Neuroprotective Effects of Metformin Versus Selegiline on Parkinson’s Disease Model By Reserpine through the Interrelation of α Synuclein and Antioxidants on Behavioral Changes in Rats

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Abstract. Aim: This study aimed to assess the neuroprotective effects of metformin compared to selegiline, (each drug alone or in combination) on Parkinson’s disease model by reserpine in rats also, it was extended to investigate the mechanisms through which metformin could produce such effect either by its antioxidant effect or genetic recognized by α synuclein and such impact on behavioral changes. Methods: Seven groups were included in the study. The first, second (control and reserpine induced). The third, fourth and fifth were treated with metformin (100, 250 mg/kg), selegiline, (0.25 mg/kg), six and seventh were treated with both selegiline and metformin both doses respectively. Drugs initiated from the first day for 21 days. Morris water maze, hang wire and forced swim tests were done on days 1, 11, 21 to estimate memory changes, motor assessment and depression respectively. Rats were then sacrificed after taking serum samples to assess serum blood glucose; their brains were dissected, homogenized to measure dopamine, α synuclein, malondialdehyde, reduced glutathione levels. Results: reserpine treated group were significant compared to control proving the induction of parkinsonian model which were improved on treatment in all groups with much more improvement in those treated with metformin than selegiline especially those treated by both metformin 100 mg/kg and selegiline. Selegiline treated showed hypoglycemia that was not observed in metformin treated. Conclusion: Metformin on low dose can serve as an add on therapy with selegiline through antioxidant and genetic mechanisms to enhance the neuroprotection in PD patients.

Keywords: Parkinson; reserpine; metformin; selegiline; antioxidant; α synuclein

1. Introduction

Parkinson’s disease (PD) is characterized by being progressive, chronic disease caused by degeneration of dopaminergic neurons leading to a decrease in the release of striatal dopamine (DA) [1]. PD is considered as the second most common neurodegenerative disease after Alzheimer’s disease. It is estimated that six million people had PD worldwide in 2016 [2]. The disease is characterized early by dopamine deficiency eventually leading to involvement of non-dopaminergic brain regions resulting in levodopa-resistant motor and non-motor symptoms. Drugs can improve quality of life of PD patients for many years [3]. Neurodegeneration related to the disease is likely to occur several decades before the onset of the motor symptoms [4]. Many neuropathological mechanisms could explain the pathogenesis of PD which include genetic or toxic disorders; oxidative stress or ischemic anomalies; neuroimmune or inflammatory reactions; that could initiate premature neuronal death [5].
Selegiline is an irreversible MAO-B inhibitor which protects animals from the prodrug MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) toxicity by inhibiting their conversion to MPP+, decreasing the oxidative deamination of dopamine to its metabolite DOPAC (3,4-Dihydroxyphenylacetic acid) and hydrogen peroxide, which leads to reduction of oxyradicals formation from hydrogen peroxide [6]. Selegiline attenuates the worsening of PD patients with early improvement in the symptoms and delayed onset of disability necessitating levodopa therapy [7]. This could be attributed to its ability to increase dopamine and to inhibit reactive oxygen species [8].

Metformin is the most widely used oral euglycemic agent recommended as first line therapy for all recently diagnosed type 2 diabetic patients. Recent data showed some benefits of this drug in neuroprotection. It produces its neuroprotective effect by inhibiting apoptosis of the neuronal cortical cells which in turn alleviates dopaminergic dysfunction and mitochondrial abnormalities [9]. Metformin releases norepinephrine (NE) indirectly by sympathomimetic-like action. This is probably due to stimulating release of endogenous NE from nerve endings or decreasing re-uptake of the exogenous NE [10]. Also, total circulating endogenous NE was notably increased within one minute after bolus IV injection of metformin at 100 mg/kg [11].

The present study was estimated to determine whether the neuroprotective effect of selegiline could be improved when added to metformin in reserpine–induced Parkinson’s disease model in rats and also to investigate the mechanisms of this effect.

