Histopathological Study of Metformin Effect on Parkinsonism Mice Model

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Abstract:

Background: Parkinson's disease is a progressive neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra. Oxidative stress and neuroinflammation are major contributors to its pathogenesis. Metformin, a widely used antidiabetic agent, has shown neuroprotective potential in several neurological conditions.

Objective: To compare the histological changes between the groups of mice that received a prophylactic dose of metformin and those that did not, as well as to compare the effect of metformin when added to standard treatment.

Methods: Thirty male albino mice were randomly divided into six groups: (Group 1) control group; (Group 2) a rotenone induction group; (Groups 3) pre-treated with metformin three days before induction (500mg/kg);(Group 4) received metformin (500 mg/kg) ; (group 5) received L-DOPA/Carbidopa (100/25 mg/kg); and (group 6) received both drugs concurrently At the end of the 47-day study period, the animals were sacrificed, and their brains were harvested and fixed in 10% neutral buffered formalin for histological examination.

Results: Histopathological analysis showed that rotenone induced classical PD-related changes, including neuronal shrinkage, pyknotic nuclei, karyorrhexis, and marked activation of microglia and astrocytes. Prophylactic metformin administration significantly ameliorated these changes, while the combined treatment group demonstrated improved neuronal preservation compared to the standard therapy alone.

Conclusions: These findings support the potential neuroprotective role of metformin, both as a prophylactic and adjunctive agent to conventional therapy in PD.

Key words: Parkinson's disease, Rotenone, Metformin, histopathological study.

دراسة النسيج المرضي لتأثير الميتفورمين على نموذج الفئران المصابة بالشلل الرعاش

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الخلاصة:

الخلفية :مرض باركنسون هو اضطراب تنكسي عصبي متقدم يتميز بفقدان الخلايا العصبية الدوبامينية في المادة السوداء . ويُعدّ الإجهاد التأكسدي والالتهاب العصبي من العوامل الرئيسية المساهمة في التسبب في الإصابة به .وقد أظهر الميتفورمين وهو عامل مضاد لمرض السكري يستخدم على نطاق واسع، إمكانيات وقائية عصبية في العديد من الحالات العصبية.

الهدف: هدفت هذه الدراسة إلى مقارنة التغيرات النسيجية بين مجموعات الفئران التي تلقت جرعة وقائية من الميتفورمين وتلك التي لم تتلقها، وكذلك مقارنة تأثير الميتفورمين عند إضافته إلى العلاج القياسي.

المنهجية :تم تقسيم ثلاثين من ذكور الفئران البيضاء عشوائياً إلى ست مجموعات :المجموعة (1)مجموعة الضابطة؛ المجموعة (2)مجموعة حث السمية بالروتينون؛ المجموعة (3) المجموعة الوقائية تلقت علاجاً مسبقاً بالميتفورمين قبل ثلاثة أيام من العلاج التحريضي (500)ملجم/كجم، المجموعة (4)تلقت الميتفورمين (500)ملجم/كجم، المجموعة (5)تلقت L-DOPA/كاربيدوبا (100/25)ملجم/كجم، المجموعة (6)تلقت كلا العقارين في وقت واحد

النتائج :في نهاية فترة الدراسة التي استمرت 47يومًا، تمت التضحية بالحيوانات وتم حصاد أدمغتها وتثبيتها في 10٪ من الفورمالين المخزن المحايد للفحص النسيجي .أظهر التحليل المرضي النسيجي أن الروتينون أحدث تغيرات كلاسيكية مرتبطة بمرض الشلل الدماغي بما في ذلك انكماش الخلايا العصبية، والنوى المتخثرة، الانشطار النووي، والتنشيط الملحوظ للخلايا الدبقية الصغيرة والخلايا النجمية .

الاستنتاج: خفف تناول الميتفورمين الوقائي من هذه التغيرات بشكل ملحوظ، في حين أظهرت مجموعة العلاج المشترك تحسناً في الحفاظ على الخلايا العصبية مقارنة بالعلاج القياسي وحده .تدعم هذه النتائج الدور الوقائي العصبي المحتمل للميتفورمين كعامل وقائي ومساعد للعلاج التقليدي في مرض الشلل الرعاشي.

الكلمات المفتاحية :مرض باركنسون، الروتينون ,المتفورمين ,دراسة نسيجية.

