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Original Article

Assessing Caffeine's Neuroprotective Effects on Rotenone-Induced Parkinson's Disease: Unravelling the Neurological Dynamics of Striatal Impact

Timileyin A. Adeyeye[1], Bamidele R. Babatunde[1], Oluwadamilola F. Shallie[1], Babatunde O. Osidele[1], Philemon D. Shallie[1]

[1]Department of Anatomy, Olabisi Onabanjo University, Ogun State, Nigeria

ABSTRACT

The alarming prevalence of Parkinson's disease (PD) has prompted a search for standard therapeutic interventions capable of altering or halting its progression. Identifying a neuroprotective or disease-modifying therapy for PD is akin to pursuing the Holy Grail. Numerous studies have affirmed the therapeutic benefits of adenosine A_{2A} receptor blockade in alleviating both motor and non-motor symptoms in PD. Furthermore, A_{2A} receptors are selectively localized in the basal ganglia. This study aimed to evaluate the impact of caffeine on the striatum of rats with PD induced by rotenone. Fifty adult male Wistar rats, weighing between 150 and 200g, were utilised in this study. The rats were randomly assigned to five groups, each consisting of ten rats: Group A (vehicle group, alcohol, 3mL/kg), group B (rotenone-only treated with 3mg/kg, ip.), group C (caffeine 30mg/kg + rotenone 3mg/kg, ip.), group D (rotenone 3mg/kg + caffeine 30mg/kg, ip.), and group E (caffeine-only treated with 30mg/kg, ip.). The findings revealed a significant ($p<0.05$) reduction in body weight and relative brain weight in response to caffeine treatments. Rotenone administration induced histological changes akin to those observed in PD, encompassing neuronal structural derangement, degenerated striatal bundled fibre, and loss of myelinated neurons and Nissl substance. This study adds to the growing body of research supporting the therapeutic potential of caffeine in PD. The results highlight caffeine's neuroprotective properties and its ability to mitigate striatal lesions. The pursuit of effective interventions for PD remains crucial, and caffeine emerges as a promising therapeutic agent in this regard.

Keywords
Caffeine, Parkinson's disease, Rotenone, Striatum

Correspondence: Philemon D. Shallie, PhD; Department of Anatomy, Olabisi Onabanjo University, P.M.B. 704, Ago-Iwoye, Nigeria. E-mail: shallie.philemon@oouagoiwoye.edu.ng; Phone Number: +2347033971642; ORCID: 0000-0001-9910-3584

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INTRODUCTION

Parkinson's disease (PD) is a complex neurological disorder characterised by motor and non-motor symptoms (Kanda and Jenner, 2020; LeWitt and Chaudhuri, 2020; LeWitt and Jenner, 2020). In PD, motor deficits result from an imbalance in the basal ganglia loop to the cortex, particularly involving the striato-nigral pathway, the direct pathway regulated by dopamine D1 receptors, and the striato-pallidal (indirect) pathway, functioning antagonistically (Schröter and Jost, 2022). The decline of nigral dopaminergic neurons in PD leads to dopamine loss, causing an imbalance with lower excitatory levels in the direct pathway and increased hyperactivity of gamma amino butyric acid (GABAergic) medium spiny neurons, exacerbating inhibitory activity in the indirect pathway (Svenningsson *et al*., 1997; Obeso *et al*., 2000; Rosin *et al*., 2003; Mishina *et al*., 2007; Obeso *et al*., 2008). This imbalance results in cardinal motor symptoms such as resting tremor, rigidity, bradykinesia, and postural instability (LeWitt and Chaudhuri, 2020; LeWitt and Jenner, 2020).

Currently, orthodox symptomatic therapy, relying on medication that either increases dopamine agonists or inhibits

depletion, remains the mainstay of PD therapy (Schröter and Jost, 2022). However, a challenge arises as higher doses of medication are required over time, leading to limitations in daily life due to intermittent on-and-off phases with dyskinesia, alternating between symptom aggravation and adequate motility (Waggan *et al*., 2021; Schröter and Jost, 2022).