2. Materials and Methods

2.1. Animals. 56 Male adult Sprague–Dawley rats weighing 150 - 200 g were used in this study. Rats were bred in the animal house at Kasr El-Ainy, Faculty of medicine, Cairo University. The animal’s treatment protocol was in accordance with the international guidelines of handling the experimental animals and conducted according to the regulations of the institutional animal care and use committee (IACUC) Cairo University, Egypt. The animals were divided into 7 groups (8 rats each), each 4 rats were housed in a cage. The rats were harbored on 12-hour light dark cycle maintained on standard rat chow with free access to water throughout the study. The room’s temperature was adjusted at 26°C. Rats were left for one week for acclimatization before exposing them to the experiment. All experiments were performed between 9 a.m. and 3 p.m. according to the rules of the committee of bioethics for animal experiments of Kasr El-Ainy.

2.2. Drugs

1-Reserpine provided by Pharco Pharmaceutical Company, Alexandria, Egypt as powder, soluble in water. It was freshly prepared to be given at a dose of 0.1 mg/kg by subcutaneous injection every other day for 21 days (10 injections) [12].

2-Metformin provided by Amoun Pharmaceutical Company, el Obour city, Egypt as powder, soluble in water. It was freshly prepared to be given as oral daily doses of 100 mg/kg and 250 mg/kg for 21 days [13]. Metformin dose 250mg/kg was calculated according to body surface area (BSA) formula by [14].

\[
\text{Human Effective Dose (HED)} = \text{animal dose} \times \frac{\text{animal Km}}{\text{human Km}}
\]

\[
= \text{animal dose} \times \frac{6}{37}
\]

3-Selegiline provided by Alfacure Pharmaceuticals, Cairo, Egypt as powder, soluble in water. It was freshly prepared to be given as oral daily doses of 0.25 mg/kg for 21 days [15]. Dose was calculated according to body surface area (BSA) formula by [14].

2.3. Experimental design. Figure (1).

The rats were subjected to the study for 21 days:

The animals were classified into the 7 groups (8 rats each):

- **Group 1**: Rats were injected with normal saline (0.1 mg/kg s.c) every other day for 21 days
- **Group 2**: Rats were injected with Reserpine (0.1 mg/kg s.c) every other day for 21 days (10 injections)
- **Group 3**: Rats were treated daily with Metformin (100 mg/kg p.o) for 21 days and reserpine as Group 2
- **Group 4**: Rats were treated daily with Metformin (250 mg/kg p.o) for 21 days and reserpine as Group 2
- **Group 5**: Rats were treated daily with Selegiline (0.25 mg/kg p.o) for 21 days and reserpine as Group 2
- **Group 6**: Rats were treated for 21 days with Metformin (100 mg/kg p.o) + Selegiline (0.25 mg/kg p.o) + reserpine as Group 2
- **Group 7**: Rats were treated for 21 days with Metformin (250 mg/kg p.o) + Selegiline (0.25 mg/kg p.o) + reserpine as Group 2

The tests were repeated on days 1, 11 and 21. Total body weight was measured at the start and end of the experiment.

2.4. Behavioral tests

2.4.1. Memory assessment

**Morris water maze test**: used to estimate spatial memory and learning of the animals [16].
2.4.2. Motor assessment

**Wire hanging test**: was used to determine a motor neuromuscular impairment, tone and motor coordination which depends on the latency of a rat to fall off a metal wire on fatigue. [17, 18].

2.4.3. Depression assessment

**Forced Stress swim test**: was used to assess depression. The rodents are exposed to a short acute period of stress with recording the time which represents their response in an active versus a passive way [19, 20].

2.5. Serum measurements. At the end of the experiment, blood samples were collected from the heart of rats which were fast for 12h. Immediately, serum was separated to measure fasting blood glucose level using glucose enzymatic-colorimetric assay kits.