Introduction

Parkinson's disease (PD) is a neurological condition that primarily affects the elderly and is slowly progressing and incurable (1). After Alzheimer's disease, PD is the second most prevalent neurological illness worldwide (2). While they typically co-occur, signs and symptoms might exist independently at the early stages of the disease (e.g., depression often occurs with anxiety, apathy overlaps with depression and cognitive impairment, and psychosis and depression can exacerbate impulse control difficulties) (3). Additionally, signs and symptoms can appear at later stages of the disease, when they are frequently most severe (4).

PD deaths in Iraq totaled 658, or 0.45% of all deaths, according to the most recent WHO data released in 2020. Iraq is ranked 59 in the world with an age-adjusted death rate of 4.74 per 100,000 population (5). In a community-based survey conducted in Al-Kadhimiya (Baghdad), the crude prevalence of Parkinson's disease was reported as 108.75 per 100,000 population, with a slightly higher rate in urban areas (114/100,000) compared to rural settings (94.3/100,000) (6). On a broader scale, Global Burden of Disease (GBD) data for the Middle East and North Africa (MENA) region estimated an age-standardized prevalence of 82.6 per 100,000 and over 309,900 prevalent cases as of 2019(7). The absence of recent national-level epidemiological studies underscores the need for regionally tailored research and therapeutic strategies.

The development of PD is strongly influenced by age, genetic predisposition, and environmental exposures, but lifestyle factors can also contribute to the disease. A state of dysbiosis will result from these factors, which will change the microbiota by increasing harmful bacteria and decreasing short-chain fatty acids (SCFAs). This impacts the intestinal barrier's integrity and function by increasing metabolites like lipopolysaccharide-binding protein (LPS-BP) and Toll-like receptors (TLRs) and decreasing bacterial lipopolysaccharide (BLPS), which in turn promotes an increase

in various pro-inflammatory cytokines can penetrate the blood-brain barrier and access the central nervous system(8) .

PD is marked by the gradual buildup of α-synuclein within Lewy bodies and the degeneration of dopaminergic neurons. (9). A pathologic change may not be evident for several years. Dopamine levels drop as a result of dopaminergic neuron death, impairing motor function and possibly adding to the cognitive abnormalities seen in certain patients(10). The elevated generation of reactive oxygen species, coupled with intensified lipid peroxidation, is indicated by a rise in lipid peroxidation byproducts. malondialdehyde (MDA) and superoxide dismutase (SOD)(11). GSK-3β is implicated in nearly all areas of brain activity, including synapse development, neuroinflammation, neuronal morphology, and neurological diseases(12). Histopathological examination remains a cornerstone in confirming PD in animal models. The typical features observed in the substantia nigra include neuronal shrinkage, pyknotic nuclei, and karyorrhexis, reflecting dopaminergic neuronal degeneration. In addition, gliosis and activation of microglia and astrocytes are commonly reported and signify ongoing neuroinflammation. The presence of Lewy body-like inclusions containing α-synuclein may also be noted in toxin-induced models. These markers are critical for validating the progression of PD and assessing the neuroprotective potential of investigational treatments (13)(14).

Scientific studies have demonstrated that Carbidopa and levodopa (L-Dopa) are drugs that doctors may prescribe to people with PD(15)(16). At current state, PD is solely treated to alleviate associated motor symptoms; progression is not inhibited. One significant unmet need in the treatment of PD is a disease-modifying strategy. New therapeutic targets are being researched in order to achieve this(17).

Metformin (MET) is an insulin-sensitizing drug that can lower insulin resistance (IR) and is used as a first-line treatment for type 2 diabetic mellitus (T2DM)(18). Through Adenosine monophosphate protein kinase (AMPK), MET promotes fatty acid oxidation, reduces ROS generation, and preserves apoptosis and energy homeostasis(19). Beyond its metabolic actions, MET has shown significant neuroprotective properties. It enhances mitochondrial function, reduces oxidative stress, and suppresses neuroinflammation by modulating microglial activation (20). In animal models of PD, MET was found to protect dopaminergic neurons, decrease α-synuclein aggregation, and improve behavioral deficits, possibly via autophagy activation and mitochondrial reactive oxygen species clearance (21).

Several experimental studies have demonstrated MET's potent antioxidant and tissue-protective effects in various organ systems. For example, it has shown efficacy in attenuating valproic acid-induced hepatotoxicity, doxorubicin-induced cardiotoxicity, and improving metabolic outcomes in polycystic ovary syndrome (22)(23)(24). Although these studies did not specifically target the nervous system, the consistent protective profile of MET across different tissues provides indirect support for its potential neuroprotective effects in neurodegenerative diseases like PD.

