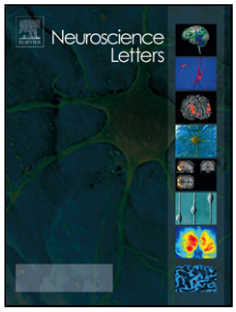


Title: Dose-Dependent Neuroprotective Effect of Caffeineon a Rotenone-Induced Rat Model of Parkinsonism: A histological study

Author: Amira M. Soliman Ahmed M. Fathalla Ahmed A. Moustafa

PII: S0304-3940(16)30279-8
DOI: http://dx.doi.org/doi:10.1016/j.neulet.2016.04.057
Reference: NSL 32007

To appear in: *Neuroscience Letters*

Received date: 19-2-2016
Revised date: 24-4-2016
Accepted date: 26-4-2016

Please cite this article as: Amira M.Soliman, Ahmed M.Fathalla, Ahmed A.Moustafa, Dose-Dependent Neuroprotective Effect of Caffeineon a Rotenone-Induced Rat Model of Parkinsonism: A histological study, Neuroscience Letters http://dx.doi.org/10.1016/j.neulet.2016.04.057

# Dose-Dependent Neuroprotective Effect of Caffeineon a Rotenone-Induced Rat Model of Parkinsonism: A histological study

**Amira M. Soliman[1], Ahmed M. Fathalla[1,] Ahmed A. Moustafa[2,3]**

[1]Department of Pharmacology, Faculty of Medicine, Suez Canal University, Ismailia, Egypt.
[2] Department of Veterans Affairs, New Jersey Health Care System, East Orange, NJ, USA.
[3] School of Social Sciences and Psychology & Marcs Institute for Brain and Behaviour, Western Sydney University, Sydney, New South Wales, Australia.

**Corresponding author:**
Amira M, Soliman[1]
Department of Pharmacology, Faculty of Medicine, Suez Canal University
Email:amiram.soliman_pgs@med.suez.edu.eg
and
Ahmed A. Moustafa
School of Social Sciences and Psychology &Marcs Institute for Brain and Behaviour, Western Sydney University, Sydney, NSW.
Email: a.moustafa@westernsydney.edu.au

**Key Words:** Parkinson's disease, rotenone model, Immunohistochemical, histological stains.

- Rotenone produces Parkinsonism
- Caffeine is neuroprotective against Parkinsonism
- Caffeine hindered dopamine reduction in Parkinsonism rats.
- A higher dose of caffeine was more effective against parkinsonism

**Abstract**

Several lines of evidence have demonstrated an inverse relationship between caffeine utilization and Parkinson's disease (PD) progression. Caffeine is a methylxanthine known as a non-specific inhibitor of adenosine($A_{2A}$ and $A_1$) receptors in the cerebrum and demonstrated to be a neuroprotective medication. In this study, the neuroprotective efficacy of two different doses of caffeine ranging above the usual consumption dose and below the toxic dose was investigated using histopathological and immunohistochemical methods. Thirty-two male rats were randomly divided into 4 groups, with 8 in each group: vehicle control (1 ml/kg/48 hfor 12 days), rotenone(1.5 mg/kg/48 h, s.c.for 12 days), low-dose Caffeine-treated: (10mg/kg IP. daily for 12 days), high-dose Caffeine-treated (20mg IP daily for 12 days). Twenty-four hours after the last rotenone injection, animals were sacrificed and brainswere sectioned and prepared for histopathological staining with hematoxylinand eosin, cresyl violet and Mallory's phosphotungestic acid haematoxylinand for immunohistochemical staining of tyrosine hydroxylase. Our study showed that the treatment with caffeine improved histopathological degeneration in the substantia nigra parts compacta (SNpc) neurons and hindered the reduction in dopamine concentration caused by rotenone. We also found that a higher dose of caffeine was more effective against histopathological degeneration.These results suggest that caffeine has a dose-dependent neuroprotective effect.

## Introduction

Parkinson's disease (PD) is a neurodegenerative disease for which no preventive or long term viable treatment methods are available. PD is characterized by a progressive reduction of dopamine in the striatum caused by death of neurons in thesubstantia nigra parts compacta(SNpc;Fahn and Sulzer 2004).PD is associated with multiple, and not a single, environmental, dietary, or life-style risk factors (Park et al., 2004). For example, studies suggest thatoxidative stress, neuroinflammation, accelerated aging,as well as other environmental factors are associated with the etiology and pathogenesis of PD(Fahn and Sulzer 2004).

Rotenone is an insecticide that occurs naturally, and is a specific inhibitor of mitochondrial complex I(Alam and Schmidt 2002).It is the most potent member of the rotenoids, a family of isoflavonoids extracted from *Leguminosae* plants(Nicolaou et al., 2000).Betarbet et al. (2000) used systemic rotenone to create a chronic progressive animal model of PD.The rotenone-induced PD model seems to replicate almost all of the hallmarks of PD including □-synuclein aggregation, lewy body formation and nigral dopaminergic neurons loss (Cannon et al., 2009), as well as motor dysfunction, as we have reported recently (Fathalla et al., 2016).

Adenosine is a neuromodulator present in the central nervous system (CNS). Its main function in the CNS is to modify neurotransmitter release, such as dopamine and glutamate (Yabuuchi et al., 2006; Courjaret et al., 2012; Hawryluk et al., 2012).In vivo and in vitro studieson adenosine-dopamine interactions were concludedthrough examinations in the basal ganglia and limbic regions because of the high expressionof A1, A2A, D1 and D2 receptors in these areas and their involvement in the pathology of PD. Adenosine $A_{2A}$ and dopamine receptors oppose each other in action, such that the activation of $A_{2A}$ receptors inhibits dopamine $D_2$ receptor

signaling. Conversely, $A_{2A}$ receptor antagonists improve $D_2$ dependent signaling (Ferré 2008).

Caffeine is a methylxanthine compound that acts as a non-specific antagonist of adenosine $A_{2A}$ and $A_1$ receptors (Derkinderen et al., 2014).Clinical studies have revealed a novel protective effect of caffeine on the pathophysiological reactions of dopaminergic nigrostriatal neurons in a mouse model of PD (Kalda et al., 2006).These ameliorative effects associated with caffeine consumption could be attributed to its effect on adenosine $A_{2A}$ receptors, which are involved in striatopallidal neuronal activity in the indirect pathway of the basal ganglia (Jenner, 2005).