2.6. Brain measurements. Under thiopental sodium anesthesia, rats were killed by decapitation to isolate their brains. All samples of brains were collected; each one was divided into 2 halves. The right half of each brain sample was homogenized in ice-cold 50mM sodium phosphate buffer (pH 7.4) containing 0.1 mM ethylene diamine tetra acetic acid (EDTA). The homogenate was centrifuged at 1000 xg for 20min at 4°C and the resultant supernatant was then frozen at −80°C for measurement of dopamine, α synuclein, MDA and GSH from the brain homogenate of each rat. While the left half was preserved in formalin 10% for pathological examination.

2.6.1. Quantitative reverse transcription polymerase chain reaction (QRT-PCR) gene expression of alpha-synuclein (as) in rat brain tissues. The brain tissue was treated for RNA extraction followed by reverse transcription (for cDNA synthesis) and quantitative real time PCR. RNasea purification reagent (Qiagen™, Valencia, CA) was used to extract the homogenate of brain tissue according to manufacturer’s instruction. cDNA was generated from 5 μg of total RNA extracted with 1 μl (20 pmol) antisense primer and 0.8 μl superscript AMV reverse transcriptase for 60 min at 37°C. Real-time qPCR amplification and analysis were performed using an Applied Biosystem with software version 3.1 (StepOne™, USA). Reaction contained SYBR Green Master Mix (Applied Biosystems), gene-specific primer pairs, cDNA and nuclease-free water. With cycling conditions (10 min at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 60°C). Analysis of the data were done using ABI Prism sequence detection system software and quantified using the v1.7 Sequence Detection Software from PE Biosystems (Foster City, CA). Relative expression of studied gene was calculated using the comparative threshold cycle method. All values were normalized to the Beta actin which was used as the control housekeeping gene. The sequences of the primers used for the real-time PCR are listed in Table 1.

Figure 1: Study design and assessments.
2.6.2. Detection of dopamine in rat brain by ELISA. This ELISA kit uses sandwich-ELISA as the method in which the samples were added to Microelisa stripplate that has been pre-coated with DA specific antibody. Then a Horseradish Peroxidase (HRP) - conjugated antibody specific for DA was added to each Microelisa stripplate well and incubated. Free components were washed away. In each well, the TMB (3, 3′, 5, 5′-Tetramethylbenzidine) substrate solution is added which will appear blue in only those that contain DA and HRP conjugated DA antibody and then turn yellow after the addition of the stop solution. The optical density (OD) is measured at a wavelength of 450 nm spectrophotometrically where its value is proportional to the concentration of DA. The concentration of DA was calculated in the samples by comparing the OD to the standard curve.

2.6.3. Measurement of reduced glutathione (GSH). The reduced chromogen is directly proportional to GSH concentration and its absorbance can be measured at 405 nm by using its kit It relied on the reduction of reduced glutathione (GSH) (DTNB) with 5,5dithiobis (2-nitrobenzoic acid) to produce a yellow compound.

2.6.4. Measurement of malondialdehyde (MDA). To measure its concentration, 100mg of the brain tissue was homogenized in 1mL PBS, pH 7.0 with micro pestle then 20% Trichloroacetic acid (TCA) was added to the homogenate to precipitate the protein, and then centrifuged. After collection of the supernatants, Thiobarbituric acid (TBA) solution was added then was boiled for 10 minutes in water bath, with measurements of the absorbance. Concentration of MDA was calculated using the standard curve in supernatants of brain homogenate.

2.7. Pathological scoring. After collection of tissue samples from the left half of the brains of rats (the upper pons, entire midbrain with the substantia nigra and ventral diencephalon), they were fixed in 10% neutral buffered formalin solution for histopathology. The specimens were treated as follows, dehydrated in ascending concentration of ethanol, cleared in xylene, embedded in paraffin wax and sectioned at thickness 5-micron and finally slides were stained by hematoxylin and eosin [21].

The severity of the PD-related Lewy body pathology was assessed semi-quantitatively (− = absent or not discernible, + = slight, ++ = moderate, +++ = severe), although the staging procedure proposed in this study does not require evaluation of lesions density [22].