Therefore, this study aimed to evaluate the potential protective effects of MET in a rotenone-induced Parkinsonism mice model, with a specific focus on its histopathological impact on dopaminergic neurons and neuroinflammatory features in the substantia nigra.

Materials and Methods

ANIMALS

Thirty male albino mice (20–30 g) were procured from the College of Pharmacy, University of Al-Farahidi. The study was conducted at the College of Pharmacy, Mustansiriyah University, over a period of 47 days. Animal handling complied with the ethical guidelines of the Pharmacy College at Mustansiriyah University. The mice were housed in groups under controlled conditions of temperature and humidity, maintained on a 12-hour light/dark cycle, with unrestricted access to standard pellet diet and water.

CHEMICALS AND DRUGS

Pure rotenone powder (Bidepharm, China) was dissolved in commercial sunflower oil. Pure MET powder was obtained from Baoji Guokang Bio-Technology Co., Ltd., China, and dissolved in distilled water. L-DOPA was purchased from Xian Sonwu Biotech Co., Ltd., China, and carbidopa was obtained from Sandoo Pharmaceuticals and Chemicals, China.

DRUG ADMINISTRATION

The mean body weight for each group was taken to determine the dose of rotenone, MET and L/DOPA/carbidopa. 10 mg of rotenone powder was dissolved in 5ml absolute ethanol. In a glass tube and mixed thoroughly by magnatic strrir until completely dissolved; then 0.5 ml from the stock solution was diluted by 4.5 ml sunflower oil to prepare working solution of concentration (1 mg /5ml). A dose of 1mg/kg (about 0.15 ml of the solution according to weight) was injected by SC injection route for each animal (25). Pure MET powder (≥98 % purity) was freshly prepared in DW at room temperature. Its high-water solubility—exceeding 300 mg/mL—ensured rapid and full dissolution (26). L-DOPA/carbidopa was prepared by dissolving 100/25 mg/kg in DW (27).

STUDY DESIGN

A- Pilot Study to Determine the Effective Dose of MET

A pilot study was conducted using fifteen male albino mice to determine the effective dose of metformin (MET). The animals were divided into five groups, each consisting of three mice. Four groups received MET orally at doses of 100, 200, and 500 mg/kg following Parkinson's disease (PD) induction using rotenone at a dose of 1 mg/kg/day, administered subcutaneously for 17 days (25).

The selection of MET doses was based on previous studies: El-Ghaiesh et al. (2020) demonstrated that 100 and 200 mg/kg/day of MET improved motor behavior and reduced oxidative and inflammatory markers in a rotenone-induced PD model in mice, while Patil et al. (2014) showed that oral administration of MET at 500 mg/kg/day provided significant neuroprotection in an MPTP-induced PD model in mice (28)(29). Lower doses (100 and 200 mg/kg/day) were included for comparison and dose optimization in the present study.

The fifth group served as a control and received the same volume of distilled water used as the vehicle for MET.

L-DOPA/carbidopa was not used in this pilot phase, as the aim was limited to assessing the effective dose of MET only.

The study lasted 47 days, including 17 days of PD induction and 30 days of MET treatment. At the end of the treatment period, SNCα was measured as it is a key PD marker, indicating neurodegeneration. Its early detection helps assess treatment effects before expanding to other biomarkers and validates the experimental protocol for the main study. When the treatment period was completed, about 1 ml of mice blood was collected in a gel tube and centrifuged to gain serum. Based on the dose-response curve, the 500 mg/kg dose

demonstrated the highest efficacy and safety, showing a greater reduction in SNCα levels compared to the 200 mg/kg and 100 mg/kg doses, as illustrated in Figure (1).