Adenosine A_{2A} receptors play a pivotal role in modulating neuronal activity and neurotransmitter release within this striato-pallidal pathway. Studies have placed particular emphasis on understanding the implications of these receptors in the context of PD. PD is characterised by the degeneration of dopaminergic neurons, affecting the balance of neurotransmitters within the basal ganglia. Levodopa, a standard therapeutic agent for PD, aims to replenish dopamine levels. However, long-term use of levodopa is associated with the development of dyskinesia, an abnormal involuntary movement that can complicate the management of PD (Calon *et al*. 2004; Vuorimaa *et al*., 2017). Activation of A_2A receptors enhances the indirect pathway, contributing to motor symptoms in PD, while their antagonism shows promise in improving motor performance in experimental models (Chase *et al*., 2003; Uchida *et al*., 2015; Mori *et al*., 2022).

Additionally, A_{2A} receptor knockout prevents the loss of dopaminergic neurons induced by PD-associated toxic agents, supporting the neuroprotective potential of A_{2A} receptor antagonists (Kachroo and Schwarzschild, 2012). This neuroprotective effect is further evidenced by studies demonstrating protection against degeneration induced by toxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 6-hydodopamine (6-OHDA), rotenone, and intracerebral injection of toxic α-synuclein particles (Fathalla *et al*., 2017; Souza *et al*., 2017; Luan *et al*., 2018).

Interestingly, epidemiological and experimental investigations have suggested that caffeine, a widely consumed psychoactive substance, may offer both neuroprotective and motor and cognitive benefits in PD (Landais *et al*., 2018; Reyes and Cornelis, 2018; Corneli 2019). Caffeine's complex pharmacological profiles, including its neuroprotective effects, are largely mediated by the adenosine A_{2A} receptor (Ikram *et al*., 2020). Recent research from our laboratory further supports the neuroprotective and therapeutic effects of caffeine in mitigating oxidative stress (Adeyeye *et al*., 2023). Despite the established link between A_{2A} receptors and PD, studies investigating the antagonistic effect of caffeine on the striatum are limited. Given this background, our study investigated caffeine's neuroprotective and therapeutic effects on the striatum in a rat PD model induced by rotenone.

MATERIALS AND METHODS

The study utilised fifty adult male Wistar rats weighing between 150 and 200 g, procured from the animal house of the Faculty of Basic Medical Sciences at Olabisi Onabanjo University. The rats were accommodated in clean, white plastic cages within a well-ventilated environment, maintaining temperatures between 24 and 28 ℃ under a 12-hour light and 12-hour dark cycle. They were provided with standard, non-pelletized rat feed and had access to water *ad libitum*. A two-week acclimatisation period preceded the initiation of experimental procedures.

Rotenone (Cat. No.: HY-B1756) and caffeine (Cat. No.: HY-103164) were procured from MedChen Express. Ethical approval for the study was obtained from the institutional Committee on Animal Care and Use in Research, Education, and Testing (ACURET) unit of Olabisi Onabanjo University, Ago-Iwoye, with the ethical code OOU/DREC/20/001. The animal experiments adhered to the guidelines outlined in the NIH Guide on Laboratory Animals for Biomedical Research (NIH, 1978) and ethical principles for investigating experimental pain in conscious animals (Zimmermann, 1983).

Experimental Design

Ten male Wistar rats were randomly assigned to one of five groups: Control group rats received an equal volume of the vehicle (alcohol, 3 mL/kg) and were provided with standard non-pelletized rat feed and *ad libitum* access to clean water; Rotenone-only treated group rats received 3 mg/kg/ day of rotenone per body weight ip. for 30 days; preventive group (Caffeine+Rotenone, Caff+Rot) rats received 30 mg/kg/day of caffeine per body weight ip. for 30 days, followed by 3 mg/kg/day of rotenone ip.; curative group (Rotenone+Caffeine, Rot+Caff) rats received 3 mg/kg/day of rotenone per body weight ip. for 30 days, followed by 30 mg/kg/day of caffeine ip.; Caffeine-only treated group rats were given 30 mg/kg/day of caffeine per body weight ip. for 30 days.