Chen et al. (2001) showed low dose of 5 mg/kg of caffeine (roughly resemble human caffeine exposure to one cup of coffee or ~ 100 mg caffeine) (Fredholm et al., 1999) to triple dopamine concentration in comparison to saline-treated group. The study also found caffeine combined with the neurotoxin, MPTP in this case, to produce a systemic toxicity at dose of 30 mg/kg and above.

So, the present study was designed to investigate the histopathological changes in a rotenone-based PD model and to evaluate the neuroprotective efficacy of caffeine usingtwo different doses (10 mg and 20 mg) ranging above the usual consumption dose and below the toxic doseon reducing the degradation of nigrostriatal neurons and improving dopamine level on a rotenone-induced PD model via histopathological and immunohistochemical methods.

## Materialsand Methods

### *Animals*

Thirty two adult male albino rats weighing 200 ± 20g were used for the current study. Animals were purchased from the National Research Centre for Experimental Animals, Cairo, Egypt. Animals were housed

under standardized conditions away from any stressful stimuli with a normal day/night cycle, 25 ± 2°Ctemperature, in plastic polyethelyne cages with free access to food and water, and were allowedtoacclimatefor one week before the start of the study. All experimental protocols were approved by the Institutional Animal Care and Use Committee at Suez Canal University. All efforts were takento reduce animal suffering and to minimize the number of animals used.

***Drugs***

Rotenone was purchased from Sigma- Aldrich (MO, USA) and dissolved in 1:1 (v/v) dimethylsulfoxide (DMSO) and polyethyleneglycol (PEG-300) (Thiffault et al., 2000). Rats received 6 subcutaneous injections of rotenone (1.5 mg/kg/48 h). Caffeine was purchased from Sigma- Aldrich (MO, USA), dissolved in saline solution and administered by intraperitonal injection (IP) Treatment injections were given 10 min before rotenone injection and continued daily for the total period of experiment. All treatments were prepared immediately before administration

***Study design***

Rats were randomly divided into 4 groups.Each group had 8 animals. All of the experimental groups, except the vehicle-control group, were injected with rotenone and/or selected drugs. So, we had the following groups: (a) vehicle-control: rats received six IP injections of the vehicle (1:1 (v/v) DMSO and PEG-300) in a volume of 1 ml/kg; (b) rotenone: rats received subcutaneous rotenone (1.5 mg/kg/48 h) and received normal saline in a volume of 1 ml/kg daily for 12 days; (c)low-dose Caffeine-treated: Rotenone treated rats received caffeine 10mg/kg IP daily for 12 days (Chen et al., 2001); (d) high-dose Caffeine-treated: Rotenone treated rats received caffeine 20mg/kgIP dailyfor 12 days (Kachroo et al., 2010).

***Processing of the brain***

Twenty-four hours after the last injection of rotenone, rats were euthanized by decapitation (Machado-Filho et al., 2014).Their brains were quickly dissected and washed with ice-cold saline. After that, the striatum was isolated and weighed. All brains were rinsed with phosphate buffered saline (PBS), fixed using 4% paraformaldehyde in 0.1 M phosphate buffer, pH= 7.2 overnight and then embedded with paraffin. All paraffin-embedded tissues were sectioned at 4 μm thickness at the SN and left to dry overnight at 37 °C. Sections were then deparaffinized, rehydrated and prepared for histopathological staining with hematoxylin& eosin (H&E), cresyl violet for Nissl-staining, Mallory's phosphotungestic acid haematoxylin (PTAH) stainingand for immunohistochemical staining of tyrosine hydroxylase (TH) (Zaitone et al., 2012).

***Immunostaining*** **(Zaitoneet al., 2012):**

Rabbit tyrosine hydroxylase (TH) antibodies delivered from Biorbyt Limited Company for biochemical kits (Cambridge, UK) were used. Sections were fixed in a 65 °C oven for 1 h and then the slides were placed in a coplin jar filled with 60 ml of triology (Cell Marque®, CA-USA) working solution and the jar was securely positioned in an autoclave. The autoclave was adjusted so that the temperature was at 120 °C. This temperature was maintained for 15 min after which the pressure was released and the coplin jar was removed to allow the slides to cool for 30 min. Sections were then washed and immersed in TBS to adjust the pH, this was repeated between each step of the immunohistochemical procedure. Quenching endogenous peroxidase activity was performed by immersing slides in 3% hydrogen peroxide for 10 min. Background staining was blocked by putting 2–3 drops of 10% goat non immune serum blocker on each slide and incubating them in a humidity chamber for 10 min. Without washing, excess serum was drained from each slide

and 2–3 drops of the ready to use rabbit monoclonal tyrosine hydroxylase primary antibodies (R&D systems®). Then, slides were incubated in the humidity chamber for 1 h. Henceforward, biotinylated secondary antibody was applied on each slide for 20 min followed by another 20 min incubation with the enzyme conjugate. Diaminobenzidine (DAB) chromogen was prepared and 2–3 drops were applied on each slide for 2 min. After that, DAB was rinsed and the slides were counterstained with Mayer's hematoxylin and cover slipping was performed as the final step before slides were examined under a light microscope.

The number TH positive neurons with distinct nuclei were estimated by counting and then divided by the total number of neurons in the examined fields to get the percentage of positive cells by using a light microscope with high resolution.

***Haematoxyline and eosin (H&E)*(Drury and Wallington, 1980):**

Sections wererehydrated in alcohol (100%, 95%, 70%) for 3mineach. Then, they were stained in Mayer’s haematoxylinfor 30sec. After that, sections werewashedand “blued” in running tap water. In the case of high background,sections were differentiated in acid alcohol 8-12 times briefly then washed in tap water, re-blued and checked under the microscope. After that, sections werestained with the 1%eosin solution for 10sec,rinsed in tap water to remove the excess stain, washed in 70% ethanol and checked microscopically until the tissue matrix and cellular components were differentiallystained. Finally, they were rapidly dehydrated through graded ethanol clear in xylene and mounted in DePeX.

***Mallory's phosphotungestic acid haematoxylin (PTAH) staining* (Adams et al., 1971):**

PTAH solution was chemically oxidized with potassium permanganate. The preparation of solution was as follows: The

haematoxylin was dissolved in 100 $cm^3$ of distilled water and400 $cm^3$ ofphosphotungestic acid. The two solutions were mixed and potassium permanganate solution was added. Sections were hydrated through graded alcohol concentrations to water. They were then placed in acid dichromate solution for 30 min [10%HCL in methylated spirit:12 $cm^3$, 3% Aqueous potassium dichromate:36 $cm^3$], washed in tap water, and then treated with acid permanganate solution for 1 min [0.5% Aqueous potassium permanganate:50 $cm^3$,3% sulphuric acid: 2.5 $cm^3$]. After that, sections were washed in tap water and bleached in 1% oxalic acid. Then, sections wererinsed in tap water and stained in Mallory's PTAH stain overnight. Finally, sections were dehydrated through graded alcohols (rapidly in order not to remove stain).

