RESEARCH PAPER

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Leflunomide exerts neuroprotective effects in an MPTP-treated mouse model of Parkinsonism

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Neuroinflammation and the immune response are recognized as significant mechanisms contributing to the progression and pathophysiology of Parkinson's disease (PD). Consequently, extensive research is being conducted on drugs targeting inflammation and immune response. Leflunomide, known for its anti-inflammatory and immunomodulatory properties, is currently used as a disease-modifying agent for the treatment of rheumatoid arthritis. The objective of this study was to investigate the effect of leflunomide on PD. The PD model was established by administering 18 mg/kg of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) intraperitoneally for 5 consecutive days. Leflunomide was administered intraperitoneally at doses of 1, 5, and 10 mg/kg for 14 days. Motor and behavioral deficits were assessed using the rotarod test, locomotor activity assessment, hanging wire test, and pole test. MPTP administration impaired motor function and locomotor activity, and caused muscle weakness and bradykinesia. Leflunomide at a dose of 10 mg/kg mitigated the severity of motor deficits and muscle weakness. Furthermore, leflunomide at a dose of 10 mg/kg suppressed the MPTP-induced elevation of interleukin-2, interleukin-6, and tumor necrosis factor-alpha levels in the brain tissue. Similarly, leflunomide attenuated the increased expression of nuclear factor kappa B and inducible nitric oxide synthase caused by MPTP treatment. Moreover, leflunomide at a dose of 10 mg/kg preserved neuronal integrity and prevented the loss of tyrosine hydroxylase expression induced by MPTP administration. Based on our findings, leflunomide exhibited a beneficial effect on the MPTP-induced PD model, potentially through modulation of anti-inflammatory mechanisms.

Key words: leflunomide, Parkinson's disease, MPTP, neuroinflammation, mice

INTRODUCTION

Parkinson's disease (PD) is an age-related neurological disorder characterized by movement abnormalities such as bradykinesia (slow-motion), resting tremor, muscle stiffness, and postural instability (Carballo-Carbajal et al., 2019). It is the second most prevalent neurodegenerative disease and is characterized by a gradual and progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Kidd & Schneider, 2011). Although several mechanisms have been proposed, the exact underlying mechanism of PD has not yet been fully elucidated. While motor symptoms can be managed with dopaminergic medications, their efficacy decreases as the severity of clinical symptoms increases due to the progressive nature of the neurodegenerative process (Schapira, 2009). Over the past two decades, our understanding of PD has significantly expanded, highlighting oxidative stress and inflammation-induced cytokine toxicity as primary

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mechanisms contributing to degeneration in the nigrostriatal system and the acceleration of disease progression in idiopathic PD patients (Tansey & Goldberg, 2010). Regardless of the disease duration, idiopathic PD patients have shown significantly increased neuroinflammation in various brain regions, including the pons, basal ganglia, striatum, frontal cortex, and temporal cortex, when compared to age-matched healthy individuals (Süß et al., 2020). Experimental studies using animal models have demonstrated that neuroinflammation plays a crucial role in disease progression, even if it is not the initial trigger (Tansey & Goldberg, 2010). Post-mortem studies and animal models have revealed the presence of inflammatory processes that can contribute to disease progression, characterized by microglial activation, cytokine accumulation, and activation of the NF-κB pathway (Shih et al., 2015; Kujawska & Jodynis-Liebert, 2018). Various mechanisms have been proposed to explain the initiation of neuroinflammation, including neuronal injury (such as brain trauma or stroke), immune challenges (bacterial or viral infections), and other factors such as chronic inflammatory syndromes (rheumatoid arthritis, atherosclerosis, type 2 diabetes, Crohn's disease, and multiple sclerosis) and exposure to environmental toxins (pesticides, etc.) (Brochard et al., 2008). Many of these events have been shown to increase the permeability of the blood-brain barrier, allowing the infiltration of lymphocytes, macrophages, and potentially environmental toxins into the brain parenchyma (Sherer et al., 2003; Goldman, 2014).

Post-mortem examination of the SNpc in PD cases has revealed the presence of activated microglial cells and elevated levels of pro-inflammatory cytokines. Over the past two decades, there has been significant advancement in our understanding of PD, with oxidative stress and inflammation-induced cytokine toxicity being recognized as the primary mechanisms contributing to degeneration in the nigrostriatal system and the acceleration of disease progression in idiopathic PD patients (Nagatsu et al., 2000). Recent preclinical studies have suggested the need for the development of new strategies to inhibit microgliosis and prevent the increase of inflammatory cytokines (Nagatsu et al., 2000; Brochard et al., 2008; Reale et al., 2009; Alcalay, 2016). These findings indicate that anti-inflammatory agents capable of halting neuroinflammation may have beneficial effects on PD pathology. Leflunomide, a drug with anti-inflammatory and immunomodulatory properties, was introduced in 1998 for the treatment of rheumatoid arthritis. Following rapid absorption, leflunomide undergoes hepatic conversion into its active form called teriflunomide. The active metabolite inhibits dihydroorotate dehydrogenase (DHODH), a mitochondrial enzyme involved in pyrimidine synthesis, thereby preventing cells from transitioning from the G1 to the S phase. Additionally, leflunomide inhibits cellular immunity and T-cell receptor response through tyrosine kinase inhibition, leading to immunomodulatory and antiviral effects (Teschner & Burst, 2010). However, while the anti-inflammatory and immunomodulatory effects of leflunomide are well-known, its potential effects on PD are still not fully understood. Therefore, the objective of the current study was to investigate the possible neuroprotective effects of leflunomide in an MPTP-induced PD model.

METHODS

Animals

The animals used in this study were sourced from the Ondokuz Mayis University vivarium in Samsun, Turkey. They were housed under standard conditions, consisting of a temperature of 22±0.5°C, humidity of 55%, and a 12-hour light/12-hour dark cycle. Throughout the study, all necessary measures were taken to minimize animal suffering, adhering to the guidelines set forth by the Canadian Council on Animal Care. Ethical approval for conducting animal experiments was obtained from the Ondokuz Mayis University Ethics Committee for Animal Experiments (HADYEK 2019-19). Thirty male C57BL/6 mice weighing between 20 and 40 grams were included in this study. Animals exhibiting abnormal motor behaviors were excluded from the study to ensure data integrity and reliability.

Chemicals and treatment groups

Leflunomide, MPTP, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Leflunomide was dissolved in a 0.1% DMSO solution and administered intraperitoneally at 1, 5, and 10 mg/kg, corresponding to the experimental groups. MPTP was dissolved in saline and administered intraperitoneally to the animals at 18 mg/kg. The control group received 200 ml of saline intraperitoneally during the experiment. The selection of leflunomide treatment doses was based on previous studies (Elshaer et al., 2019; El-Sherbiny et al., 2021).