Neurobehavioural Tests

After the induction period, neurobehavioral tests were conducted, including:

Open Field Test

The open field test assessed locomotion, exploration, and anxiety. Parameters measured included line crosses and rearing frequency (Sturman *et al*., 2018). The Open Field Test provides simultaneous locomotion, exploration, and anxiety measures. The number of line crosses and the frequency of rearing are usually used as measures of locomotor activity exploration and anxiety. A high frequency of these behaviours indicates increased locomotion and exploration and a lower level of anxiety, while a high level of these behaviours depicts decreased locomotion and exploration and a higher level of anxiety (Tatem *et al*. 2014; Sturman *et al*. 2018).

Static Rod Test

The static rod test evaluated motor coordination capabilities. Rods of varying diameters were used to refine the test. Subjects were assessed on two rods of different diameters (22 and 28 mm). The orientation and transit times were recorded, with a maximum score of 120 sec. allocated for each (Deacon, 2013). Orientation time is the time taken to orientate 180º from the starting position toward

the shelf, while the transit time is the time taken to travel to the shelf end (nose beyond the 10 cm mark from the shelf end of the rod). An animal that is able to orientate in a short time after being placed on the rod signifies good motor coordination, and an animal that takes a longer time before orientation and transiting the rod signifies bad motor coordination. A successful transit is recorded when the subject travels the rod in an upright position as it indicates good motor coordination (Breuss *et al*., 2017). A subject that traverses the rod in an upright position has better motor coordination than a subject that orients upside-down on the rod (Horiuchi *et al*., 2017).

Tissue Sample Preparation
At the study's conclusion, the Wistar rats were euthanized with chloroform. The whole brain was extracted, and coronal sections of the brain in the region of the striatum were fixed in 10% phosphate-buffered formalin (PBF) and processed for histological analysis.

Haematoxylin and Eosin Routine Staining
Tissue sections underwent routine staining with haematoxylin and eosin following standard procedures (Ortiz-Hidalgo and Pina-Oviedo, 2019).

Bielschowsky's Silver Staining Protocol
Sections were stained with Bielschowsky's silver stain following established protocols (Nissl, 2014).

Luxol Fast Blue Counter Staining with Nissl Stain
Tissue sections were stained using Luxol-fast blue and counterstained with Nissl stain according to Mitroi *et al*. (2022).

Photomicrography
Photomicrographs were obtained using an Omax LED digital microscope. At ×400 magnifications, the histoarchitectural features of the striatum were assessed.

Statistical Analysis
The data underwent analysis of variance (ANOVA) using GraphPad (5.1) (Geoff *et al*., 2007), and the results are presented as mean ± SEM. Statistical significance was defined as a p-value < 0.05.

RESULTS

Neurobehavioural Results
Caffeine Ameliorates Motor Deficit Associated with Parkinson's Disease
The mean line crossing and rearing across the groups revealed a significant interaction ($F = 13.22$; $p < 0.0001$). There was a significant reduction ($p < 0.0001$) in locomotive activity, as demonstrated by fewer line crossings and rearing behaviours, observed in the rotenone treatment group. Conversely, there was a significant increase ($p < 0.0001$, $p < 0.001$, and $p < 0.0001$) in locomotive activity observed in the Caff+Rot, Rot+Caff, and Caffeine-only groups, respectively, when compared to the Rotenone-only group (Fig. 1).