Color index: Neuroglia fibers: dark blue, Nuclei: blue, Myelin: lighter blue, Collagen: deep brownish red, Cytoplasm: pale pinkish brownish red.

***Cresyl fast violet [Nissl]*** (**Drury and Wallington, 1967):**

Sections were dewaxed with xylol and washed with water, then covered with filtered cresyl fast violet stain for 20-30 min. Sections were rinsed in distilled water and left in 96% alcohol until most of the stain had been removed. After that, sections were passed through absolute alcohol into xylene, left for a few minutes and rinsed in absolute alcohol to remove the stain.The differentiation is speeded up when rinsing in absolute alcohol, therefore xylene was added to arrest the differentiation. Importantly, color index is as follows. Nissl substance: purple /dark blue. Neurons: purple / blue. Cell nuclei: purple / blue.

**Histopathological data analysis**

The frequency distribution of the histopathological changes was determined for each change in all studied groups(Hoglinger et al., 2004). The boundaries of the SNpc were chosen on three consecutive sections

corresponding to a representative median plane of the SNpc. Each midbrain section was viewed at low power (×10 magnification) and the SNpc was outlined. Digitized tissue histopathology was quantified using computer-assisted image analysis (Image-Pro plus software; Media Cybernetics) which was calibrated for color and measuring parameters before use.

***Statistical analysis:***

Data were expressed as mean ± SEM and analyzed using the Statistical Package of Social Sciences (SPSS program, version 17, SPSS Inc., Chicago, IL, USA). A one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test were conducted to examine the mean values among groups. $P< 0.05$ was considered significant.

## Results

***TH Immunohistochemical stain:***

Positive brownish staining for TH was detected in the pars compacta cells of the vehicle-controltreated rats (Fig. 1i), while in the rotenone group there was a decreased staining of the soma and processes of all pars compacta cells in all animals(Fig. 1ii). Treatment with caffeine (10 mg and 20 mg) led to an increase in the positive brownish staining compared to rotenone group (Fig.1iiiandFig.1iv).

The mean optical density of TH activity in pars compacta cells of thevehicle control group was 0.92 ± 0.008, while the optical density of TH activity in rotenone group was 0.63 ± 0.008. After treatment with caffeine (10 mg and 20 mg), the mean optical density of TH activity in pars compacta cells was 0.69 ± 0.004, 0.76 ± 0.008 respectively (Fig 2).

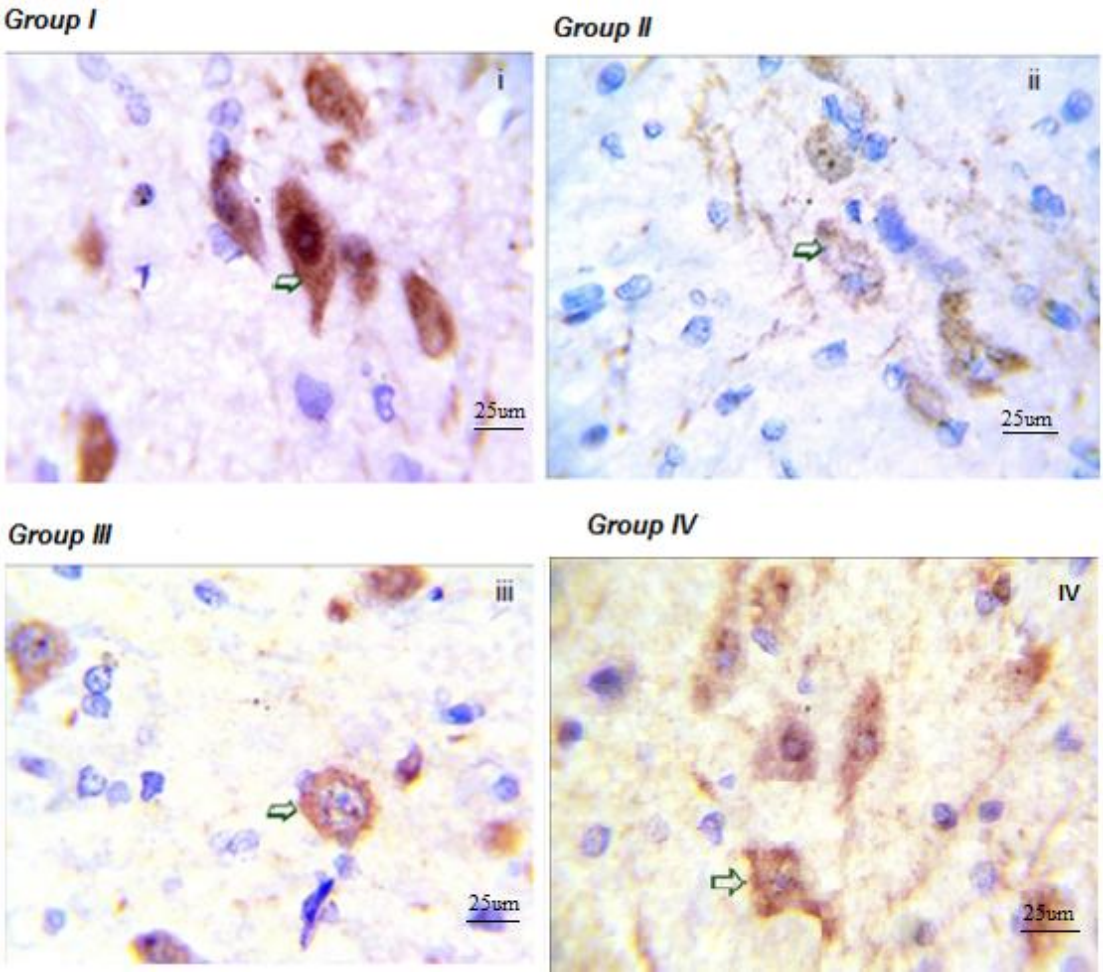


**Figure (1):i)**A section in SNPc of the mid brain of a vehicle-control treated rat. A positive brownish immunoreaction appeared in some cells in the SNPc. **ii)** A section in theSNPc of the mid brain of a rat from therotenone group.Thereis a decrease in the number and size of positive brownish immunoreactive cells in the SNPc. **iii)** Section in SNPc of the mid brain of a rat treated with low dose caffeine (10 mg)shows an increase in the number and size of positive brownish immunoreactive cells in the SNPc compared to the rotenone group. **iv)**High dosecaffeine(20mg)showed an increase in the number and size of positive brownish immunoreactive cells in the SNPccompared to the rotenone group. **(TH x 100)**.