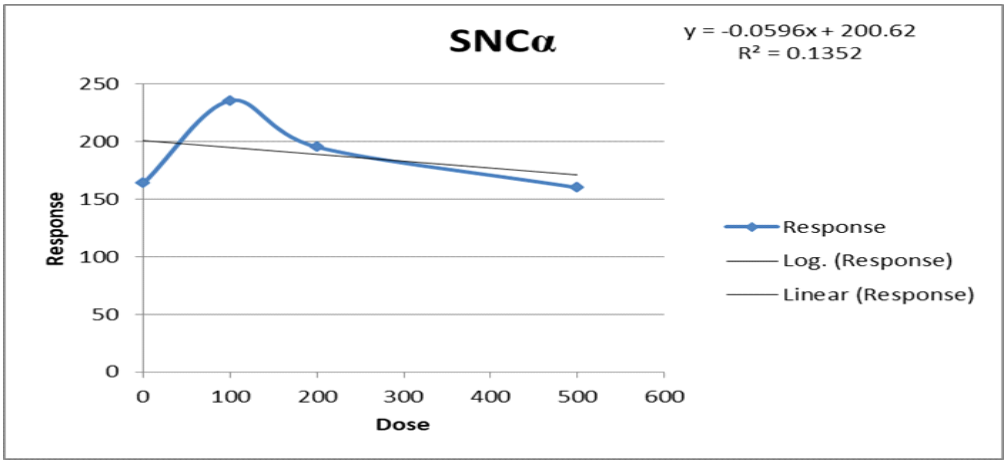


Figure (1): Preliminary study for selecting the effective MET dose for treatment.

B- Main Study

Thirty healthy male albino mice weighing 25–30 grams were randomly assigned to six experimental groups (n = 5 per group) as follows:

Group 1 (control group): Nine injections of the vehicle (sunflower oil, 4 ml/kg) subcutaneously were given to the mice every 48 hours.

Group 2 (Induction group): received nine doses of rotenone (1 mg/kg, administered every other day subcutaneously) dissolved in 99% pure ethanol and subsequently diluted in sunflower oil (25).

Group 3 (MET pre-treatment group): received oral MET once daily for three consecutive days prior to the initiation of rotenone administration, and continued to receive MET (500 mg/kg, dissolved in distilled water) throughout the 17-day period of rotenone exposure; the dose was selected based on the pilot study.

Group 4 (MET treatment group): This group received nine subcutaneous doses of rotenone (1 mg/kg, administered every other day) over a 17-day period, followed by a daily oral dose of MET (500 mg/kg, dissolved in distilled water) for 30 consecutive days. The duration of MET treatment was based on a previous study by Ryu et al. (2020), in which MET was administered orally for 4 weeks in a 6-OHDA-induced Parkinson's model and showed significant neuroprotective effects (30).

Group 5 (L-DOPA/carbidopa treatment group): received nine doses of rotenone (1 mg/kg, every other day, subcutaneously), followed by daily oral administration of L-Dopa (100 mg/kg) with Carbidopa (25 mg/kg) diluted in distilled water for 30 days (27).

Group 6 (MET/L-DOPA/carbidopa treatment group): received nine doses of rotenone (1 mg/kg, every other day, subcutaneously) followed by MET 500 mg/kg + L-Dopa (100 mg/kg). Carbidopa (25 mg/kg) administered orally every day for 30 days. The total duration of the experiment lasted 47 days.

HISTOPATHOLOGICAL STUDIES

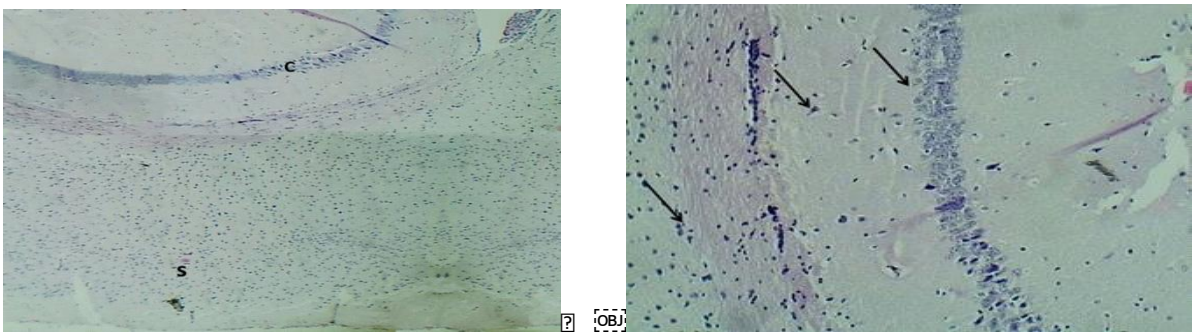
The substantia nigra was immediately dissected post-mortem from each animal. The tissues were fixed in 10% neutral-buffered formalin for at least 72 hours. After rinsing with tap water, the specimens were dehydrated, cleared with xylene, and embedded in paraffin. Histological analysis was conducted by sectioning the paraffin blocks and staining the tissue sections with hematoxylin and eosin (H&E)(31)(32).