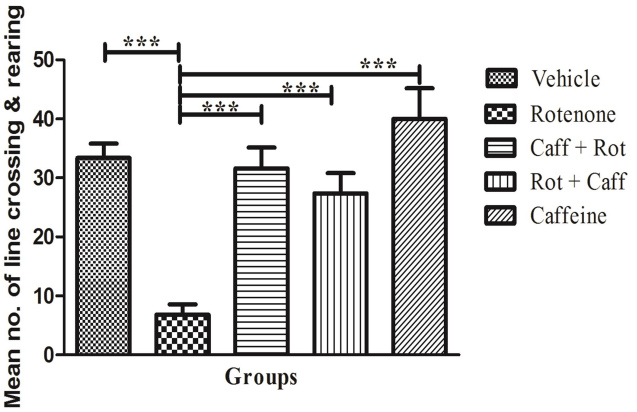

Fig. 1: The mean line crossing and rearing for the experimental groups. The results of the one-way ANOVA revealed a significant interaction ($F = 13.22$; $p < 0.0001$) in the mean ± SE line crossing and rearing across the groups.

There was a significant interaction between the orientation times on 22 mm and 28 mm ($p<0.0001$) rods, as well as a significant effect of treatment with caffeine ($p<0.0001$). Bonferroni post-hoc testing showed a significant decrease ($p<0.0001$) in motor coordination in the Rot+Caff group on both the 22 mm and 28 mm rods compared to the control. Conversely, there was a significant increase in motor coordination of orientation time ($p < 0.0001$, $p < 0.001$, and $p < 0.0001$) in the Caff+Rot, Rot+Caff, and Caffeine groups, respectively, on both the 22 mm and 28 mm rods, when compared to the Rotenone-only treatment group, illustrating the ameliorative effect of caffeine (Fig. 2).

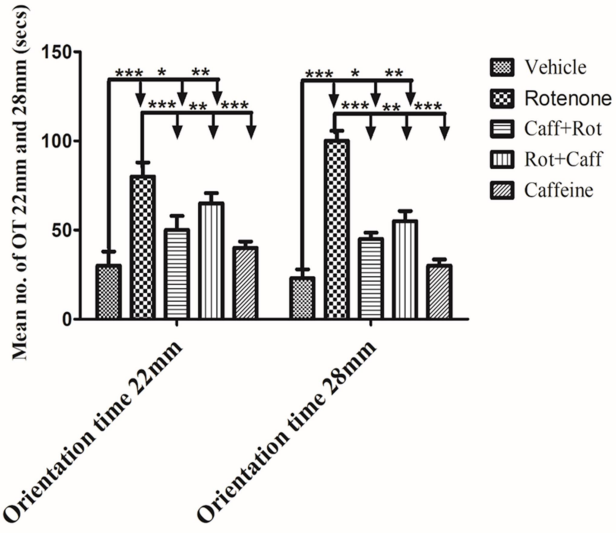

Fig. 2: The mean orientation time for the experimental groups using 22 mm and 28 mm rods. The results of the two-way ANOVA indicated a significant interaction ($F = 4.84$, $p < 0.0001$), a significant main effect of the orientation time of 22mm and 28mm ($F = 0.22$, $p < 0.001$), and a significant effect of treatment ($F = 74.10$, $p < 0.0001$).

Likewise, there was a significant interaction ($p<0.0001$) between the transit time on 22 mm and 28 mm rods, alongside a significant effect of caffeine ($p<0.0001$). Bonferroni post-hoc analysis demonstrated a significant decrease ($p < 0.0001$) in motor coordination in the Rotenone treatment group and the Rot+Caff groups on both the 22 mm and 28 mm rods. Conversely, there was a significant increase in motor coordination ($p < 0.0001$, $p < 0.001$, and $p < 0.0001$) in the Caff+Rot, Rot+Caff, and Caffeine groups, respectively, on both the 22 mm and 28 mm rods, when compared to the Rotenone-only treatment group, highlighting the ameliorative effect of caffeine (Fig. 3).