Experimental design

Before the start of the experiments, all animals were randomly assigned to five groups: control (n=6),

MPTP (n=6), MPTP + Leflunomide 1 mg/kg (n=6), MPTP + Leflunomide 5 mg/kg (n=6), and MPTP + Leflunomide 10 mg/kg (n=6). A rotarod test using the rotarod apparatus (Ugo Basile, Italy), was performed on all animals at the onset of the study (designated as day 0) to verify that all animals had normal motor function and did not have pre-existing impairments that could interfere with the experimental model. As all animals exhibited normal motor function, no exclusion was necessary at this stage. Immediately following the rotarod tests on day 0, the experimental treatments commenced.

During the first five days (days 0-4), the control group received daily intraperitoneal injections of 200 ml of saline, which served as vehicle control, while the other groups were administered MPTP intraperitoneally at 18 mg/kg per day to induce a PD model, as described previously (Meredith & Rademacher, 2011).

On day 5, treatment for the experimental groups began. The control group continued to receive daily saline injections for 14 days, while the MPTP group received intraperitoneal injections of 0.1% DMSO, used as a solvent for leflunomide, for the same duration. Concurrently, the other groups were treated with leflunomide at doses of 1, 5, and 10 mg/kg for 14 days.

The final round of behavioral tests was performed 24 h after the last drug administration (on day 19). After completing the behavioral assessments, all animals

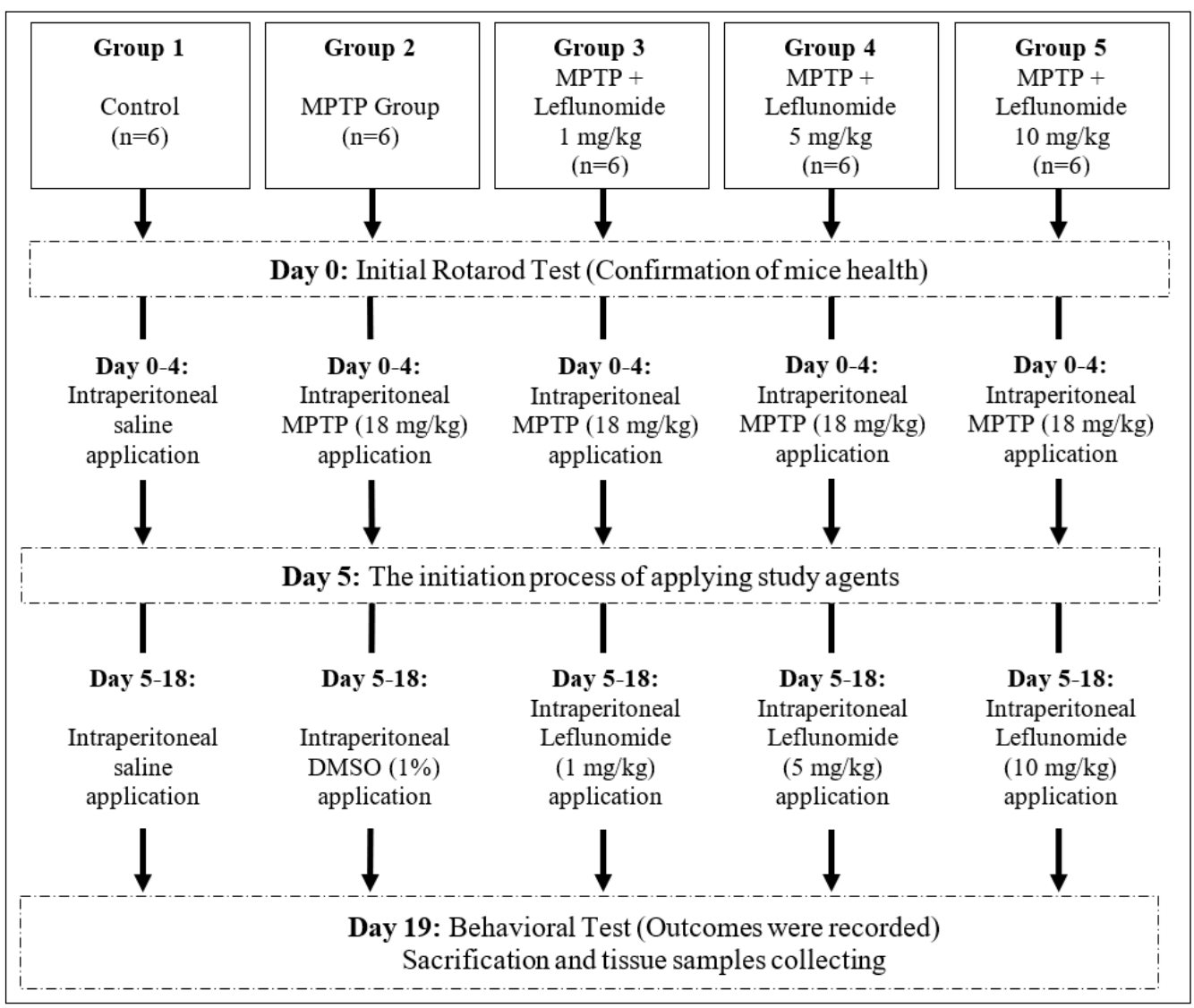

Fig. 1. Study flowchart. The flowchart outlines the experimental design of the study, detailing the allocation of experimental groups, treatment regimen, and timeline of behavioral tests and tissue collection. Group 1 served as the control, receiving saline throughout the study period. Group 2 received MPTP injections to induce a PD model. Groups 3, 4, and 5 received MPTP, followed by treatment with leflunomide at doses of 1, 5, and 10 mg/kg, respectively. Behavioral tests were conducted on day 19 to assess motor function, while tissue samples were collected on day 19 post-sacrifice for further analysis.

were euthanized by decapitation, and blood and tissue samples were collected for subsequent biochemical and histopathological analyses. The study procedure is summarized in Fig. 1 as a flowchart.

Behavioral Tests

Rotarod Test

Twenty-four hours after the completion of drug treatments, rotarod tests were conducted for motor coordination and balance. The animals were pre-trained on the rotarod for two consecutive days before testing. During the test, the animals were placed on the rod, which rotated at a constant speed of 20 rpm. The test was repeated three times per animal, with at least a 30-min rest interval between each trial. The latency to fall was recorded for each trial, and the result was calculated as the average of the three measurements. This average value was used for statistical analysis. The rod was cleaned between trials to avoid odor-related biases.

Locomotor Activity Test

The locomotor activity of the animals was assessed using a locomotor activity cage equipped with horizontal and vertical infrared beams. Each animal was placed in the cage, and its movement was recorded for five minutes. The locomotor activity was measured based on the number of beam breaks during this period. The cage was cleaned with a 10% ethanol solution before each trial to eliminate any odor-related factors that might affect behavior.

Hanging Wire Test

Muscle strength and endurance were evaluated using the hanging wire test. Each animal was gently placed on a wireframe cover and encouraged to grasp the wire. Once the animal was holding on, the cover was inverted, suspending the animal approximately 50 cm above a soft bedding surface. The latency to fall was recorded. The test was repeated three times for each animal, with at least a 30-min interval between each trial. The average of the three trials was calculated for analysis.