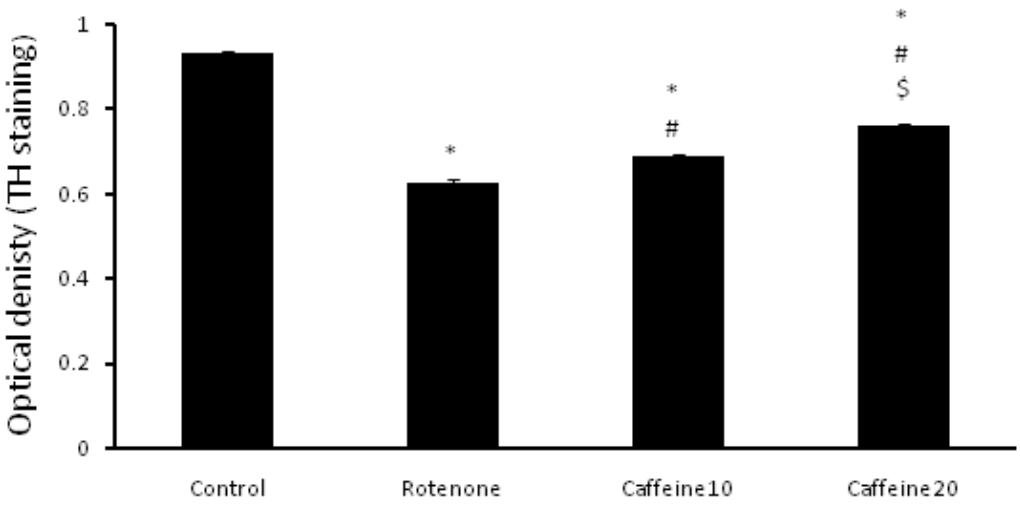


**Figure (2):**Optical density (TH staining).The rotenone group showed a decrease in optical density. Treatment with caffeine (10 mg and 20 mg) caused an increase in optical density. Data was analyzed using a one way ANOVA followed byBonferronipost hoc test, *n*=8, expressed as mean ± SE. $^{*}P < 0.05$ compared to the vehicle treated group, $^{\#}P < 0.05$ compared to the rotenone group, $^{\$}P < 0.05$ compared to the caffeine (10 mg) treated group.

**H&E stain:**

Examination of striatal sections which were stained with H&E showed scattered large multipolar neurons with vesicular nuclei and the surrounding neuropil of pars compacta appeared in the vehicle-control group (Fig3 i). On the other hand, in the rotenone group, neurons appeared widely displaced as a result of a complete loss of some cells and others were distorted, angulated and shrunken (Fig3 ii).Treatment with low-dose caffeine (10 mg) resulted in the restoration of the architecture of the pars compacta cells (i.e., 10 mg of caffeine led to a large multipolar cell with vesicular nuclei, less than that of the vehicle treated group. There were a higher number of the neurons. Some degenerating multipolar cells were shown, leaving vacuoles in intercellular space) (Fig3 iii), while

SNpc cells in the high-dose caffeine-treated (20 mg) group were nearly similar to the vehicle control group (Fig.3iv).

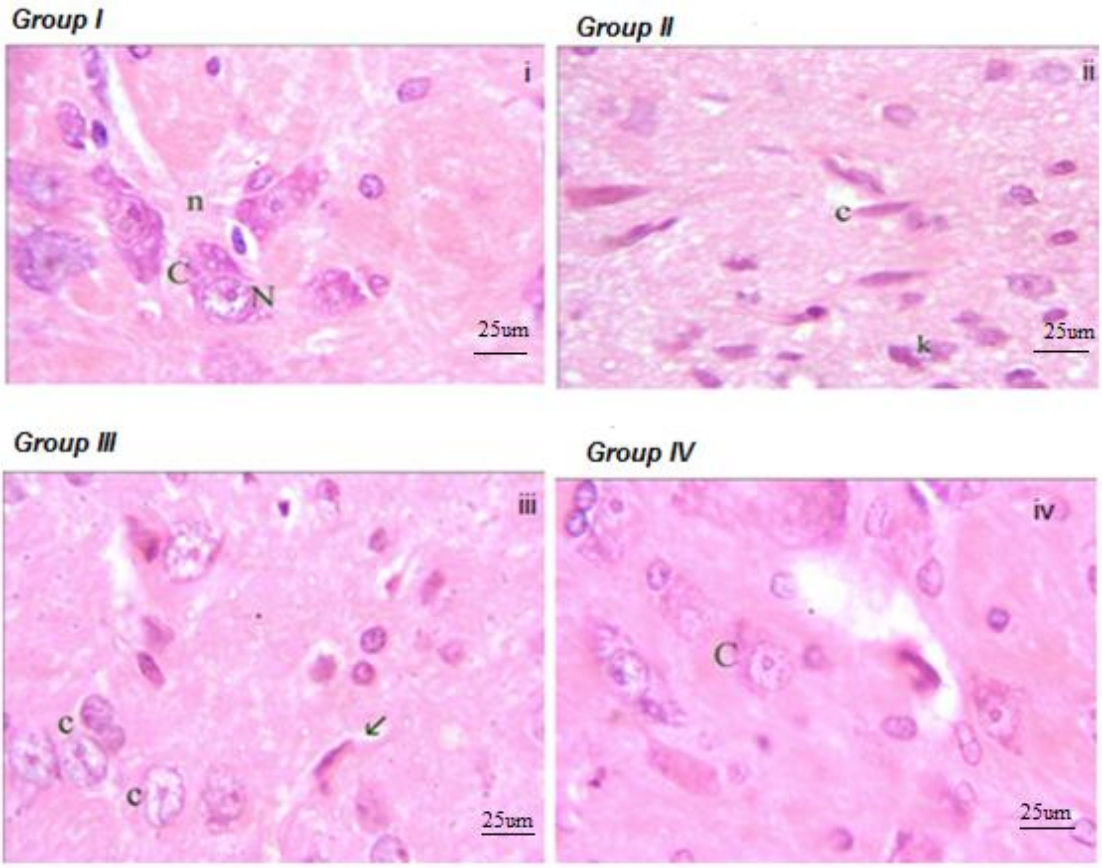


**Figure (3):**i) section in SNPc in the vehicle-control group showing the region of SN in the mid brain that contain scattered large multipolar neurons (c) with vesicular nuclei (N) and the surrounding neuropil (n). ii) A decrease in density of neurons was obtained in the rotenone group, the pars compacta cells are widely displaced and distorted and shrunken. One pars compacta cell has dark stained cytoplasm and deep stained nucleus (c). Another cell has karyolytic nucleus (k). iii)Caffeine (10 mg) treated group shows restoration of the architecture of the pars compacta cells; large multipolar cells with vesicular nuclei (c) are seen. There are a higher number of neurons. Some degenerating multipolar cells are shown, leaving vacuoles (→) in the intercellular space. iv) High dose caffeine (20 mg) treated group, shows scattered large multipolar cells (C) and surrounding neutrophil are nearly similar to that of the vehicle treated group.**(H&E x 100)**.