2.8. Statistical methods. Data were coded and entered using the GraphPad Prism version 7. Then, they were summarized using mean and standard deviation. One way analysis of variance (ANOVA) with multiple comparisons using Tukey’s test was used to compare between groups. P values less than or equal 0.05 were considered statistically significant [23].

3. Results

3.1. Pharmacological data

3.1.1. Morris water maze test. There was significant increase in all groups including reserpine group in mean time spent to reach hidden platform compared to control group but all treated groups were significantly decreased compared to reserpine group (P value < 0.05) (Figure 2).

3.1.2. Wire hanging test. There was significant decrease in mean time spent hanged to the wire in reserpine group and all treated groups compared to control group but they were significantly increased compared to reserpine group (P value < 0.05) (Figure 2).

3.1.3. Forced swim test. There was significant increase in mean despair time in all groups including reserpine group compared to control group but all treated groups were significantly decreased compared to reserpine group (P value < 0.05) (Figure 2).

3.2. Biochemical data

3.2.1. Brain hemolysate study.

1. Rat Brain Dopamine ELISA

There was significant decrease in mean brain dopamine level in reserpine treated group compared to control group (P value < 0.05) (Table 2)

2. Rat Brain Malondialdehyde (MDA)

There was significant increase in mean brain MDA level in reserpine treated group compared to control group while there was significant decrease in all treated groups compared to reserpine group, for combined
3. Rat Brain Reduced Glutathione (GSH)

There was significant decrease in mean brain GSH level in reserpine treated group compared to control group and to all treated groups except groups S+ML+R and S+MH+R while there was significant increase in all treated groups compared to reserpine group in addition to combined groups they were significantly increased to both metformin low dose and selegiline treated groups (P value < 0.05) (Table 2)

4. Rat Brain α Synuclein

There was significant increase in mean brain α synuclein level in reserpine group, ML+R, S+R group compared to control group, while there was significant decrease in all treated groups compared to reserpine group (P value < 0.05) (Table 2)

Table 2: Biochemical tests in Brain hemolysate study (mean ± SD) of different groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups (n. 8 rats in each group)</th>
<th>1 Control</th>
<th>2 Reserpine</th>
<th>3 ML+R</th>
<th>4 MH+R</th>
<th>5 S+R</th>
<th>6 S+ML+R</th>
<th>7 S+MH+R</th>
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<tr>
<td>Dopamine (pg/ml)</td>
<td></td>
<td>115 ± 16.76</td>
<td>62.85 ± 10.3</td>
<td>75 ± 16.28</td>
<td>84.58 ± 10.48</td>
<td>71.75 ± 17.1</td>
<td>88.33 ± 13.57</td>
<td>85.68 ± 16</td>
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<td></td>
<td>(mean ± SD)</td>
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<tr>
<td>MDA (nmol/ml)</td>
<td></td>
<td>13.12 ± 3.09</td>
<td>82.85 ± 17.68</td>
<td>44.02 ± 9.61</td>
<td>37.93 ± 5.703</td>
<td>49.1 ± 5.359</td>
<td>19.4 ± 3.51</td>
<td>0.33 ± 3.59</td>
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<tr>
<td></td>
<td>(mean ± SD)</td>
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<tr>
<td>GSH (nmol/ml)</td>
<td></td>
<td>61.18 ± 4.44</td>
<td>26.22 ± 5.31</td>
<td>41.9 ± 5.319</td>
<td>42.68 ± 3.955</td>
<td>39.5 ± 7.227</td>
<td>52.72 ± 6.206</td>
<td>52.13 ± 4.06</td>
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<td></td>
<td>(mean ± SD)</td>
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<tr>
<td>α Synuclein</td>
<td></td>
<td>1.01 ± 0.014</td>
<td>4.95 ± 0.21</td>
<td>2.58 ± 0.679</td>
<td>2.18 ± 0.18</td>
<td>3.085 ± 0.02</td>
<td>1.85 ± 0.49</td>
<td>2.1 ± 0.424</td>
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<tr>
<td></td>
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3.2.2. Serum measurements: Serum glucose. Only the group treated with selegiline alone showed significant decrease of glucose compared to control, reserpine, ML+R and MH+R groups (Figure 3) which was elevated when combined with metformin.