Pole Test

Bradykinesia, or slowness of movement, was assessed using the pole test, as previously described by Ogawa et al. (1985). Each animal was placed on top of a rough-surfaced, stainless-steel pole measuring 1 meter in height, with its forelimbs facing upwards. The time taken for the animal to descend to the bedding surface was recorded. The pole test was conducted three times per animal, and the average time was used for analysis.

Biochemistry and Western blot

Following the behavioral tests, the animals were anesthetized and trans-cardially perfused with heparinized phosphate-buffered saline (PBS, pH 7.4) to remove blood from the tissues. The brain tissues were rapidly isolated, and one hemisphere was homogenized using liquid nitrogen. Additionally, liver tissues were collected to evaluate potential drug-induced damage, which is a known effect of leflunomide (Gupta et al., 2011; Elshaer et al., 2019). The brain tissue samples were reconstituted with PBS to evaluate cytokine levels and RIPA buffer for western blot experiments. The total protein content of the samples was determined using Lowry's method and the Bicinchoninic Acid assay (BCA) (Lowry et al., 1951). The levels of cytokines, including IL-2, IL-6, and TNF-α, were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits.

For Western blot analysis, equal amounts of protein from the samples were loaded onto 4-20% Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. After electrophoresis, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes and blocked with 5% skim milk powder for one hour. The membranes were then incubated with primary antibodies against NF-κB (#66535-1-Ig, Proteintech, Rosemont, US) and iNOS, #18985-1-AP, Proteintech, Rosemont, US) overnight at 4°C. After washing, the membranes were incubated with secondary antibodies (#31460, Thermo Scientific, US) for 2 hours. Protein bands were visualized using a chemiluminescent agent (SuperSignal® West Pico Chemiluminescent Substrate, #34579, Thermo Scientific, US) and imaged with ChemiDoc. During the quantitative analysis, the protein expression for each mouse was first normalized to the corresponding beta-actin levels. The normalized values for each mouse were then averaged from three repeated measurements per parameter. Group averages were subsequently determined using these calculated values. Following this, the protein expression levels for all groups were referenced to the control group, and the changes were expressed as fold differences relative to the control. The relative protein expression levels were quantified using ImageJ software (NIH, US).

Histopathological and immunohistochemical analysis

The remaining hemispheres and livers from the perfused samples were immediately fixed in a 4% paraformaldehyde solution for 24 h at 4°C. After fixation, the samples were washed under running water and subjected to a routine histological protocol as described previously (Avcı et al., 2020). Paraffin wax-embedded samples were cut into 5-µm thick sections using a rotary microtome (Leica, RM2125). The sections were then stained with hematoxylin and eosin for examination under a light microscope (Nikon Eclipse E400, Japan). This staining allows for the visualization of cellular and tissue structures. For the liver tissues, a careful histological examination was performed to evaluate the presence of inflammation, necrosis, and fibrosis.

Immunohistochemical (IHC) staining was carried out to assess TH immunopositivity on 5-µm sections using the streptavidin-biotin-immunoperoxidase complex (Lab Vision™ UltraVision™ LP Detection System, Thermo Fisher Scientific). The sections were deparaffinized and dehydrated through graded alcohols. Endogenous peroxidase activity was blocked using a 3% hydrogen peroxide solution. Antigen retrieval was performed by heating the sections in citrate buffer (pH 6.0) using a microwave. Following a blocking step to prevent nonspecific binding, the sections were incubated with the primary antibody against TH (anti-TH antibody; Elabscience, Cat No: E-AB-70077; dilution: 1/300) overnight at 4°C. Subsequently, the sections were incubated with a biotinylated secondary antibody, followed by streptavidin-peroxidase incubation. The labeling was visualized using 3-amino-9-ethyl carbazole (AEC) as the chromogen. Counterstaining with Mayer's hematoxylin was performed, and the sections were examined under a light microscope (Nikon Eclipse E400, Japan). TH immunopositivity was evaluated under ×40 and ×100 high-power magnification fields per brain sample.

Statistical analysis

All experimental results were analyzed using Prism 9 (v. 9.5.1, GraphPad, Boston, MA) and SPSS (v. 21.0, SPSS Inc., Chicago, IL). The normality of data distribution was assessed using the Shapiro-Wilk test. For comparisons among multiple groups, Brown-Forsythe and one-way analysis of variance (ANOVA) was applied to data following a normal distribution. Dunnett T3 and Tukey's *post hoc* test were employed for pairwise comparisons. Descriptive statistics of the quantitative variables in the study are given as mean and standard deviation. P-values less than 0.05 were considered significant.

RESULTS

Leflunomide attenuated MPTP-induced motor impairment and muscle weakness

The results of the behavioral tests are as follows:

Rotarod test and locomotor activity (Fig. 2): MPTP treatment significantly decreased falling latency (29 ± 7.2) and locomotor activity (190 ± 7.6) compared to the control group (93.2 ± 5.2 and 303 ± 56.3, respectively; $p<0.001$). Leflunomide treatment at doses of 1 and 5 mg/kg did not affect rotarod performance (29.7 ± 4 and 33.8 ± 6.1, respectively) and locomotor activity (194 ± 8.4 and 194.1 ± 10.8, respectively). Leflunomide at a dose of 10 mg/kg significantly prevented the MPTP-induced decrease in falling latency (73.0 ± 2.8, $p<0.001$) and locomotor activity (255.4 ± 32.2, $p=0.007$).

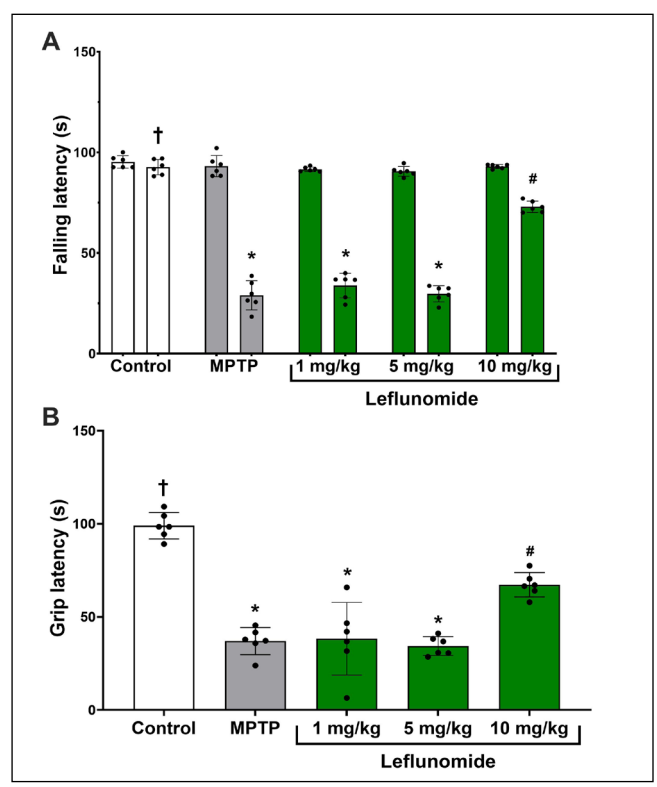

Fig. 2. Rotarod test and locomotor activity. Motor coordination (A) and activity (B) were investigated using the rotarod test in all groups. The bar on the left of the graph represents the pre-treatment fall duration, while the bar on the right illustrates the post-treatment fall duration after medication administration. MPTP decreased motor coordination, which was significantly reversed with 10 mg/kg leflunomide treatment (A). Locomotor activity results demonstrated that MPTP decreased total ambulation in the activity cage (B). Leflunomide 10 mg/kg (n=6) significantly inhibited MPTP-induced decrease (B). All data are expressed as mean ± SD. †, *, # The different symbols on the column indicate that this group is statistically significantly different from the other group(s).