**PTAH stain:**

Delicate blue dendritic arborizations of pars compacta cells appeared in the vehicle-control group (Fig. 4i). Systemic IP. rotenone injection resulted in a marked decrease in dendrite length of pars compacta arborizations (Fig. 4ii). Low-dose caffeine-treated (10 mg) group showed increased in dendrite length compared to the rotenone group, but less than the vehicle-control group (Fig 4iii). Regarding the high dose caffeine-treated 20 mg group, the dendrite lengths were increased compared to the rotenone group and was nearly similar to the vehicle control group (Fig.4 iv).

The mean length of the dendrites in the vehicle control group was 0.53 ± 0.008, while in the rotenone group it was markedly decreased compared to that of thevehicle-treated group (0.32± 0.008) (Fig 5).The mean length of the dendrites in the group treated with low dose caffeine (10 mg) was increased compared to therotenone group, but was less than the vehicle treated group (0.46 ± 0.004). It also increased in the high dose caffeine (20 mg) group in comparison to therotenone group with a value nearly similar to that of thevehicle-control treated group (0.48 ± 0.008) (Fig. 5).

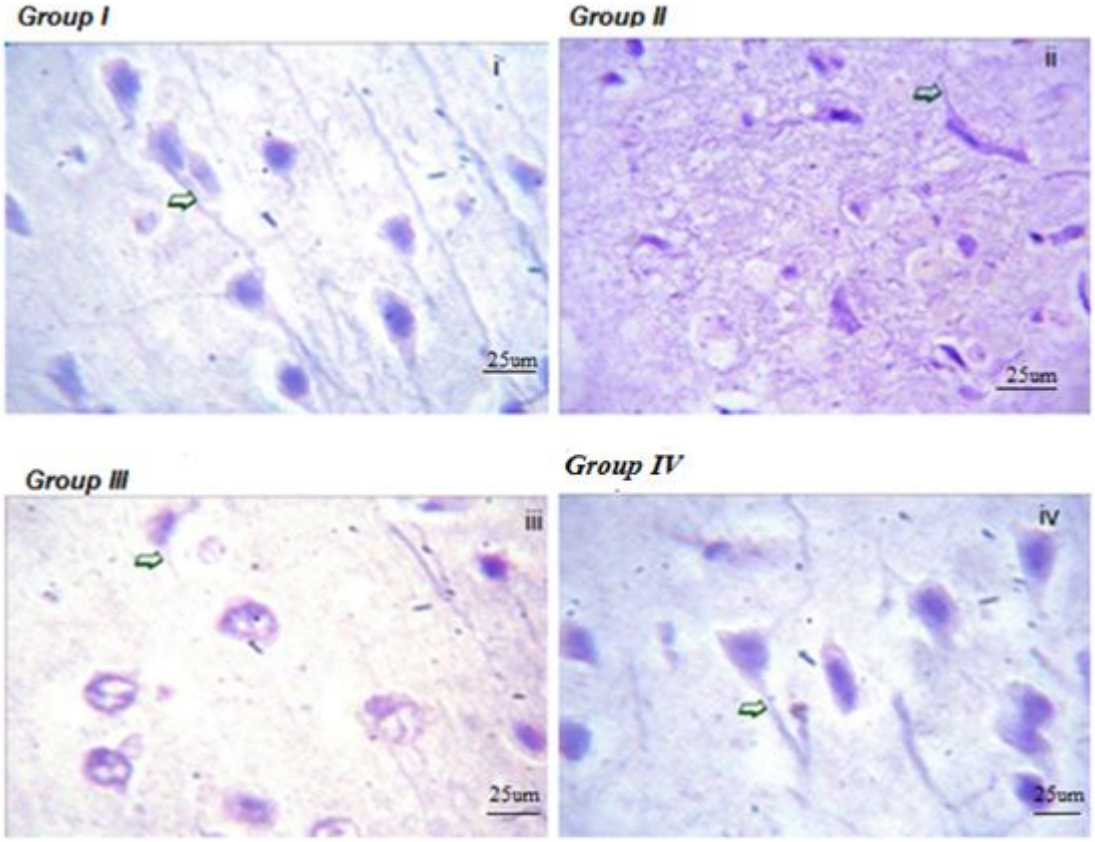


**Figure (4):** i) A section in SNPc of the mid brain of a rat from the vehicle-controltreated group showing delicate dendritic arborization ( ⇨) of pars compacta cells.ii) A section in SNPc of the mid brain of a rat from therotenone group. There was a decrease in length of pars compacta dendrites ( ⇨).iii) Showing a section in SNPc of the mid brain of a rat treated with low dose caffeine (10 mg) that shows an increase in dendrite length ( ⇨) of pars compacta cells compared to that of the rotenone group. iv) A section in SNPc of the mid brain of a rat treated with high dose caffeine (20 mg) that shows an increase in dendrite length ( ⇨) of pars compacta cells, compared to that of the rotenone group.**(PTAH x100)**