3.3. Pathological data

3.3.1. Microscopic findings. Histologically, substantia nigra area of control group consisted of perikaryon cells with short shrunken primary dendrites (Photo 1-a Figure 4).
Figure 3: Mean rat serum glucose level (mg/dl) in different groups (n. 8 rats in each group). Data are presented as mean ± SD. *# @ $: Statistically significant compared to corresponding value in control, reserpine, Low dose Metformin + Reserpine group, High dose Metformin + Reserpine, Selegiline + Reserpine group respectively. Level of significance is set at P value < 0.05. ML: Metformin low dose, MH: Metformin high dose, S: Selegiline

Figure 4: Shows photos of substantia nigra animals of control group 1(a,b), and that treated with reserpine 2 (a,b,c,d) where (1a) showed swollen perikaryon cells with short shrunken primary dendrites arrow (H&E X200) (1b) pyramidal or ovoid neuronal cells arrow (H&E X400), 2(a,b) showed neurodegeneration and necrosis of nigral neurons arrow (H&E X200) (2c) showed granular, pale-staining eosinophilic Lewy bodies(H&E X400) (2d) showed neuronal death, extracellular deposit Lewy bodies (H&E X400).

Their cell body had branching pattern of the dendrites. Mainly three thin dendrites emerged from the perikaryon was seen. The distal dendritic portions characterized by axon-like delicate filiform processes. Medium-sized fusiform, ovoid or pyramidal neurons were localized mainly in the pars compacta and pars reticulata (Photo 1-b Figure 4). Lewy bodies (− = absent or not discernible).

Parkinson’s disease induced reserpine group showed degeneration and necrosis of nigral neurons accompanied by reactive changes including astrogliosis and microglial cell activation (Photo 2-a, b Figure 4). The affected cortical areas have neuronal loss, spongiosis, and gliosis with swollen achromatic or ballooned neurons. The cortical substantia nigra usually shows moderate to severe neuronal loss with extra-neuronal neuro-melanin released from dead neurons.
Figure 5: photos of substantia nigra of animals treated with metformin low and high dose where 3 (a,b) are that of low dose in which (3a) showed neuronal degeneration arrow (H&E X200), (3b) neuronal necrosis with extra-cellular Lewy bodies arrow while 4(a,b) that treated with high dose in which (4a) showed mild degenerative changes of neuronal cells arrow (H&E X200) (4b) Swelling of neuronal cells with shorting of dendritic branches extra-cellular Lewy bodies arrow (H&E X400).

and was free within the neurophil and taken up by microglia cells. Lewy bodies which appeared as pale, granular bodies with ill-defined rounded areas of granular, pale-staining eosinophilic material that also seen in pigmented neurons of the substantia nigra and locus ceruleus (Photo 2-c Figure 4). In case of neuronal death, Lewy bodies detected as extracellular deposit in the neuropil (Photo 2-d Figure 4) Lewy bodies (+++ = severe).

Animals treated with (Metformin low dose) showed neuronal degeneration in substantia nigra (Photo 3-a Figure 5). Neuronal necrosis with extra-cellular Lewy bodies was seen (Photo 3-b Figure 5). Also, gliosis and spongiosis were observed with low grade in comparison with those treated with reserpine. Lewy bodies (+ = slight).

On the other side, Animals group treated with (Metformin high dose) showed mild degenerative changes of substantia nigra neuronal cells with relative giosis in comparison with animal’s groups treated with low dose metformin (Photo 4-a Figure 5). Swelling of neuronal cells with shorting of dendritic branches was seen (Photo 4-b Figure 5). Lewy bodies were detected in both extra/intra-neuronal cells. Lewy bodies (+ = slight).

Animals group treated with selegiline only showed mild degenerative changes of substantia nigra neuronal cells with giosis (Photo 5-a and b Figure 6) in comparison with animal’s groups treated with metformin high dose.