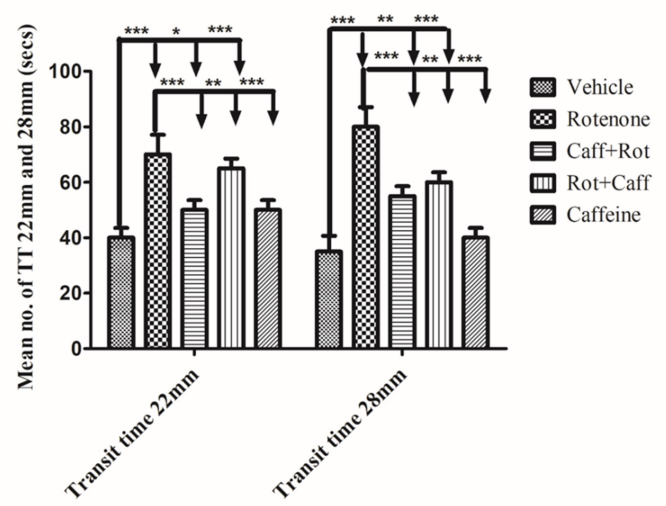

Fig. 3: The average transit time for the experimental groups using 22 mm and 28 mm rods. The results of the two-way ANOVA showed a significant interaction ($F = 4.91$, $p < 0.0001$), a significant main effect of the transit time of 22mm and 28mm ($F = 0.11$, $p < 0.01$), and a significant effect of treatment ($F = 63.03$, $p < 0.0001$).

Caffeine Confers Protection against PD-induced Striatal Lesions

The results of the histological analysis using H&E staining of the striatum are as follows: (a) The striatum of the vehicle group exhibits normal striatal bundles (light blue circle) and well-defined neuronal nuclei (black arrow). (b) In contrast, the striatum of the PD rat group displays shrunken striatal bundles (green arrow), reduced fibre density, increased nuclei density (white arrow), and eosinophilia (yellow double arrow). (c) Following post-treatment with caffeine in PD rats, there is some preservation of the striatum observed. (d) Pre-treatment with caffeine in PD rats shows disorientation of the striatal fibres. (e) Lastly, the striatum of rats exposed solely to caffeine shows preserved striatal bundles (Fig. 4).

The results from the silver stain of the striatum are detailed as follows: (a) The striatum of the vehicle group exhibits normal, well-organized striatal fibre bundles (red arrow). (b) However, in the PD rat group, the striatum appears distorted with significant degeneration of striatal fibre bundles, as evidenced by vacuolation (white arrow). (c) Pre-treatment with caffeine in PD rats demonstrates some preservation of striatal fibre bundles. (d) Post-treatment with caffeine in PD rats also shows some degree of preserved striatal fibre bundles. (e) Furthermore, the striatum of rats exposed solely to caffeine exhibits a limited number of striatal fibre bundles (Fig. 5).

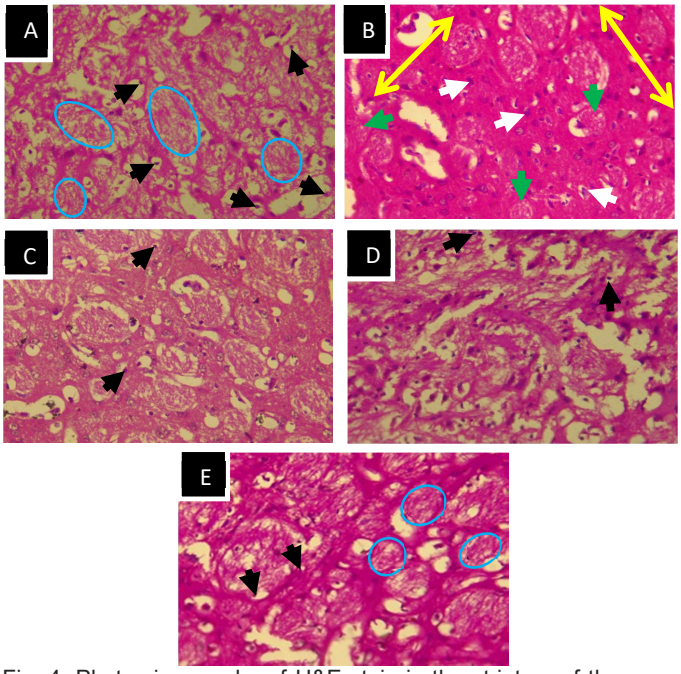

Fig. 4: Photomicrographs of H&E stain in the striatum of the experimental groups at ×400 magnification.