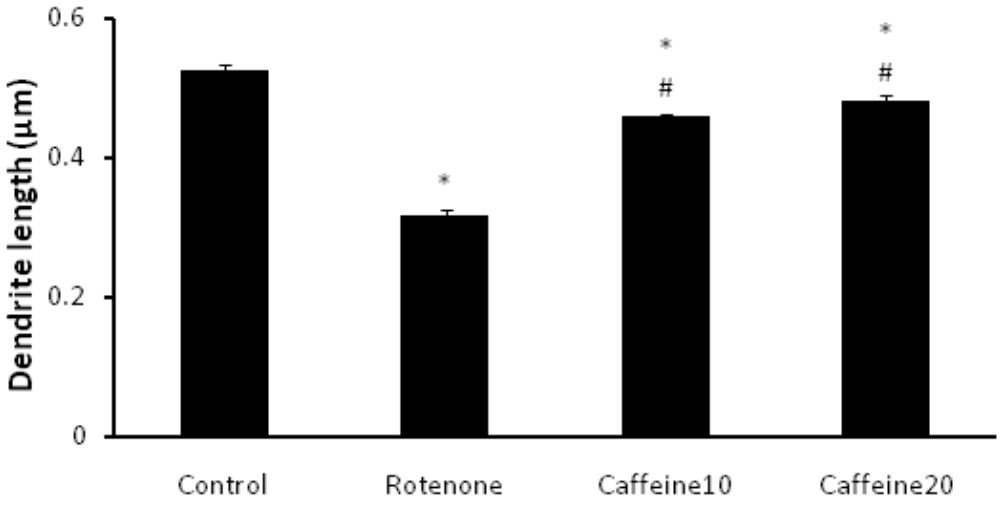


**Figure (5):**The dendrite length of the substantia nigra neurons among the groups.The rotenone group showed a decrease in dendrite length compared to the vehicle (control) group. The low dose caffeine (10 mg) treated group showed an increase in dendrite length, but less than thehigh dose caffeine- treated(20 mg) group. Data wasanalyzed using a one way ANOVA followed byBonferronipost hoc test, *n*=8, expressed as mean ± SE. $^{*}P < 0.05$ compared to the vehicle treated group, $^{\#}P < 0.05$ compared to the rotenone group.

***Cresyl fast violet stain:***

Nissl's granules are the site of protein synthesis in neurons and its presence indicate healthy tissues (John and James 2009).

Examination of the cresyl fast violet stain in the vehicle-control treated group showed aggregates of purple granules (Nissl's granules) within the perikarya of pars compacta cells(Fig 6i),while in the rotenone group it was markedly decreased (Fig 6ii). Groups treated with Caffeine (10 mg and 20 mg)showed a marked increase of the Nissl's granules (Fig 6iii and iv).

Image analysis showed that the mean optical density of Nissle granules in the vehicle group was 0.4 ± 0.004 and this was markedly decreased in the rotenone group compared to the vehicle treated group(0.122 ± 0.004). After treatment with caffeine (10 mg and 20 mg)the mean optical density was markedly increased (0.2 ± 0.004) and (0.29 ±

0.004), respectively compared to therotenone group, but was less than the vehicle treated group (fig 7).

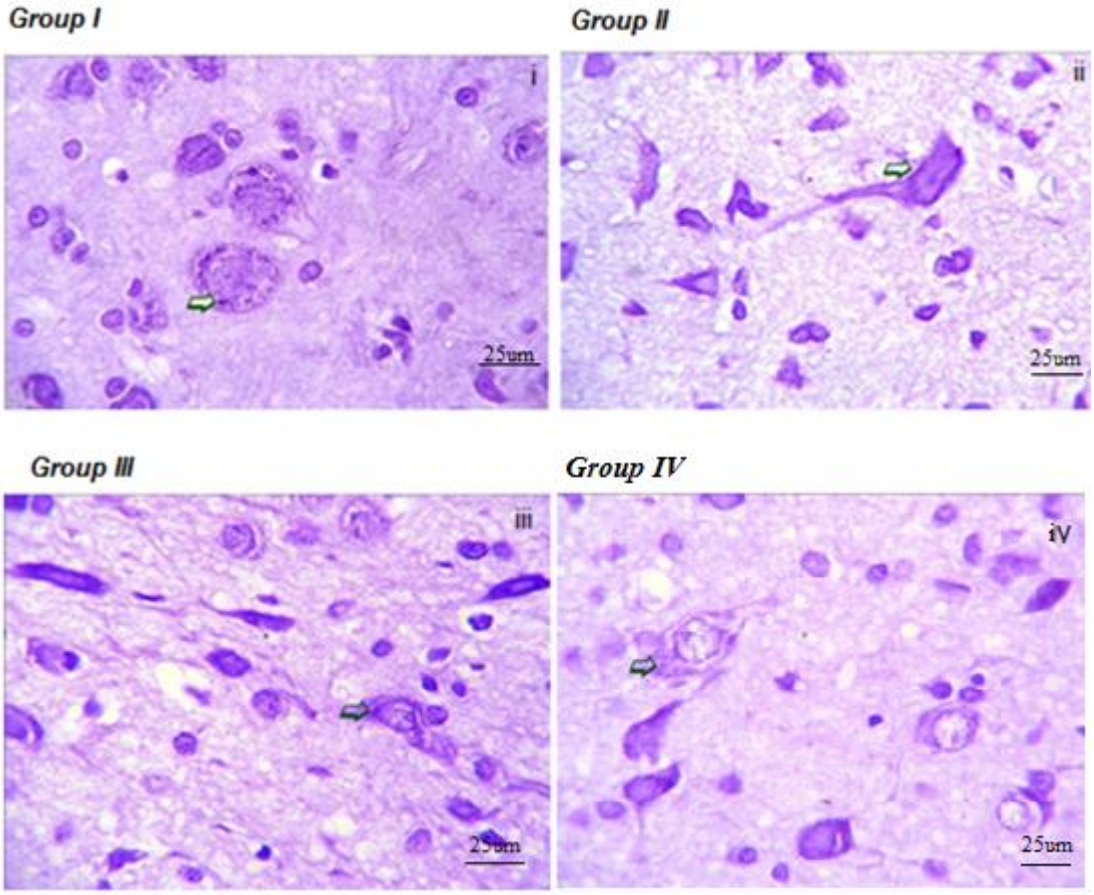


**Figure (6):**i) A section in SNPc of the mid brain of a rat from the vehicle-conttrol treated group illustrates the purple Nissl granules ( ⇨ ) in the perikarya of SNPc cells. ii)A section in SNPc of the mid brain of a rat from the rotenone group illustrates the decrease in the purple Nissl granules ( ⇨) in the perikarya of SNPc cells. iii). There was an increase in purple Nissl granules (⇨ ) in the perikarya of SNPc cells of the rats treated with low dose (10 mg) ofcaffeine, compared to that of the rotenone group. iv)A section in SNPc of the mid brain of a rat treated with high dose (20 mg) of caffeine shows an increase in purple Nissl granules (⇨ ) in the perikarya of SNPc cells, compared to that of the rotenone group. **(Cresyl fast violet x 100)**.