Results

Metformin treatment results on histopathological changes displayed in the figures below.

Control group

The histopathological figures of the cerebral cortex showed normal appearance of cerebral cells layers, also the basal ganglia showed normal parts with normal neurons and glial cells (Figure2 A and B).

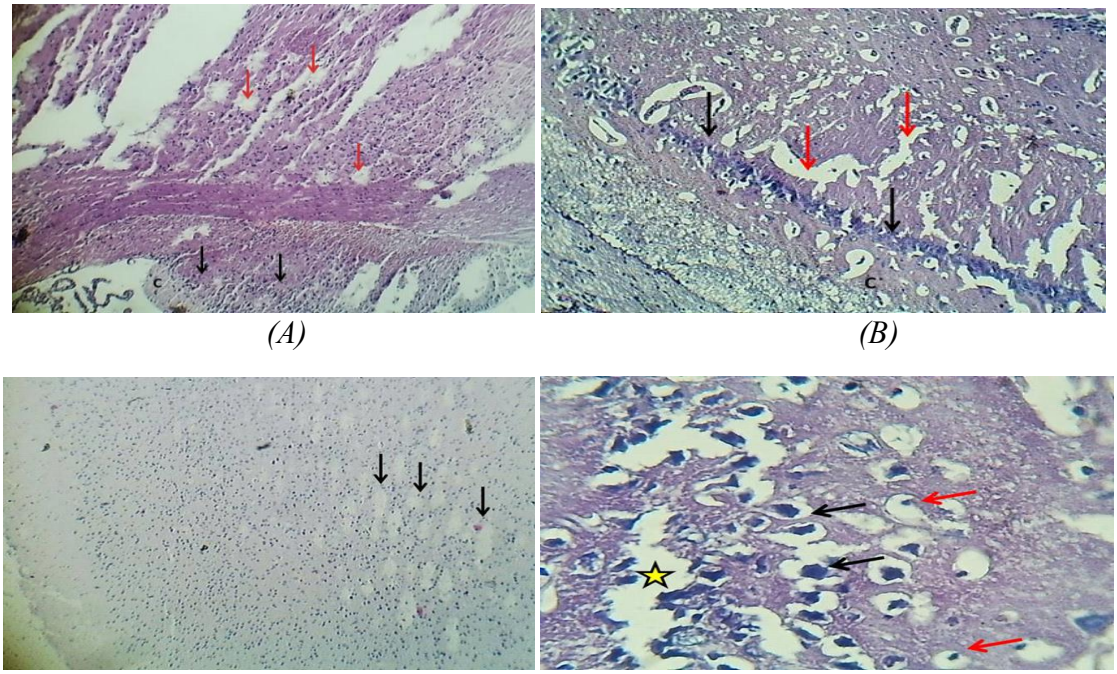


(A) *(B)*

Figure 2: (A) section of basal ganglia (control) shows normal neurons of caudate nucleus C and cerebral cortex layers S.H&E.40x. (B) section of basal ganglia (control) shows normal neurons of caudate nucleus, cerebral cortex layers (Black arrows) & glial cells mH&E.40x.

Induction group

The histopathological figures of the substantia nigra revealed multiple vaculated foci associated with necrosis of glial cells and tissue depletion (Figure3 A), other figures revealed severe degeneration (vacular changes) with severe degeneration and nuclear pyknosis of neurons (Figure.3 B & C). The cerebral cortex showed normal appearance of external granular, external pyramidal and internal granular layer cells while the multiform layer cells showed multiple focal vacular area that associated with necrosis and atrophy of glial cells (Figure.3 D).



