Protein samples extracted from the substantia nigra of different Parkinson's disease conditions were analyzed using the anti-NURR1 antibody. In control tissues, NURR1 was highly expressed, and after Parkinson's disease progression, NURR1 showed a gradual downward-regulated pattern. Treatment with ibuprofen under initial conditions of Parkinson's showed an increase (p=0.027) in the NURR1 expression pattern, but showed no significant (p=0.419) NURR1 expression under treatment conditions with ibuprofen in the advanced stage of PD (Fig. 6, Table 6).

In this study, protein samples extracted from the substantia nigra were analyzed using an anti-α-synuclein antibody (Fig. 6). A significant reduction in α-synuclein expression was observed in control tissues, and the expression was increased significantly with Parkinson's disease progression in the early (p=0.022) and advanced (p=0.006) stages of the disease. Treatment with ibuprofen under the initial conditions of Parkinson's disease showed a marked decrease (p=0.043) in α-synuclein expression. But, there was substantial (p=0.100) upregulation in the expression of α-synuclein under treatment conditions with ibuprofen in the advanced stage of PD, indicating no difference in treatment outcome (Fig. 7, Table 7).

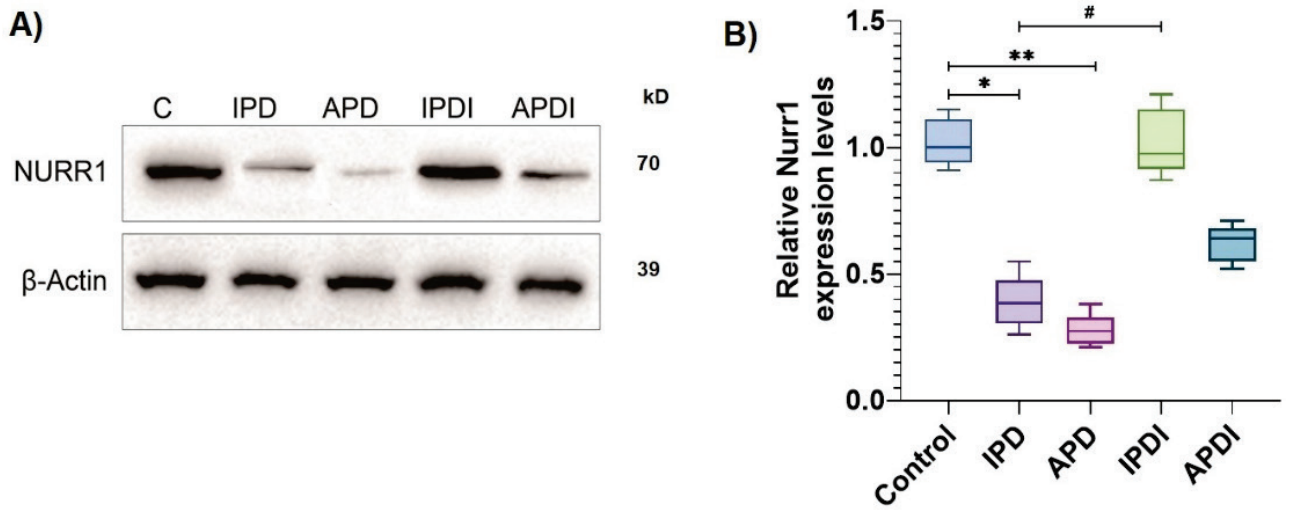

Fig. 6. (**A**) Comparing NURR1 expression in ibuprofen treated and non-treated groups using Western blotting: C represents control substantia nigra tissue showing significantly high expression of NURR1. IPD represents initial Parkinson's disease tissue and shows a significantly decrease in NURR1 expression. APD represents advanced Parkinson's disease with least expression of NURR1. IPDI represents Initial Parkinson's disease treated with ibuprofen showing significantly high up-regulation of NURR1. APDI represents advanced Parkinson's disease treated with ibuprofen and represents significantly low expression of NURR1. For the loading control β-actin was used (n=9). (**B**) Quantitative analysis of Western blots for comparing NURR1 expression. The obtained results are represented in mean ± SD. * p<0.05, ** p<0.01 was considered as statistically significant when compared to control. # p<0.01 when compared to initial stage of Parkinson's disease (IPD).