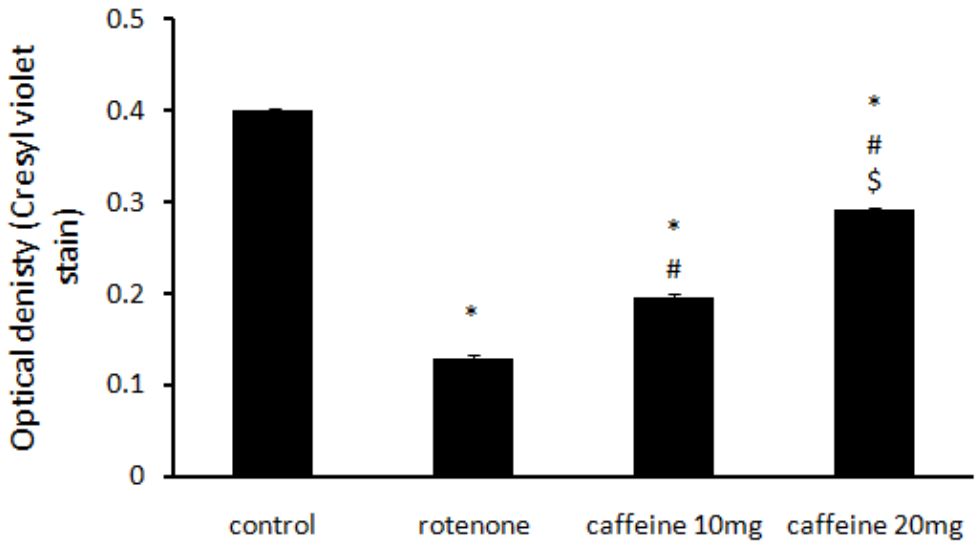


**Figure (7):**Optical Density (Cresyl fast violet stain). The rotenone group showed a decrease in optical density, while the low dose caffeine treated(10 mg) group showed an increase in optical density compared to the rotenone group. Treatment with high dose caffeine (20 mg) showed an increase in optical density compared to the rotenone group and caffeine (10 mg) group. Data was analyzed using a one way ANOVA followed byBonferronipost hoc test, *n*=8, expressed as mean ± SE. $^{*}P < 0.05$ compared to the vehicle treated group, $^{\#}P < 0.05$ compared to the rotenone group, $^{\$}P < 0.05$ compared to the caffeine (10 mg) treated group.

## Discussion

Rotenonewas used to create an animal model of PD as rotenone administration has previously shown tocause most of histopathological signs of Parkinsonismsuch asdamage to central dopaminergic neurons, clear degeneration and apoptosis of SN dopaminergic neurons (Ayala et al., 2007; Betarbet et al., 2000), as well as motor abnormalities (Fathalla et al., 2016).

Johnson et al.(2015)reported insignificant changes in TH levels in the SNpc and striatum in rotenone model of PD in rats.In contrast, our animal model injected with rotenone showed a significant decrease in the number and size of positive brownish immunoreactive staining of the soma and processes of all pars compacta cells with TH

immunostaining.This result are in line withZaitone et al., (2012); Sarkar et al. (2015) who showed a similar decrease in TH levelusing this low dose rotenone PD model. The discrepancy in results could be because of the use of different methods of assessing TH expression within the same population of dopaminergic cells.

In this context, Gammella et al. (2010) demonstrated that the pathophysiology of PD is in great part, a result from a decrease in the synthesis, activity, and TH expression, as observed in the striatum of PD patients. Also Zhu et al., (2012), Walker et al., (2016)displayed that, TH is the rate limiting step, in catecholamine synthesis and that the reduced TH expression observed in PD leads to a reduced DA synthesis in the striatum.

Histopathological examination of striatal sections revealed thatrotenone injection was associated with neuropathological changes in the SN. This is a result of a complete loss of some cells, others were distorted, angulated, and shrunken as shown with the H&E stain, and there was a decrease in dendritic arborizations and Nissl's granules of pars compacta cells with PTHA and cresyl violet stains, respectively ($p< 0.05$).Thisis in line with the study of Ludviget al., (2008),which found that rotenone-treated rats had DAergic degeneration and cytoplasmic inclusions in nigral neurons. Hematoxylin and eosin staining showed different signs of degenerative and necrotic changes in the form of deeply eosinophilic cytoplasm, dense cytoplasmic inclusions in the cytoplasm of nigral neurons; such inclusions were frequently clustered in the cytoplasm.

Caffeine is a methylxanthine compound that actsby implication to potentiate dopaminergic signaling (Machado-Filho et al., 2014). It is known as a non-selective inhibitor of $A_{2A}$ and $A_1$ adenosine receptors in the brain and shown to be a neuroprotective drug (Derkinderen et al.,

2014; Rivera-Oliver and Díaz-Ríos, 2014).The present study showed that caffeine exposure reduced the loss of TH neurons, and that the high dose was more effective in increasing the TH positive dopaminergic neurons more than the low dose. In agreement with this study, Chen et al., (2008); Machado-Filho et al., (2014) demonstrated that caffeine had a neuroprotective effect (Bagga et al., 2016) as it blocked the decrease in the number of TH-positive dopaminergic neurons in mice striata. Another study showed that chronic infusion of MPTP into the rat brain showed a progressive loss of DA and TH in the striatum andSN and that consumption of caffeine prevented dopaminergic degeneration in the SN (Sonsalla et al., 2012).

The histopathological results revealed that caffeine (20mg) was more effective in restoring the architecture of the pars compacta cells andincreasing the number of neurons more than the lower caffeine dose did as shownwith the H&S stain. They also revealed an increase in length of dendritic arborizationsand in density of Nissl's granules of pars compacta cells as shown with PTHA stain and cresyl violet stain ($p < 0.05$).

The results of this study suggest that caffeine administration could provide a dose-dependent therapeutic effect on a rat rotenone model of PD.

## References

1. Adams CW, Bayliss OB, Hallpike JF, Turner DR (1971). Histochemistry of myelin. XII. Anionic strainingof myelin basic roteins for histology, electrophoresis andelectron microscopy. J Neurochem 18(3):389-94.
2. Alam M, Schmidt WJ (2002). Rotenone destroys dopaminergic neurons and induces parkinsonian symptoms in rats. Behav Brain Res. 2002 Oct 17;136(1):317-24.

3. Ayala A, Venero JL, Cano J (2007). Mitochondrial toxins and neurodegenerative diseases. Front Biosci (12): 986-1007.

4. Bagga P, Chugani AN, Patel AB (2016). Neuroprotective effects of caffeine in MPTP model of Parkinson's disease: A (13)C NMR study. Neurochem Int 92:25-34.

5. Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT (2000). Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci 3: 1301-1306.