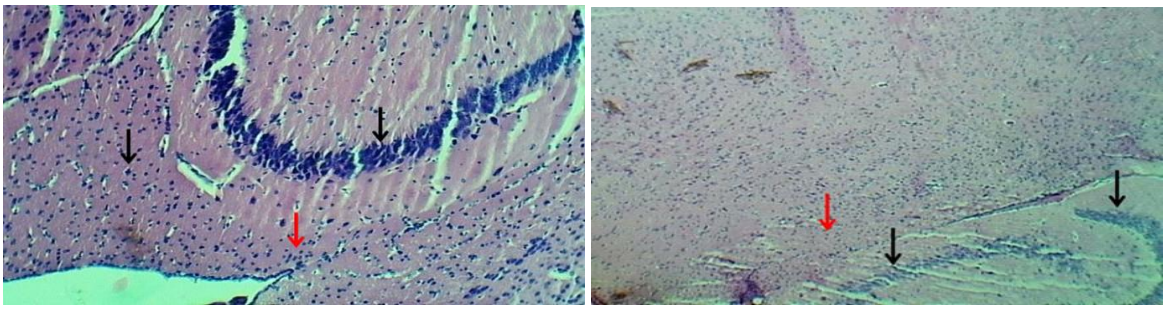
(A) *(B)*

© *(D)*

Figure3: (A): section of substantia nigra (Induction) shows multiple vaculated foci associated with tissue depletion of glial cells (Red arrows) with severe degeneration with nuclear pyknosis of neurons (Black arrows).H&E.40x. (B): section of substantia nigra shows sever vaculation within glial cells of substantial nigra region (Red arrows), with nuclear atrophy and pyknosis of neurons (Black arrows)E.100x. (C): section of substantia nigra shows numerous vaculated neurons (Black arrows) and glial cells (Red arrows) with tissue depletion (Asterisk).H&E.400x. D: section of cerebral cortex shows normal appearance of external granular, external pyramidal and internal granular layer cells while the multiform layer cells showed multiple focal vacular area (arrows) H&E.100x.

Pre-treatment group

The histopathological figures of the basal ganglia of all subgroups revealed normal histological features of neurons and glial cells. within substantial nigra regions, and normal histological features of neurons and glial of layers of cerebral cortex (Figure 4 A and B).



(A) ***(B)***

Figure 4: (A): Section of substantia nigari shows normal histological features of neurons and glial cells. within substantial nigra regions.H&E.100x. (B) section of substantia nigra shows normal appearance of neurons (Black arrow) and glial cells (Red arrow) within substantial nigra region .H&E.100x

MET group

The histopathological figures of the basal ganglia of all subgroups revealed normal histological features of neurons and glial cells within substantial nigari regions, and normal appearance of neurons and glial of layers of cerebral cortex (Figure5).

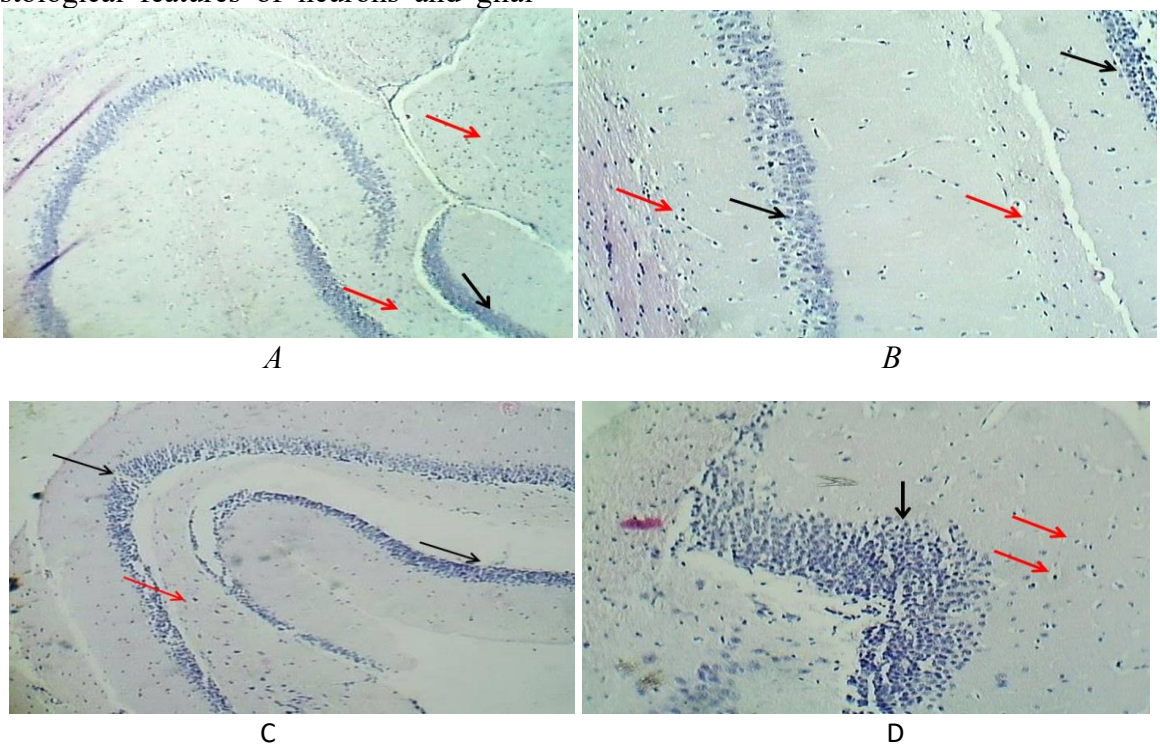
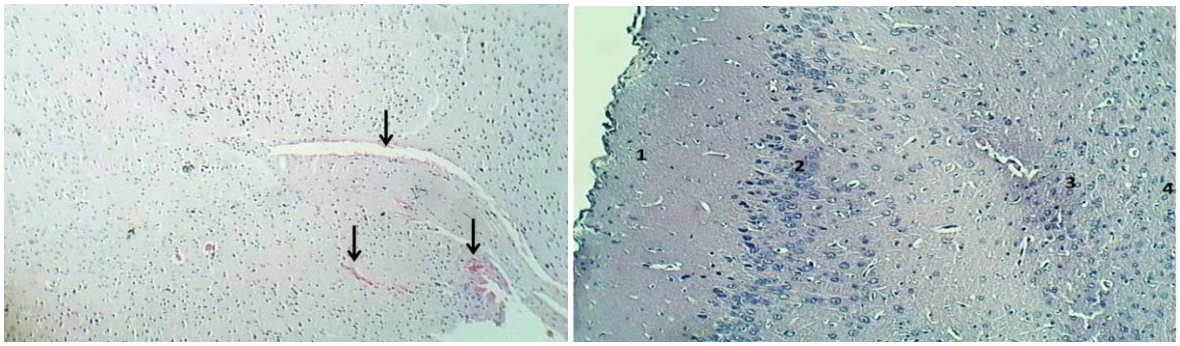