Lastly, the findings from the Luxol-fast stain reveal the following: (a) The striatum of the vehicle group is deeply stained, indicating the presence of normal, myelinated neuronal fibres (white circle). (b) In contrast, the striatum of the PD rat group displays relatively less densely stained fibres (red circle), indicative of demyelination, along with numerous vacuolations (white arrow). (c) Pre-treatment with caffeine in PD rats shows some degree of preservation of myelin. (d) Post-treatment with caffeine in PD rats displays a decrease in the intensity of staining, along with vacuolations. (e) Lastly, the striatum of rats exposed solely to caffeine demonstrates a certain degree of myelin preservation (Fig. 6).

These histological findings provide insights into the effects of PD, caffeine treatment, and their interactions on the morphology and integrity of the striatum in the rat model.

DISCUSSION

In our investigation, we delved into the intricate dynamics of the striatum, a pivotal brain region with chief functions attributed to the direct and indirect pathways. These pathways finely regulate motor activity, either amplifying or dampening it. The relevance of this system in the context of Parkinson's disease (PD) has been extensively documented, with a focus on the progressive damage to dopa-

minergic neurons in the substantia nigra pars compacta projecting to the striatum, a hallmark characteristic of PD. This damage triggers functional modifications within the basal ganglia circuitry, leading to the manifestation of motor deficits observed in PD (Mishina *et al*., 2007; Obeso *et al*., 2008; Mori *et al*., 2022; Schröter and Jost, 2022).

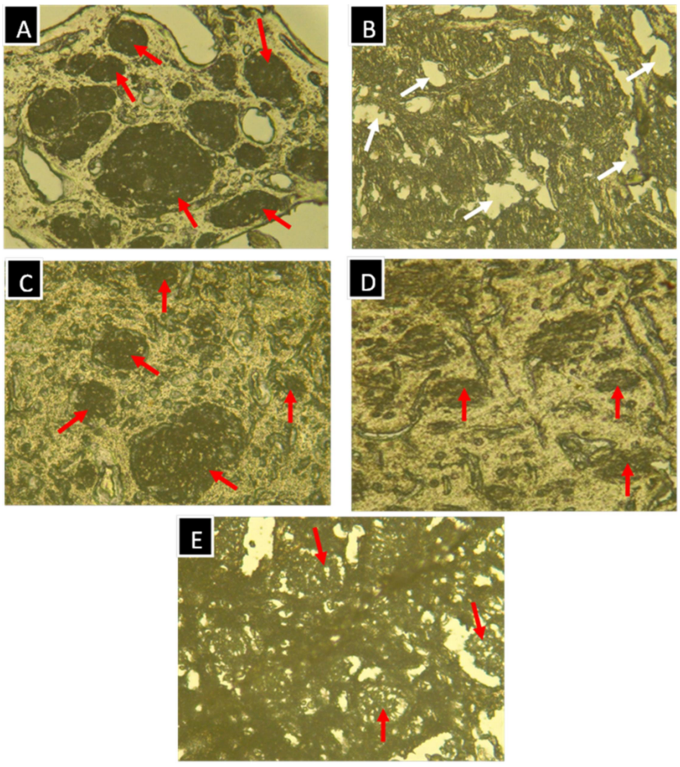

Fig. 5: Photomicrographs of Bielschowsky's Silver Stain in the striatum of the experimental groups at ×400 magnification.