Ballooning of neuronal cells with shorting of dendritic branches was seen (5-b Figure 6). Lewy bodies detected in both extra/intra-neuronal cells. Few neuronal losses in the substantia nigra especially in the ventrolateral neurons of the pars Compacta. Lewy bodies (++ = moderate).

Animals group treated with selegiline with low dose metformin showed mild degenerative changes of substantia nigra neuronal cells with giosis (Photo 6-a and b Figure 6). Lewy bodies weren’t detected in both extra/intra-neuronal cells, Lewy bodies (− = absent or not discernible).

Animals group treated with selegiline with high dose metformin showed neurodegenerative changes characterized by neuronal swelling and shorting of dendritic branches (Photo 7-a Figure 6). Brain hemorrhage was seen in some examined animals (Photo 7-b Figure 6), Absence of Lewy bodies (− = absent or not discernible).

4. Discussion

Parkinson’s disease is a multisystem chronic, progressive neurodegenerative disorder which is presented with a wide variety of symptoms and signs that can greatly affect patients’ quality of life [24]. In the present study, the neuroprotective effects of metformin combination with selegiline (each one alone and in combination) in Parkinson disease (PD) model in rats which is the main neurotoxic effect of reserpine were investigated to identify their efficacy in ameliorating the condition either only symptomatically as detected by behavior tests or also improved biochemically and pathologically which wasn’t studied before. Reserpine blocks irreversibly the vesicular monoamine transporters 1 in the neuroendocrine and 2 in the neurons (VMAT-1 and VMAT-2) and by the blockade of VMAT2 prevents the uptake and decreases the store of monoamines (catecholamines; norepinephrine, dopamine, and serotonin) into the vesicle, resulting in their consumption in both central and peripheral neurons by their accumulation in the synaptic terminal subjecting them to monoaminoxidase that led to their metabolism in addition the body takes long time days to weeks to to replenish the depleted VMATs, so reserpine’s effects are long-lasting.
Figure 6: photos of substantia nigra of animals treated with selegiline where (5 a,b) are treated with selegiline only so that (5a) showed mild neurodegeneration and gliosis arrow (H&E X200) (5b) showed ballooning of neuronal cells with shorting of dendritic branches and Lewy bodies detected in both extra-neuronal cells arrow (H&E X400) while (6a,b) are those combined with low dose metformin where (6 a) showed degenerative changes of neuronal cells arrow (H&E X200),(6 b) swelling of neuronal cells with gliosis (H&EX400) and (7a,b) are those combined with high dose metformin in which (7a) showed neurodegenerative changes characterized by neuronal swelling and shorting of dendritic branches arrow (H&EX200) (7b) focal hemorrhagic area (H&EX200).

[25, 26]. So, reserpine was used to simulate parkinsonian manifestations

Our results were in accordance with Fernandes et al. [27] who reported that repeated administration of low dose reserpine (0.1 mg/kg) in rats induced a gradual appearance of motor signs, determined by catalepsy behavior also Neisewander et al. [28] observed that injection of rats daily with reserpine (1.0 mg/kg) for 6 weeks showed gradual alterations of motor behavior evaluated by measuring the times of occurrence protrusion of tongue. Similarly, Taylor et al. [29] showed that reserpine depleted biogenic amines such as norepinephrine, 5–hydroxytryptamine and dopamine in the brain with accumulation of α-synuclein [30]. Also, Fernandes et al. [27] stated that reserpine increased lipid peroxidation in the striatal, which represented damage of neurons by oxidative stress in addition to the presence of Lewy bodies at the substantia nigra and locus coeruleus in the pathological examination of rat brains this was in accordance with Schulz-Schaeffer [31] who confirmed that parkinson’s disease is usually associated with loss of substantia nigra neurons with the presence of Lewy body inclusions in some of the remaining neurons These represented the pathological signs present in the end stages of the disease.