Muscle weakness (hanging wire test, Fig. 3A): The administration of MPTP (37.0 ± 7.3) resulted in a considerable decrease in hanging time when compared to the control group (99.0 ± 7.0, p<0.001). However, treatment with leflunomide at doses of 1 mg/kg (38.2 ± 19.5) and 5 mg/kg (34.3 ± 5.0) did not demonstrate a statistically significant impact on muscle weakness compared to the MPTP group (p>0.05). On the other hand, treatment with leflunomide at a dose of 10 mg/kg (68.5 ± 6.9) exhibited a significant effect on muscle weakness (p<0.05).

Bradykinesia (pole test, Fig. 3B): MPTP treatment (24.0 ± 3.2) caused a significant increase in time to downward movement compared to the control group (10.5 ± 1.8, p<0.001). Leflunomide treatment at a dose of 10 mg/kg (15.5 ± 1.1) significantly prevented this prolongation (p<0.001), but this effect was not observed at doses of 1 mg/kg (23.7 ± 1.0) and 5 mg/kg (21.0 ± 1.4, p>0.05).

For detailed statistical analysis and specific tests performed, please refer to Tables 1-3.

Leflunomide inhibited MPTP-induced increase in pro-inflammatory cytokines

The results of the ELISA analysis for IL-2, IL-6, and TNF levels in brain tissues are as follows:

IL-2 levels (Fig. 4A): MPTP treatment caused a pronounced increase in IL-2 levels (758.0 ± 96.0) compared to the control group (43.9 ± 18.9, p<0.001). Leflunomide treatment at doses of 1 mg/kg (744 ± 77.6) and 5 mg/kg (705 ± 68) did not substantially impact the MPTP-induced rise in IL-2 levels (p>0.05). However, at 10 mg/kg, leflunomide notably reduced the elevated IL-2 levels (456 ± 83.3), showing a significant difference compared to the MPTP group (p<0.001).

IL-6 levels (Fig. 4B): MPTP administration resulted in a marked increase in IL-6 levels (714.0 ± 69.3) compared to the control group (43.8 ± 12.7, p<0.001). Leflunomide at 1 mg/kg (724 ± 37.9) and 5 mg/kg (654 ± 57.8) did not lead to a statistically significant reduction in IL-6 levels (p>0.05). However, the 10 mg/kg dose of leflunomide considerably lowered IL-6 levels (445 ± 55.5) relative to the MPTP group (p<0.001).

TNF levels (Fig. 4C): TNF levels significantly increased after MPTP treatment (617.0 ± 67.3) compared to the control group (95.1 ± 12.2, p<0.001). Leflunomide at doses of 1 mg/kg (600.1 ± 20.1) and 5 mg/kg (592 ± 48.3) did not show a substantial effect on MPTP-induced TNF elevation (p>0.05). In contrast, treatment with leflunomide at 10 mg/kg led to a clear decrease in TNF levels (457 ± 43.7), showing a statistically significant difference compared to the MPTP group (p<0.001). For detailed statistical analysis, please refer to Table 2.

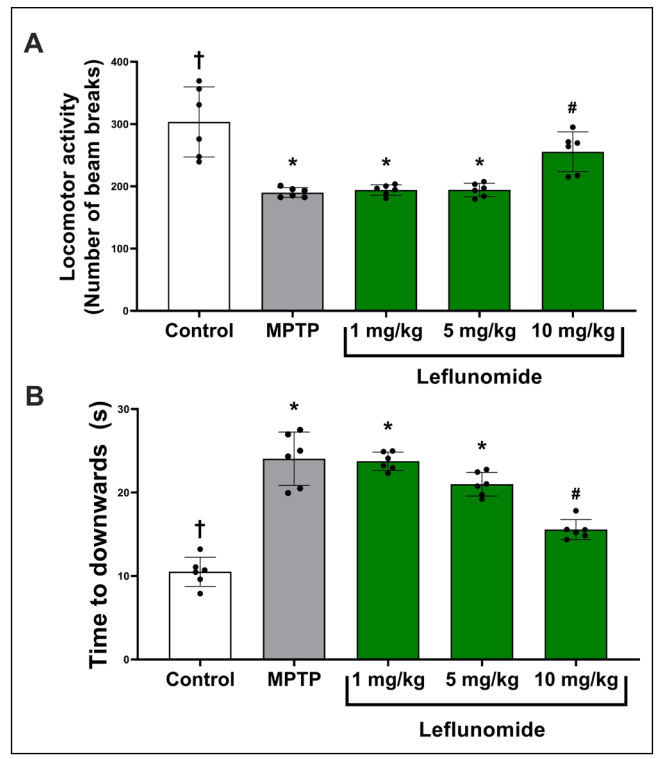

Fig. 3. Hanging wire test and pole test. Muscle weakness and bradykinesia were evaluated with grip strength (A) and pole tests (B). MPTP caused a significant decrease in muscle weakness (A) and bradykinesia (B). Leflunomide at the dose of 10 mg/kg (n=6) alleviated muscle weakness (A) and bradykinesia (B). All data are expressed as mean ± SD. †, *, # The different symbols on the column indicate that this group is statistically significantly different from the other group(s).

Table 1. The statistical differences between the experimental groups in terms of behavioral tests.

	Behavioral Tests		
	Locomotor Activity*	Time To Downwards**	Grip Latency*
p value	<0.001	<0.001	<0.001
F value	17.1	57	42.4
DFn	4	4	4
DFd	25	25	25

*Mann-Whitney U; **One-way ANOVA.