Our study meticulously employed various motor neurobehavioral tests to unravel the impact of rotenone, a common PD-inducing model in rats. The unequivocal results exposed severe motor deficits in PD rats following rotenone administration, marked by a significant reduction in line crossing and rearing behaviour in the open field test. Intriguingly, our findings took an optimistic turn when both pretreated and post-treated PD rats with caffeine exhibited a substantial increase in these motor activities. This hints at caffeine's potential as a neuroprotective agent, countering the detrimental effects of rotenone.

Expanding our exploration to motor coordination, we scrutinized the effects of rotenone on orientation and transit times in a static rod test. This meticulous test, designed to gauge motor coordination, illuminated a significant increase in orientation time on both 22 and 28 mm rods, indicative of poor motor coordination. Additionally, an augmented transit time further signalled motor deficits in rotenone-treated PD rats. The static rod test, with its parameters of orientation time and transit time, provided valuable insights into the motor deficits, unveiling prolonged times for rats to orientate and transit the rod.

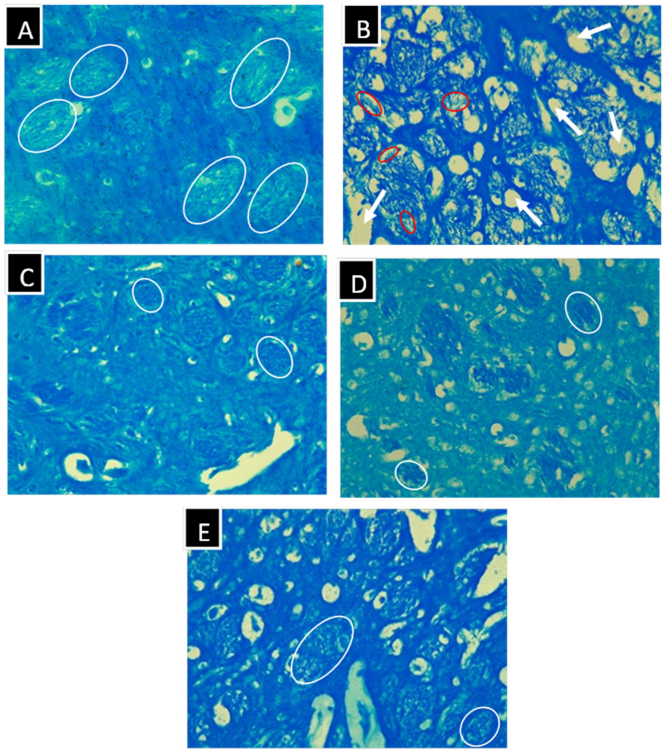

Fig. 6: Photomicrographs of Luxol-fast blue counterstained with Nissl stain in the striatum of the experimental groups at ×400 magnifications

Additionally, we assessed several morphological indices, including histoarchitecture, fibres, and myelin sheath. Our findings revealed contracted and degenerated striatal fibres, heightened nuclei density, and eosinophilia in the Parkinson's disease (PD) group. Interestingly, the ingestion of caffeine demonstrated varying degrees of protection, preservation, and restoration of the observed lesions in the PD group.

Reduced striatal fibres and increased nuclei in the striatum can stem from various factors, with one prominent cause being Parkinson's disease, a progressive neurodegenerative disorder characterised by the selective loss of dopamine in the striatum. A previous research identified a continuous decline in specific striatal uptake of 99 mTc-TRODAT-1 with advancing disease severity in PD patients (Huang *et al*., 2001). Brain-derived neurotrophic factor, a member of the neurotrophin family, plays a crucial role in promoting the survival and proper function of striatal neurons (Baydyuk and Xu, 2014).

Injuries to the striatum prompt the migration of neuroblasts from the subventricular zone to the striatum, where they undergo differentiation into adult neurons (Kernie and Parent, 2010). Our results also indicated increased eosinophilia, commonly associated with inflammation. Neuroinflammation, denoting inflammation of the nervous system, can manifest as a response to various triggers such as infection, traumatic brain injury, toxic metabolites, or autoimmunity. This process is characterised by the activation of the brain's resident immune cells, microglia, and the infil-

tration of peripheral immune cells, including eosinophils, into the brain and spinal cord.