Table 6. Relative expression of NURR1 levels in mice.

Groups	Number of animals	Mean	Median	S.D.	S.E.	Lower 95 % CI	Upper 95 % CI	P-value
Control	6	1.018	1	0.091	0.037	0.922	1.114	-
IPD	6	0.391	0.385	0.105	0.042	0.281	0.501	0.016*
APD	6	0.28	0.275	0.061	0.025	0.215	0.344	0.002**
IPDI	6	1.015	0.975	0.128	0.052	0.88	1.15	0.027#
APDI	6	0.623	0.64	0.070	0.028	0.548	0.697	0.419

IPD – initial Parkinson's disease; APD – advanced Parkinson's disease; IPDI – initial Parkinson's disease treated with ibuprofen; APDI – advanced Parkinson's disease treated with ibuprofen. * p<0.05 & ** p<0.01 vs. control group; # p<0.05 vs. IPD.

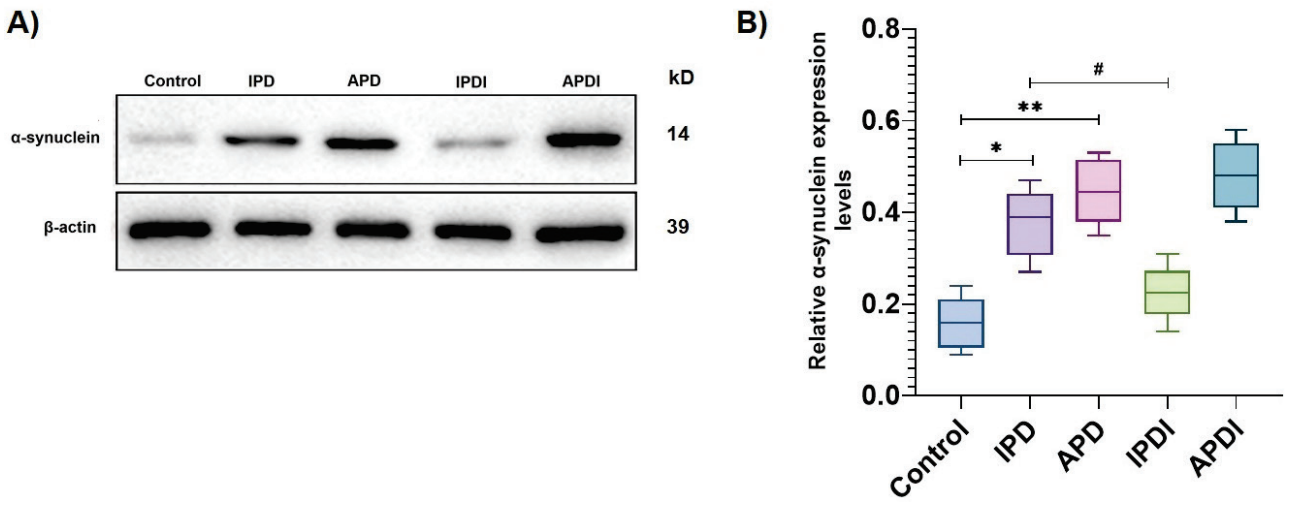

Fig. 7. (**A**) Comparing α-synuclein expression in Ibuprofen treated and non-treated groups using Western blotting: C represents control substantia nigra tissue significantly lower expression of α-synuclein. IPD represents initial Parkinson's disease tissue and shows a significantly increase in α-synuclein expression. APD represents advanced Parkinson's disease significantly higher expression of α-synuclein. IPDI represents Initial Parkinson's disease treated with Ibuprofen showing significantly lower expression of α-synuclein. APDI represents advanced Parkinson's disease treated with Ibuprofen and represents significantly higher expression of α-synuclein. For the loading control β-actin was used. (n=9). (**B**) Quantitative analysis of Western blots for comparing α-synuclein. The obtained results are represented in mean ± SD. * p<0.05, ** p<0.01 was considered as statistically significant when compared to control. # p<0.01 when compared to initial stage of Parkinson's disease (IPD).