	Falling Latency***	
	F (DFn, DFd)	P value
Interaction	$F_{(4,50)}$=135	P<0.001
Row Factor	$F_{(1,50)}$=1455	P<0.001
Column Factor	F(4,50)=163	P<0.001

***Two-way ANOVA.

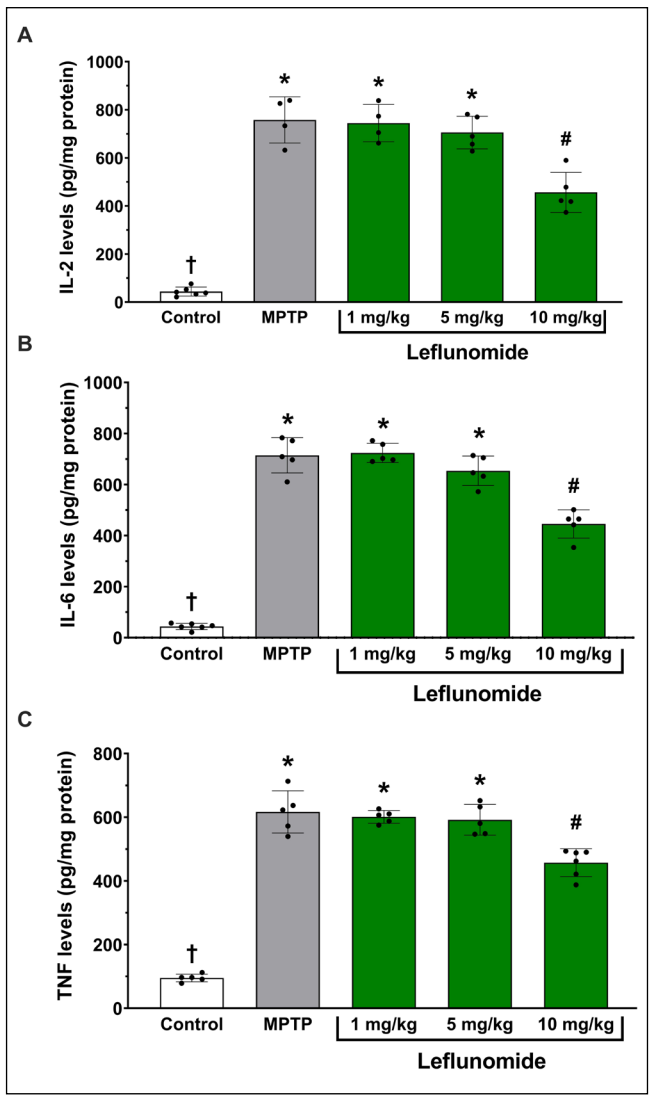

Fig. 4. IL-2, IL-6, and TNF levels. ELISA results of brain tissue analysis of IL-2 (A), IL-6 (B), and TNF (C) levels in all groups. MPTP caused a significant increase in IL-2 (A), IL-6 (B), and TNF (C) levels, as expected. Leflunomide prevented MPTP-induced increase in these cytokines. All data are expressed as mean ± SD. †, *, # The different symbols on the column indicate that this group is statistically significantly different from the other group(s).

Table 2. The statistical differences between the experimental groups in terms of biochemical parameters.

	Biochemistry*		
	IL-2	IL-6	TNF-α
p value	<0.001	<0.001	<0.001
F value	100	190	132
DFn	4	4	4
DFd	19	21	21

*One-way ANOVA.

Leflunomide suppressed MPTP-induced increase in NF-κB and iNOS expression

The results of the Western blot analysis for NF-κB and iNOS expression levels are as follows:

NF-κB expression (Fig. 5B): MPTP treatment induced a significant increase in NF-κB expression (4.2 ± 0.2 fold change) compared to the control group (normalized to 1.0, $p<0.001$). Leflunomide treatment at doses of 1 mg/kg (4.2 ± 0.3 fold change) and 5 mg/kg (4.2 ± 0.3 fold change) did not show a significant effect on the MPTP-induced increase in NF-κB expression ($p>0.05$). However, leflunomide at a dose of 10 mg/kg significantly alleviated the MPTP-induced increase in NF-κB expression (2.2 ± 0.2 fold change) compared to the MPTP group ($p<0.01$).

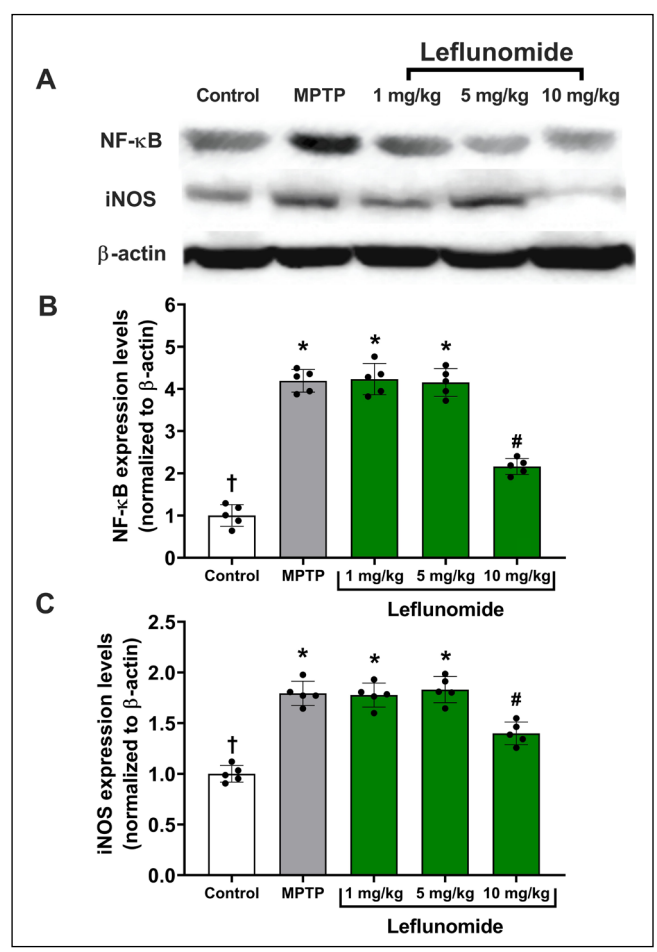

Fig. 5. NF-κB, iNOS expression levels. Western blot results of brain tissue analysis of NF-κB (A, B) and iNOS (A, C) in all groups. MPTP caused a significant increase in NF-κB (A, B) and iNOS (A, C) expression. Leflunomide prevented MPTP-induced increase in NF-κB (A, B) and iNOS (A, C) expression. All data are expressed as mean ± SD. †, *, # The different symbols on the column indicate that this group is statistically significantly different from the other group(s).

iNOS expression (Fig. 5C): MPTP treatment induced a significant increase in iNOS expression (1.8 ± 0.1 fold change) compared to the control group (normalized to 1.0, $p<0.001$). Leflunomide treatment at doses of 1 mg/kg (1.8 ± 0.1 fold change) and 5 mg/kg (1.8 ± 0.1 fold change) did not show a significant effect on the MPTP-induced increase in iNOS expression ($p>0.05$). However, leflunomide at a dose of 10 mg/kg significantly alleviated the MPTP-induced increase in iNOS expression (1.4 ± 0.1 fold change) compared to the MPTP group ($p<0.01$). For detailed statistical analysis, please refer to Table 3.

Table 3. The statistical differences between the experimental groups in terms of Western blot analysis.

	Western Blot*	
	iNOS	NF-κB
p value	<0.001	<0.001
F value	50.1	131
DFn	4	4
DFd	20	21

*One-way ANOVA.