6. Cannon JR, TapiasV, HonickAS, DroletRE, GreenamyreJT (2009). A highly reproducible rotenone model of Parkinson's disease. Neurobiol Dis 34(2): 279-290.

7. Chen H, O'Reilly EJ, Schwarzschild MA, Ascherio A (2008). Peripheral inflammatory biomarkers and risk of Parkinson's disease. American J Epidemiol 167 (1): 90-95.

8. Chen JF, Xu K, Petzer JP, StaalR, Xu YH, Beilstein M, Sonsalla PK, Castagnoli K, CastagnoliN JR, Schwarzschild MA (2001). Neuroprotection by caffeine and A(2A) adenosine receptor inactivation in a model of Parkinson's disease. J Neurosci 21(10): 1-6.

9. Courjaret R, Miras-Portugal M, Deitmer J (2012). Purinergic Modulation of Granule Cells. The Cerebellum 11:62-70.

10. Drury RA, Wallington EA (1967). Carleton's Histological Techniques. 4th ed 214-215.

11. Drury RA, Wallington EA (1980). Carlton's histological techniques.5th Ed. Oxford univ press LondonNew York Toronto 1:653-661.

12. Fahn S, Sulzer D (2004). Neurodegeneration and neuroprotection in Parkinson disease. NeuroRx 1(1):139-54.

13. Fathalla, A., Soliman, A., Hammdi, M., Moustafa, A.A. (2016). Adenosine A2A receptor blockade prevents rotenone-induced Motor

Impairment in a Rat Model of Parkinsonism. Frontiers in Behavioral Neuroscience 10:35.

14. Ferré S (2008). An update on the mechanisms of the psychostimulant effects of caffeine. J Neurochem 105(4):1067-1079.

15. Fredholm BB, Battig K, Holmen J, Nehlig A, AvartauEE (1999). Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacol Rev 51(1): 83-133.

16. Gammella E, Cairo G, Tacchini L (2010). Adenosine A2A receptor but not HIF-1 mediates tyrosine hydroxylase induction in hypoxic PC12 cells. J Neurosci Research (88): 2007-2016.

17. Hawryluk JM, Ferrari LL, Keating SA, Arrigoni E (2012). Adenosine inhibits glutamatergic input to basal forebarin cholinergic neurons. J Neurophysiol. 107(10):2769-2781.

18. Hoglinger GU, Rizk P, Muriel MP, Duyckaerts C, Oertel WH, Caille I (2004). Dopamine depletion impairs precursor cell proliferation in Parkinson disease. Nat Neurosci 7:726–735.

19. Jenner P (2005). Istradefylline, a novel adenosine A2A receptor antagonist, for the treatment of Parkinson's disease. Expert OpinInvestig Drugs 14(6): 729-738.

20. John H., James L (2009). From Molecules to Networks: An Introduction to Cellular and Molecular Neuroscience. Academic Press. pp. 20–. ISBN 978-0-12-374132-5.

21. Johnson ME, Lim Y, Senthilkumaran M, Zhou XF, Bobrovskaya L (2015). Investigation of tyrosine hydroxylase and BDNF in a low-dose rotenone model of Parkinson's disease. J Chem Neuroanat 70:33-41

22. Kachroo A, Irizarry MC, Schwarzschild(2010). Caffeine protects against combined paraquat and maneb-induced dopaminergic neuron degeneration. ExpNeurol 223(2): 657-661.

23. Kalda A, Yu L, Oztas E, Chen JF (2006). Novel neuroprotection by caffeine and adenosine A(2A) receptor antagonists in animal models of Parkinson's disease. J Neurol Sci 248(1-2):9-15.

24. Ludvig N, Sheffield LG, Tang HM, Baptiste SL, Devinsky O, Kuzniecky (2008). Histological evidence for drug diffusion across the cerebral meninges into the underlying neocortex in rats. Brain Res 1188: 228-232.

25. Machado-Filho J, Correia A, Montenegro A, Nobre M, Cerqueira G, Neves K, Naffah-Mazzacoratti , Cavalheiro E, de Castro Brito ,de Barros Viana (2014). Caffeine neuroprotective effects on 6-OHDA-lesioned rats are mediated by several factors, including pro-inflammatory cytokines and histone deacetylase inhibitions. Behav Brain Res 264:116-125.

26. Nicolaou K, Pfefferkorn J, Schuler F, Roecker A, Cao G, Casida J (2000). Combinatorial synthesis of novel and potent inhibitors of NADH: ubiquinone oxidoreductase. Chem Biol 7(12): 979-992.

27. Park SW, Kim SH, Park KH, Kim CD, Kim JY, Baek SY, Chung BS, Kang CD. Preventive effect of antioxidants in MPTP-induced mouse model of Parkinson’s disease. NeurosciLett 2004; 363: 243-246.

28. Rivera-Oliver M, Díaz-Ríos M (2014). Using caffeine and other adenosine receptor antagonists and agonists as therapeutic tools against neurodegenerative diseases: a review. Life Sci 101(1-2):1-9.

29. Sonsalla PK, Wong LY, Harris SL, Richardson JR, Khobahy I, Li W (2012). Delayed caffeine treatment prevents nigral dopamine neuron loss in a progressive rat model of Parkinson’s disease. ExpNeurol 234 (2): 482-487.

30. Thiffault C, Langston JW, Di Monte DA. (2000).Increased striatal dopamine turnover following acute administration of rotenone to mice. Brain Res 885 (2): 283-288.

31. Walker DG, Lue LF, Serrano G, Adler CH, Caviness JN, Sue LI, Beach TG (2016). Altered Expression Patterns of Inflammation-Associated and Trophic Molecules in Substantia Nigra and Striatum Brain Samples from Parkinson's Disease, Incidental Lewy Body Disease and Normal Control Cases. Front Neurosci 14;9:507.

32. Yabuuchi K, Kuroiwa M, Shuto T, Sotogaku N, Snyder GL, Higashi H, Tanaka M, Greengard P, Nishi A (2006). Role of adenosine A1 receptors in the modulation of dopamine D1 and adenosine A2A receptor signaling in the neostriatum. Neuroscience 141(1):19-25.

33. Zaitone S, Abo-Elmatty M, Shaalan A (2012). Acetyl-L-carnitine and α-lipoic acid affect rotenone-induced damage in nigral dopaminergic neurons of rat brain, implication for Parkinson's disease therapy. Pharmacology, Biochemistry and Behavior 100 (2012) 347–360.

34. Zhu Y, Zhang J, Zeng Y (2012). Overview of tyrosine hydroxylase in Parkinson’s disease. CNS NeurolDisord Drug Targets 11(4): 350-358.