Table 7. Relative expression of α-synuclein in mice.

Groups	Number of animals	Mean	Median	S.D.	S.E.	Lower 95 % CI	Upper 95 % CI	p-value
Control	6	0.16	0.16	0.055	0.022	0.101	0.218	-
IPD	6	0.378	0.39	0.073	0.030	0.300	0.455	0.022*
APD	6	0.445	0.445	0.069	0.028	0.372	0.517	0.006**
IPDI	6	0.225	0.225	0.058	0.023	0.163	0.286	0.043#
APDI	6	0.48	0.48	0.076	0.031	0.400	0.559	0.100

IPD – initial Parkinson's disease; APD – advanced Parkinson's disease; IPDI – initial Parkinson's disease treated with ibuprofen; APDI – advanced Parkinson's disease treated with ibuprofen. * p<0.05 & ** p<0.01 vs. control group; # p<0.05 vs. IPD.

Discussion

Parkinson's disease (PD) is a neurodegenerative disease that affects motor and nonmotor neuronal connection [32]. Two major pathophysiological conditions associated with Parkinson's are neuronal degeneration and Lewy body structure formation [7]. In the present study, we observed the neuronal degeneration of neurons, which is an initial step in the formation of Lewy bodies observed in the initial stages of MPTP-treated initial Parkinson disease developed mice. The formation of Lewy bodies occurs due to the deposition of a misfolded protein, α-synuclein, which later forms an aggregate and causes dopaminergic degradation [33]. Lewy body formation is a sign of widespread damage that occurs in a specific part of the brain called the substantia nigra, and we observed more Lewy body formation in the advanced stages of PD-developed mice.

In addition to the formation of Lewy bodies and neuronal degeneration, the Parkinson disease condition involves an inflammatory response, mitochondrial dysfunction, and synaptic changes [34]. Currently, there are no drugs available to cure Parkinson's disease, but the available drugs help slow the progress of disease conditions, including anti-inflammatory drugs, neurotrophic neurogenesis factors, and drugs that target mitochondrial dysfunction [35]. NSAIDs are widely used as anti-inflammatory drugs with potential therapeutic value in decreasing the risk of Parkinson's disease [36]. It is accepted that, among the NSAIDs, ibuprofen is the most widely used as an anti-inflammatory drug in addition to aspirin and acetaminophen. However, further investigation is needed to determine the risk related to its neuroprotective properties in Parkinson's disease [20]. In our study, MPTP-treated mice recovered significantly from neuronal degeneration after receiving ibuprofen, and α-synuclein expression was significantly enhanced. The protective effects were more evident in the early stages of neuronal injury than in the later (advanced) stages. One of the limitations of this study is the use of only one dose (50 mg/kg, i.p.) in this investigation, which is one of the limitations of this study. It is believed to play a crucial role in decreasing injury to neuronal endings in the striatum, stimulating the recovery and regeneration phase, thus preventing MPTP toxicity.

NURR1 is a neuroprotective and anti-inflammatory transcription factor. Several studies [22] have highlighted the relevance of NURR1 in Parkinson's disease, multiple sclerosis, and Alzheimer's disease, indicating that NURR1 could serve as a therapeutic target for neurodegenerative diseases, including PD disease, Alzheimer's disease, and multiple sclerosis [37]. NURR1 has a variety of biological functions that involve survival and development of dopaminergic neurons [38], regulation of inflammatory-related gene [39], inducing a cancer environment by suppressing the apoptosis process [40], showing a neuroprotective effect in midbrain microglial cells [41]. Furthermore, mutations in NURR1 are related to many neurological diseases, such as schizophrenia [42], PD, and manic depression [43,44]. There remains a lack of knowledge about NURR1's molecular mechanism of action, as well as the availability of NURR1 modulators. This has promoted studies on the mechanistic aspects of NURR1 function and the exploration of new NURR1 modulators for functional studies.

We observed that ibuprofen had a neuroprotective effect in the initial stage of Parkinson's disease but showed no neuroprotective activity in the advanced stage of Parkinson's disease, affecting NURR1 expression.