Leflunomide protected neuronal integrity and MPTP-induced loss of TH immunoreactivity

The brain evaluations examined the striatal area and SNpc for TH immunoreactivity. TH immunoreactivity was analyzed in the SNpc across four areas from four sections per mouse at ×400 magnification by two blinded researchers. In the striatal area, TH immunoreactivity was quantified as the integrated density using ImageJ software (v1.54g, Wayne Rasband and contributors, National Institutes of Health, USA) (Wen et al., 2021). Among the preparations where the striatal areas were examined, a decrease and loss of immune reaction in TH-positive areas were observed in the MPTP group compared to the other groups. However, the leflunomide 1, 5, and 10 mg/kg groups showed similarity to the control group. Although the prevalence of immune-positive areas in the striatal area decreased in the MPTP group compared to the other groups, no prominent difference was observed among the treatment groups (Fig. 6A and Fig. 7B). In the preparations where the SNpc was examined, there was a decrease in the number of regional neurons in the MPTP group. However, the leflunomide 1, 5, and 10 mg/kg groups did not exhibit a decrease in the number of neurons. The leflunomide 10 mg/kg group yielded results closest to the control group (Fig. 6B and Fig. 7A).

Additionally, histological examination of the liver tissue confirmed that leflunomide, even at the highest treatment dose of 10 mg/kg, did not cause any obvious signs of inflammation, steatosis, or necrosis (Fig. 8). These findings suggest that leflunomide did not induce hepatic injury in the experimental model.

Effect of Leflunomide on Liver Tissue

Liver evaluations were based on the scoring criteria published in the European Journal of Nuclear Medicine and Molecular Imaging (2006). The classical hepatic lobule structure, centered on the central vein, was examined in 5 different areas on 10 liver sections taken from each subject. Lobules were examined in terms

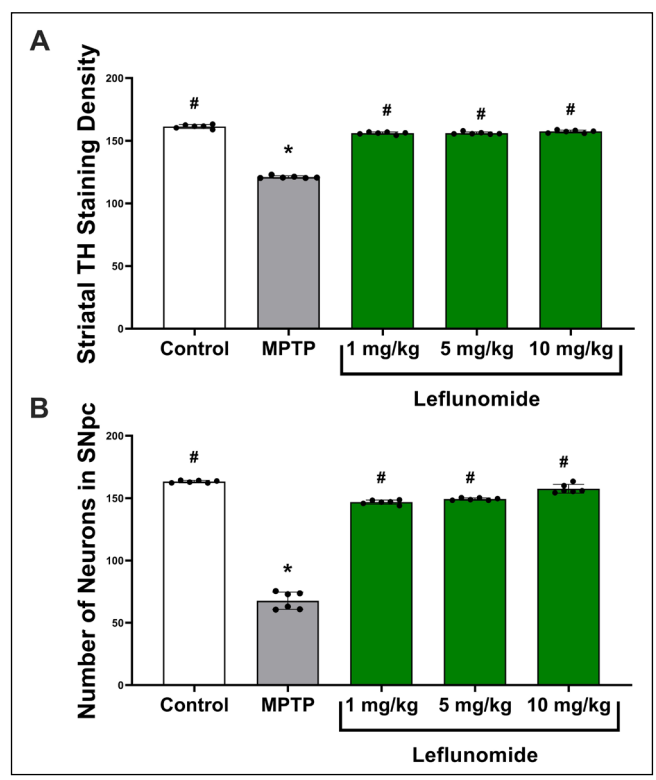

Fig. 6. Comparison of striatal TH staining density and number of neurons in SNpc between groups. Results of immunohistochemical analysis of striatal TH staining density (A) and number of neurons in SNpc (B) between groups. (A) Striatal TH staining density was significantly decreased in the MPTP group compared to the control group ($p<0.05$). Leflunomide treatment at doses of 1, 5, and 10 mg/kg prevented the MPTP-induced reduction in TH staining density ($p<0.05$), resulting in levels similar to the control group. (B) The number of neurons in the SNpc was significantly reduced in the MPTP group compared to the control group ($p<0.05$). Leflunomide treatment at doses of 1, 5, and 10 mg/kg significantly prevented the decrease in the number of neurons in the SNpc ($p<0.05$), resulting in values comparable to the control group. All data are expressed as mean ±S D. †, *, # The different symbols on the column indicate that this group is statistically significantly different from the other group(s).

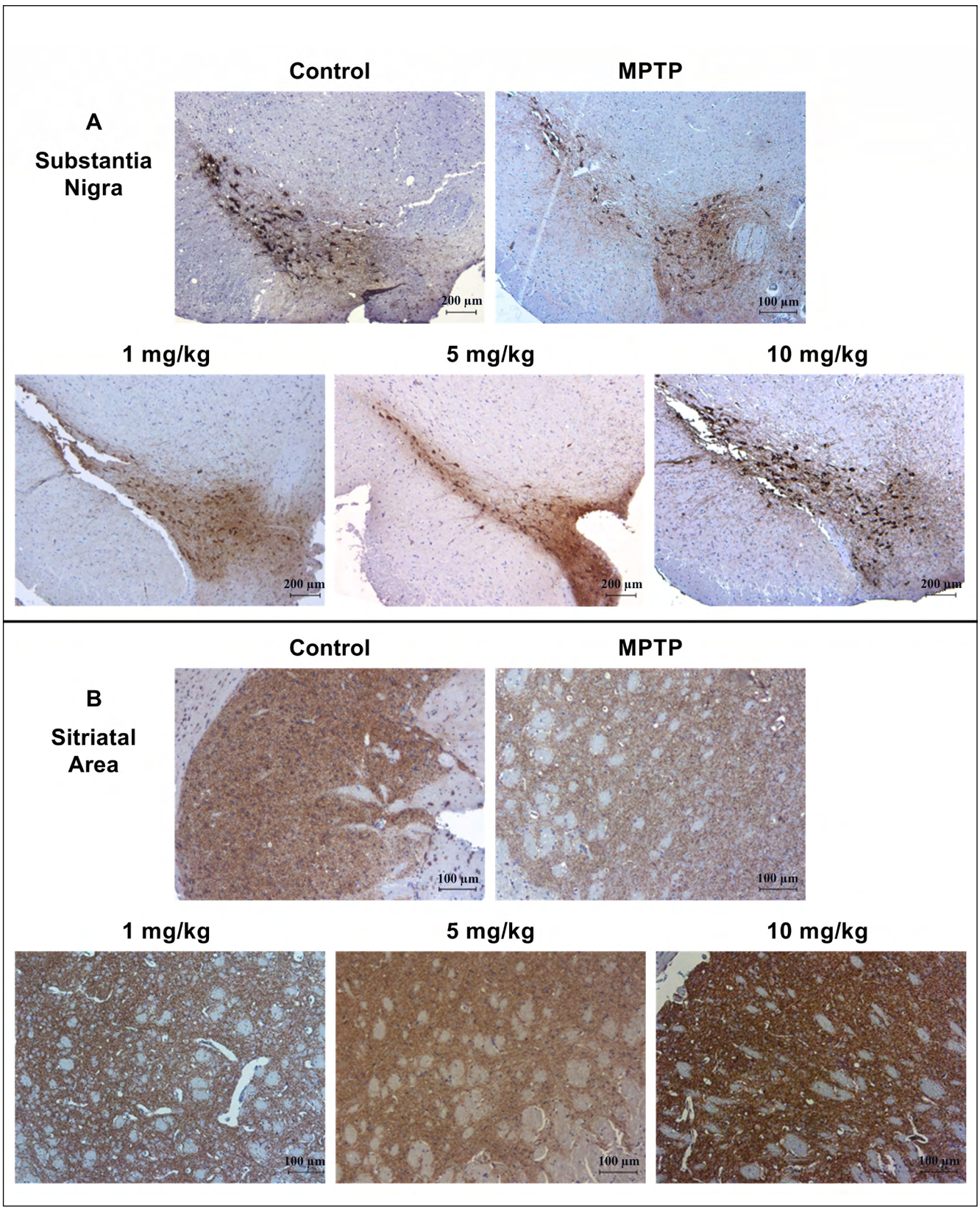

Fig. 7. Immunohistochemical staining on SNpc and striatal areas. All groups were investigated with immunohistochemical staining on the SNpc and striatal regions. MPTP caused a reduction in TH immunopositivity. Leflunomide at the dose of 10 mg/kg (n=6) partially rescued the loss of TH immunopositivity. Arrows and stars indicate TH immunopositivity.