Figure 5: (A) Section of substantia nigra shows normal appearance of neurons (Black arrows) and supporting tissue with glial cells (red arrows) within substantial nigra regions.H&E.40x. (B) Section of substantia nigra shows normal appearance of neurons (Black arrows) and supporting glial cells (Red arrows) within substantial nigari regions.H&E.400x. (C) Section of substantia nigra shows normal appearance of neurons (Black arrows) and supporting glial cells (Red arrow) within substantial nigari regions.H&E.40x. (D) Section of substantia nigra shows normal appearance of neurons (Black arrow) and supporting glial cells (Red arrows) within substantial nigari regions.H&E.400x

L-DOPA group

The histopathological figures of the basal ganglia regions revealed normal appearance of neurons and glial cells within substantial nigari region, with mild multiple focal hemorrhages and normal appearance of layers of cerebral cortex Figure 6 A and B

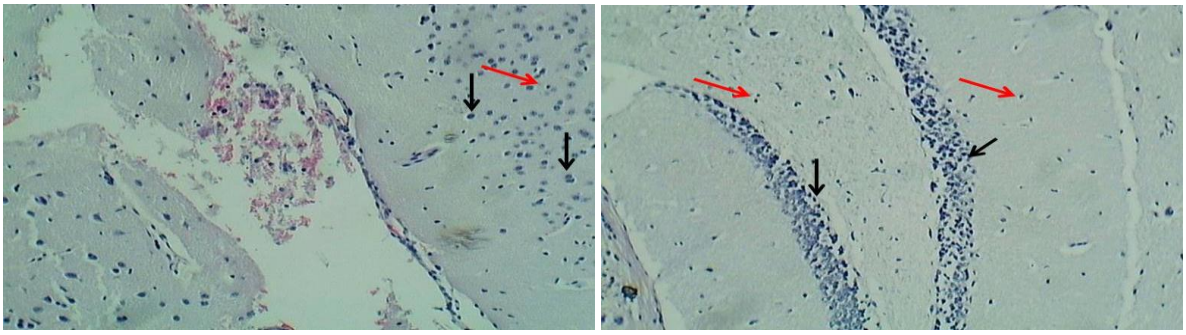


(A) *(B)*

Figure 6: (A) section of basal ganglia shows normal appearance of neurons and glial cells with mild hemorrhage changes (arrows).H&E.40x. (B) section of the cerebral cortex from the Levodopa group displays normal architecture of the cortical layers, including the molecular layer (1), external granular layer (2), external pyramidal layer (3), and internal granular layer. (4). H&E.100x

MET/L-DOPA/Carbidopa group

The histopathological figures of the basal ganglia revealed normal histological features of neurons and glial cells. within substantial nigari regions, and normal appearance of neurons and glial of layers of cerebral cortex (Figure7)



A *B*

Figure 7: (A) Section of substantia nigra shows normal appearance of neurons (Black arrows) and supporting glial cells (Red arrow) with little focal hemorrhage H&E.400x. (B) Section of substantia nigra shows normal appearance of neurons (Black arrows) and supporting glial cells (Red arrows) within substantial nigari regions.H&E.400x.