The literature corroborates our findings, with several studies reporting that rotenone induces motor deficits in PD rats through neurochemical disruptions in the nigrostriatal dopamine pathway. This disruption leads to hyperactivity of GABAergic medium spiny neurons (MSN) in the indirect pathway, upsetting the balance in striatal output from both direct and indirect pathways projecting to the cortex motor neurons. This cascade of events contributes to a functional imbalance, manifesting as a 'No-Go' signal from the globus pallidus externus to the thalamus. This ultimately results in a reduction in activity loops supporting movement (Go) and an increase in loops inhibiting movement (No-Go). This functional imbalance aligns with our study's outcomes, resulting in poor orientation and transit times and subsequent poor motor coordination (Svenningsson *et al.*, 1997; Rosin *et al*., 2003; Mori *et al*., 2022).

The therapeutic spotlight on A_{2A} receptors in the striatum emerges as a pivotal aspect. A_{2A} receptor activation reinforces the 'No-Go' signal by increasing the excitability of the indirect path and reducing D2 activity. Multiple studies substantiate the efficacy of A_{2A} receptor antagonists, including caffeine, as a promising therapeutic window in PD. These antagonists confer motor benefits by protecting against the degeneration of dopaminergic neurons induced by toxins like MPTP, 6-OHDA, and rotenone, along with the intracerebral injection of toxic α-synuclein particles (Chase *et al*., 2003; Kachroo and Schwarzschild, 2012; Uchida *et al*., 2014; Uchida *et al*., 2015).

Moreover, our study aligns with existing research, establishing that rotenone exposure reproduces key PD features in rats, inducing selective dopaminergic neurotoxicity through complex inhibition and triggering oxidative stress and subsequent damage. The micro-anatomical morphological observations in our study mirrored the neurotoxic effects of rotenone, including degeneration of striatal fibre bundles, demyelination, densely packed neuronal fibres, degenerated neurons, and vacuolated cytoplasm. Encouragingly, caffeine, acting as an A_{2A} receptor antagonist, demonstrated a capacity to mitigate these alterations, consistent with the microanatomical changes observed in our study (Fathalla *et al*., 2017; Souza *et al.*, 2017; Luan *et al*., 2018).

Epidemiological and animal studies underscored the broader neuroprotective effects of caffeine, extending beyond dopaminergic neurodegeneration to include cognitive benefits. Genetic knockout mouse studies highlighted that caffeine's action is predominantly mediated by the brain adenosine A_{2A} receptor, offering neuroprotection through the modulation of oxidative stress. Caffeine's antioxidant properties, as reported by Khadrawy *et al*. (2017), were reinforced in our study, where daily intraperitoneal administration of caffeine in PD rats protected against the adverse effects of rotenone (Adeyeye *et al*., 2023).

Conclusion

In conclusion, our present investigation reinforces the neuroprotective and therapeutic actions of caffeine on PD, acting through antagonistic mechanisms on A_{2A} receptors in the striatum. The antioxidant effects of caffeine, particularly in mitigating elevated oxidative stress, position it as a promising complementary approach in the prevention and treatment of devastating neurodegenerative conditions such as PD. While our findings, along with those of existing studies, indicate the strong antioxidant and neuroprotective effects of caffeine, further research is imperative to fully explore its efficacy as a complementary therapeutic strategy in the management of neurodegenerative diseases like PD.

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No funding was received for this research.

Conflict of Interest

None declared.

Authors' Contribution

TAA: Data curation, Formal analysis, Investigation, Software, Validation, Visualization, Writing – original draft. BRB: Formal analysis, Funding acquisition, Investigation, Methodology, Validation. BOO: Formal analysis, Investigation, Methodology. OFS: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing - review & editing. PDS: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing - review & editing.

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