of steatosis, inflammation, necrosis, and fibrosis, and evaluations were made between 0-3 according to the presence and severity of these criteria. According to this: Control: 0-1, MPTP: 2-3, Leflunomide 1 mg/kg: 1, Leflunomide 5 mg/kg: 1, Leflunomide 10 mg/kg: 0.

No damage was observed in the control group. Intense inflammation and accompanying cell damage were observed in the MPTP group. An average of more than 50% overall damage and disruption of the typical radial hepatocyte alignment around the central vein were observed in the examined areas. Inflammation was found to be high in portal areas. A small amount of inflammation foci was found in the leflunomide 1 mg/kg group. These foci constituted less than 10% of the studied areas. In addition, few necrotic hepatocytes were found. In the leflunomide 5 mg/kg group, less than 10% inflammation was seen only in the portal area. It was observed that the foci of inflammation were below 10% in the leflunomide 10 mg/kg group, but settled in smaller and scattered clusters compared to the leflunomide 5 mg/kg group. When evaluated in terms of other scoring criteria, the results of this group are similar to the control group (Fig. 8).

DISCUSSION

In this study, we investigated the potential neuroprotective effect of leflunomide in MPTP-induced neurodegeneration, which mimics PD. Our findings demonstrate that leflunomide, particularly at the 10 mg/kg dose, effectively mitigated MPTP-induced dopaminergic neuronal loss and significantly ameliorated motor deficits and muscle weakness caused by MPTP. Additionally, leflunomide treatment suppressed the elevated levels of pro-inflammatory cytokines and protein expression induced by MPTP in the brain tissue.

The neurotoxin MPTP is considered the gold standard for studying dopaminergic neuron death in PD (Lin et al., 2020). In C57BL/6 mice, doses of 20-25 mg/kg have been shown to induce dopaminergic neuron loss, a decrease in TH-positive cells in the striatum, and significant impairments in motor functions, as observed in tests such as the rotarod, pole test, grid test, and beam walk test. This model is effective in replicating Parkinson-like symptoms and is utilized for developing treatment strategies. It is also

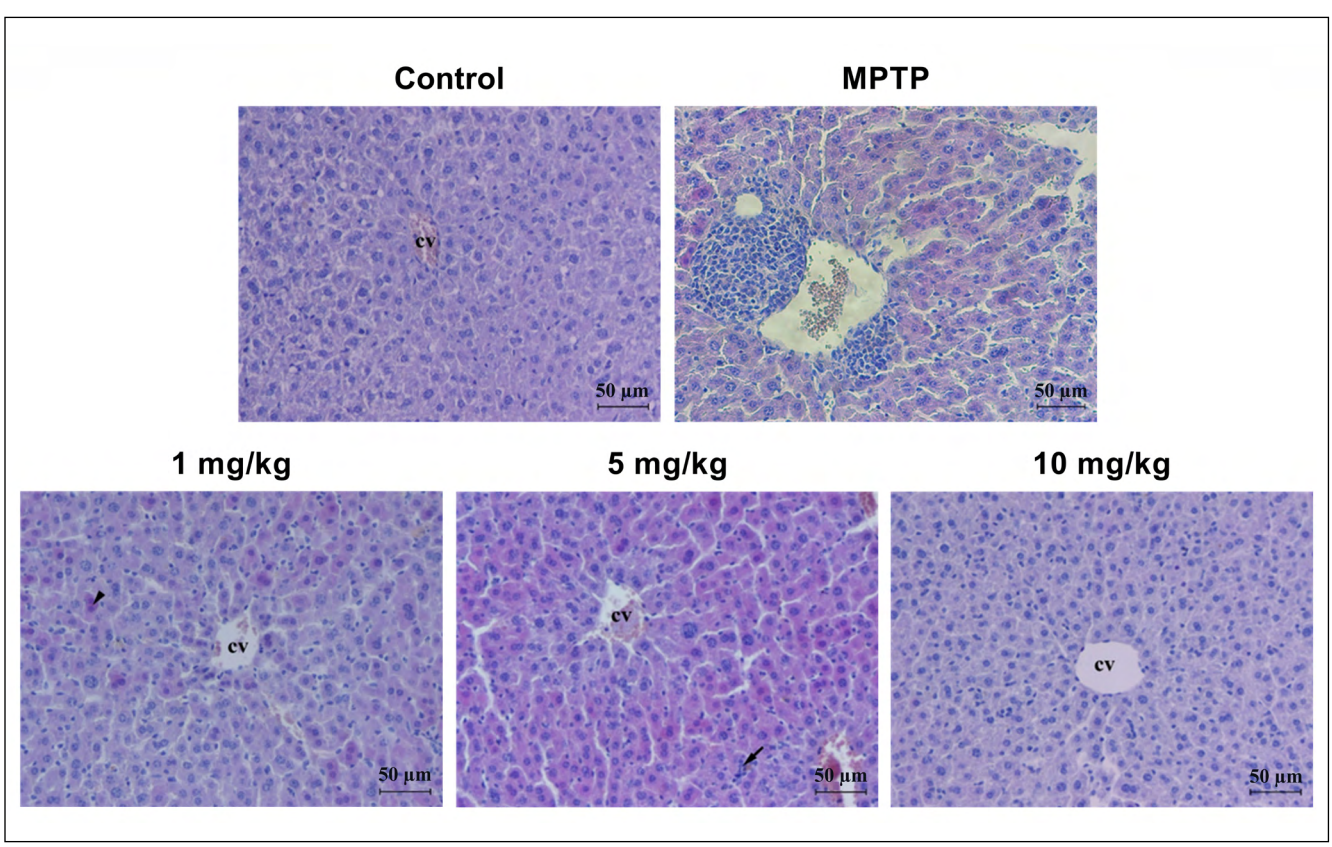

Fig. 8. Histological examination of hepatic tissues: Histopathological analysis of liver tissue in all groups. All sections were stained with hematoxylin & eosin (H&E). CV: Central vein. Images were captured at x200 magnification. Arrowheads indicate areas with minimal hepatocyte damage, while arrows point to regions with limited inflammatory cell infiltration.

commonly used to investigate mechanisms related to PD, including oxidative stress and neuroinflammation (Langston, 2017; Mustapha & Taib, 2021).