Discussion

PD is driven by a dysregulation of dopamine and catecholamines in the nigrostriatal pathway, ultimately resulting in neuronal death (33). Aggregated α-synuclein deposition is linked to PD, a progressive neurodegenerative illness (34). Presently available treatments are only able to alleviate symptoms; they are unable to stop, slow, or prevent the neurodegenerative process of PD (35). Rotenone liquified in sunflower oil was injected subcutaneously into male albino mice in this investigation to create an experimental PD model (36). Our results showed that rotenone resulted in areas of substantial Neuronal loss observed within a markedly vacuolated neuropil. and a large number of dark pyknotic neurons near infiltrating glial cells this agree with a previous study (37). Treatment with MET and L-dopa/Carbidopa helped to mitigate

these alterations, and treatment with MET/L-dopa/Carbidopa resulted in a significant increase in the amount and size of pigmented neurons.

Although the substantia nigra is the primary site of Parkinsonism pathology, the cerebral cortex is also significantly impacted. When rotenone was administered, cytoplasmic vacuolations and focally apoptotic neurons were seen (38). Treatment with MET and L-dopa/Carbidopa helped to mitigate these alterations, and treatment with MET/L-dopa/Carbidopa resulted in a significant increase in the amount and size of pigmented neurons.

When compared to the bare L-Dopa and MET therapy groups, the pretreatment with MET and combined treatment group had the strongest neuroprotective effect against rotenone toxicity, as evidenced by the reversal of pathological alterations and structural repair. The results agree with a recent study which suggests repurposing MET as a neuroprotective medication since MET has alleviated the neuronal loss caused by lipopolysaccharide (LPS) exposure in all areas of the frontal cortex and hippocampal regions (39). Current researches has demonstrated that MET promotes the neurogenesis, proliferation, self-renewal, and differentiation of adult brain precursor cells by activating 5′ adenosine monophosphate-activated protein kinase (AMPK)(40)(41). Moreover, several recent studies confirm its neuroprotective effects in PD models. El-Ghaiesh et al. reported that MET protects against rotenone-induced nigrostriatal neuronal death through AMPK-FOXO3 signaling and inhibition of angiogenesis in mice (28). Mendonça et al. found that MET enhances autophagy in the substantia nigra and hippocampus, improving motor and neuropsychiatric deficits in rotenone-treated animals (42). A 2024 study also showed that MET delays astrocyte senescence, reducing neuroinflammation and neuronal loss in MPTP-injected mice (20). Finally, a review of neurological disorders highlighted MET's potential in enhancing mitochondrial biogenesis, autophagy, and neurotrophic support—further encouraging its repurposing as a neuroprotective agent in PD (43).

This study has several limitations that should be acknowledged to provide a comprehensive interpretation of the findings. Firstly, the use of a rotenone-induced PD model in mice, while well-established, may not fully replicate the complexity of the human condition, limiting the generalizability of the results. Secondly, the study duration was relatively short (30 days), which may not adequately reflect the long-term effects of MET treatment or the chronic progression of PD. Thirdly, it is suggested to consider using a reduced dose of MET (e.g., 250 mg/kg) when co-administered with L-DOPA/carbidopa, as this lower dose might be sufficient to induce histological improvements without the need for the full dose.

Future work should evaluate the effect of lower MET doses when combined with L-DOPA/carbidopa and consider longer treatment durations. It is also recommended to include histological examination of other brain regions affected in PD, such as the striatum, to better understand the impact of treatment on the nigrostriatal pathway.

Conclusion:

The current study's histological observations showed that the MET treatment alleviated the neuronal loss brought on by the rotenone exposure. The most effective of the three MET dosages was 500 mg/kg body weight. The study's findings support the repurposing of MET as a neuroprotective agent in neuroinflammatory conditions, and may offer promising directions for neurodegenerative disease treatment. According to these results, MET demonstrated additive neuroprotective effects to L-dopa and ameliorated rotenone-induced neurotoxicity and motor impairments in mice.

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