In recent years, there has been increasing interest in NURR1's potential neuroprotective effects of NURR1. Several studies have presented evidence suggesting that reducing NURR1 expression can result in the release of proinflammatory cytokines from microglia, as well as the production of more hazardous inflammatory reactions, such as the release of reactive oxygen species or radical oxygen species, which could further magnify the inflammatory response, ultimately leading to neuronal death in Parkinson's disease [9,10,21]. The NURR1 gene, which is expressed in glia, has been found to be a strong inhibitor of the inflammatory cytokines that are secreted by activated microglia and astrocytes. Microglia are important for inhibiting the expression of pro-inflammatory neurotoxic molecules to decrease the level of inflammation [45].

From our results, we understand that NURR1 shows constant downregulation as Parkinson's disease progresses [46], which may be due to the damage response and its neuroprotective role. Treatment with ibuprofen restored neurons in the initial stages of Parkinson's disease because NURR1 expression increased, indicating that it provided neuroprotection in the initial stages of Parkinson's disease. However, conditions with NURR1 expression after treatment in advanced stages of Parkinson's showed a significant decrease in NURR1 expression. This may be because ibuprofen maintained the neuroprotective effects of

NURR1 overexpression in the initial stages of Parkinson's, but not in the late (advanced) stage of Parkinson's disease. This is particularly true in the case of PD, which is characterized by the selective death of dopaminergic neurons in the SN as well as the presence of Lewy bodies that contain α-synuclein aggregates. The pathogenesis of Parkinson's is also characterized by the association of Lewy bodies with the inclusion of a protein called α-synuclein, which plays an important role in disease development. Therefore, in the present study, we evaluated the amount and aggregation of α-synuclein. A significant increase in the level of α-synuclein was observed when we compared brain tissue from IPD and APD mice treated with MPTP with brain tissue from the control group. Compared with mice with IPD, ibuprofen (50 mg/kg, i.p.) significantly reduced α-synuclein aggregates. However, no significant changes were observed after ibuprofen treatment in APDI mice. According to the results of a published study [47], we were able to demonstrate in MPTP models of Parkinson's disease that temsirolimus showed strong neuroprotective effects that resulted in a significant reduction in synuclein aggregation, which is the hallmark of Parkinson's disease.

The α-SYN gene is expressed in various cell types and tissues [13]. This study shows that ibuprofen suppresses several dopaminergic-associated genes through its effects on α-SYN (Fig. 7). It appears that NURR1 inhibition by increase expression levels of α-SYN contributes to Parkinson's disease-related dopaminergic dysfunction.

It is obvious from the Western blot results that α-SYN is localized in both the nucleus and cytoplasm whereas NURR1 is most commonly located within the nucleus [11]. NURR1 is hypothesized to contribute to PD pathogenesis. NURR1 is also considered a crucial target for α-SYN overexpression. A feedback loop is suspected between α-SYN and NURR1 [48]. According to our findings, the results are similar to those reported in other studies with cell lines and animals [11,13].

Limitations of the study

1. In our study, only one dose of ibuprofen (50 mg/kg, i.p.) was administered.
2. *In vitro* NURR1 and ibuprofen functional studies should be performed.
3. Further studies involving inflammatory mediators that affect NURR1 must be performed in the presence of ibuprofen.
4. A detailed study involving several biomarkers that show an effect on dopaminergic neurons in Parkinson models is required, which involves the comparison between two and four weeks of MPTP effect and protection of ibuprofen on biomarkers such as TH neurons other than NURR1 expression.

Conclusions

In conclusion, we developed an effective mouse model using the MPTP protocol. Using this model, it can be shown that both neuronal degeneration and Lewy body formation are initiated at the onset of Parkinson's and at advanced stages. Ibuprofen treatment was effective for initial Parkinson's disease, although there was no positive response in advanced-stage PD. NURR1 expression shows constant upregulation as Parkinson's disease progresses and may help to assess the risk associated with anti-inflammatory drug treatment. Based on these results, we can gain a deeper understanding of NURR1's activity. To validate NURR1 as a future drug target, we need to develop several types of potent NURR1 modulator tool compounds that address various binding sites and distinctive receptor responses.

Conflict of Interest

There is no conflict of interest.

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