Moreover, publications indicate that while subacute MPTP treatment can largely replicate the clinical and pathological features of PD in mice, it often fails to produce Lewy body-like inclusions (Blandini & Armentero, 2012). Despite these discrepancies in the literature, many studies support the sustained motor function impairments observed in our PD model. For instance, Wang et al. (2018) reported motor dysfunction in animals following 5×30 mg/kg MPTP administration. Similarly, Rai et al. (2019) observed motor activity loss in mice treated with 2×30 mg/kg. Li et al. (2019) demonstrated Parkinsonism features in movement tests with 5×25 mg/kg. Xue et al. (2020) successfully established a Parkinson's model by administering 2×22 mg/kg MPTP, followed by 2×18 mg/kg two weeks later. Haga et al. (2020) also reported motor activity loss in mice with 2×25 mg/kg MPTP. These findings confirm that the dose of MPTP used in our study is appropriate and in line with the commonly used protocol in the literature. Additionally, studies have shown the infiltration of CD4 and CD8 T lymphocytes, which are key components of the adaptive immune response, in the SNpc of PD patients and MPTP-treated mice (Tansey & Goldberg, 2010; Brochard et al., 2008). Therefore, the modulation of inflammation holds significant therapeutic potential for PD, and the investigation of anti-inflammatory agents has gained substantial attention in recent decades. Our study reveals that leflunomide, a systemic anti-inflammatory agent, effectively reduces CNS inflammation and neuronal loss, which are prominent features of PD patients (Pajares et al., 2020). Furthermore, leflunomide prevented the decline in locomotor activity and ameliorated Parkinsonian behavioral abnormalities in our mouse model. To the best of our knowledge, this study is the first to investigate the potential of leflunomide in PD.

In the presence of neurodegeneration, microglia undergo activation and transition to an ameboid form, leading to the secretion of various chemokines and cytokines (Schetters et al., 2018; Ferreira & Romero-Ramos, 2018). These activated microglia have been shown to produce and release harmful compounds such as reactive oxygen species (ROS), reactive nitrogen species (RNS), and pro-inflammatory cytokines (He et al., 2001). In our study, the observed increase in IL-2, IL-6, and TNF levels in the MPTP-induced Parkinson's model indicates enhanced microglial activation secondary to neurodegeneration. Previous studies have suggested potential mechanisms involving toll-like receptors (TLRs), NF-κB, iNOS, cyclooxygenase 2 (Cox-2), and the interleukin cytokine family in mediating microglia-associated degeneration of dopaminergic neurons (Hickman et al., 2018).

Pro-inflammatory cytokines can directly activate the NF-κB pathway by binding to surface receptors on dopaminergic neurons, potentially leading to neuronal apoptosis (Liu et al., 2017). Ros-Bernal et al. (2011) provided evidence that NF-κB transcriptional activity increased in the SNpc and modulated microglia-mediated neuronal death through glucocorticoid receptors in microglia in the MPTP-induced PD model. The same study reported increased microglial iNOS and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity in the SNpc of MPTP-treated mice compared to the control group. iNOS produces nitric oxide (NO) and superoxide radicals, which exert toxic effects. Similarly, the NADPH oxidase system serves as the primary source of glial-derived ROS production. In a lipopolysaccharide-induced PD model, it has been demonstrated that activated microglia secrete IL-1β, which initiates CD23 surface receptor expression in microglia (Hunot et al., 1999). CD23 expression then activates microglia to produce NO and pro-inflammatory cytokines.

In our study, we observed a significant decrease in motor coordination and exacerbated bradykinesia in MPTP-treated animals compared to the control group. Histopathological analysis revealed a reduction in the number of regional neurons in the SNpc of MPTP-treated mice. Examination of striatal sections from MPTP-treated mice showed a significant decrease in TH activity, suggesting heightened microglial activation and potential exacerbation of neuronal degeneration. This is further supported by increased pro-inflammatory markers such as iNOS and NF-κB in whole-brain molecular analyses, potentially linking elevated microglial activity to neurodegeneration in the striatum.

This study revealed a significant decrease in IL-2, IL-6, and TNF levels, as well as NF-κB and iNOS expressions, in the leflunomide 10 mg/kg group compared to the control and MPTP groups. Leflunomide acts by inhibiting the formation of receptor signal responses in T cells, thereby blocking their progression to mitosis (Teschner & Burst, 2010; Gupta et al., 2011). The decreased T cell activity associated with leflunomide leads to its anti-inflammatory and immunomodulatory effects. Previous research by Kraan et al. (2000) demonstrated that leflunomide treatment in rheumatoid arthritis patients resulted in reduced inflammatory cell count, and decreased expression of adhesion molecules such as ICAM-1, metalloproteinases, IL-2, IL-6, and IL-10. They also observed a significant decrease in NF-κB and cyclooxygenase activity,

and immunoglobulin synthesis in memory T cells and dendritic cells (Kraan et al., 2000; Cutolo, 2003).

When it comes to the limitations of our study, we first induced Parkinsonism symptoms in animals by administering MPTP to observe the effects of leflunomide on Parkinson's patients. However, we did not include a separate animal group receiving leflunomide alone. Therefore, we could not obtain data on the specific effects of anti-inflammatory agents like leflunomide on the brain tissue of a healthy animal when administered independently. Additionally, we only examined hepatotoxicity indicators through histopathological examination and did not compare them by taking blood samples for liver function tests. Another limitation is related to motor memory; repeating motor tests, such as the rotarod at the end of the experiment, may have introduced a learning component that could confound the results. Addressing these issues will be among our primary objectives in the next phase of our study.

In this study, leflunomide may have blocked T cell activity, thereby inhibiting the inflammatory response, cytokine production, and ROS generation secondary to MPTP-induced neuronal degeneration. Although the effective dose of the drug was not reached in the 1 mg/kg and 5 mg/kg leflunomide groups, the administration of 10 mg/kg leflunomide may have achieved an effective concentration in the brain, leading to a decrease in pro-inflammatory cytokines and iNOS and NF-κB activity.

CONCLUSIONS

Our study suggests that systemic anti-inflammatory agents, such as leflunomide, may be effective in mitigating dopaminergic neuronal loss and motor deficits in subacute models of PD. However, in many preclinical studies conducted thus far, anti-inflammatory agents have been administered concurrently or immediately after the neurodegeneration agent, as in our study. It is important to note that neuronal degeneration and cellular debris formation have already begun in PD patients before the onset of Parkinsonian symptoms. Unfortunately, it is currently not feasible to detect the disease and initiate anti-inflammatory treatment before the clinical manifestations appear.

Nevertheless, it is crucial to acknowledge that PD has a complex multifactorial nature. As etiological factors and early diagnostic approaches continue to advance in the coming years, it is conceivable that anti-inflammatory agents will prove highly effective in the treatment of neurodegenerative diseases, both in terms of disease modification and symptomatic relief.

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