Treatment with selegiline in our study was proved by previous studies which indicated that selegiline reversed memory impairments associated with aging [32] or drug administration [33]. Many different concepts might be related to increased cognition of selegiline as increased dopamine and norepinephrine in the synaptic cleft, which in turn enhanced consolidation of memory through activation of the cyclic AMP/protein kinase signaling pathway through the stimulation of beta-adrenergic and D1/D5 dopaminergic receptors [34]. Also, antioxidant actions of selegiline might contribute to its efficiency on memory impairment due to involvement of oxidative stress in the decline of cognition associated with disorders of Neurodegeneration [35]. This was in accordance to Bisht et al. [36] who declared that selegiline administration significantly decreased lipid peroxidation and nitrite concentration with increasing the activity of GSH in MPTP-treated rats. Besides, selegiline restored dopamine and its metabolite content as compared to the MPTP-administered group. Also, Rinne et al. [37] stated that the number of Lewy bodies were fewer in PD patients who had been treated with selegiline than those treated with levodopa. This suggested that selegiline treatment retarded the death of nigral neurons which was in agreement to our results.

On the other hand, Vaglini et al. [38] contradicted our results as they stated that pretreatment with selegiline
in the hippocampus. Moreover, Lu et al. [47] demonstrated that metformin encouraged neurogenesis in both human and rodent neurons by stimulating the atypical protein kinase C-CREB binding protein (PKC-CBP) pathway, which was important for differentiation of neural precursors. In another study by Chen et al. [48] in mice, found that metformin improved memory impairment, inhibited apoptosis of neurons and accumulation of amyloid beta (Aβ) in the hippocampus. Moreover, Lu et al. [41] proved the neuroprotective effects of metformin in PD by ameliorating MPTP-induced motor deficits which was detected through increased performance on a rotarod apparatus together with increased level of dopamine in the striatum. Also Guo et al. [42] reported that chronic treatment with metformin for 24 weeks improved cognition evaluated by the Wechsler Memory Scale–Revised. Similarly, Lu et al. [41] proved the neuroprotective effects of metformin in PD through increased dopamine level in the striatum in addition to 47.3% decrease of α-synuclein positive cells. Also, Ma et al. [43] proved that metformin was capable of decreasing malondialdehyde, as well as increasing superoxide dismutase activity. Patil et al. [13] also found significant decrease in the level of GSH in the MPTP group in comparison to normal mice which was elevated significantly by metformin pre-treatment.

Opposing to our results, Thangthaeng et al. [44] who observed that metformin had a harmful effect on visual acuity and spatial memory evidenced by reduced SOD activity in brain regions.

Based on the results of our study, reserpine and metformin treated groups showed non-significant changes in serum blood glucose level but hypoglycemia was observed in selegiline treated groups which was more profound in selegiline group than in groups combined with metformin (S+ML and S+MH groups). compared to the control group. Bacha & Klinepeter Bartz [45] agreed that metformin induced little effect on blood glucose in normoglycemic states without affecting the release of insulin or other islet hormones and so rarely causes hypoglycaemia except in cases of decreased feeding, alcohol administration or co-administration with anti-diabetic drugs causing hypoglycaemia [46]. Ibrahim et al. [47] reported a case of a patient with recurrent hypoglycemic events following the introduction of the MAOI rasagiline which was terminated after withdrawal of the medicine. Also, Rowland et al.[48] reported hypoglycemia with selegiline where it produced evident hypoglycemia in a 70 years old man with Parkinson disease. Similarly, Murad et al., [49] reported many drugs causing hypoglycemia, including MAOIs. In addition selegiline was concluded in the list of drugs that could cause hypoglycemia in Diabetes in Control.com [50].

Although animals treated with selegiline combined with metformin showed better pathological features, yet combination of selegiline with high dose metformin (250 mg/kg) used in our study revealed hemorrhage in pathological samples taken from rat brains.

There are some previous reports which mentioned that platelet function perversion was occurred by metformin and undesirable adverse effect in some cases especially those who take excessive unneeded dose. This was also agreed by previous studies that indicated the fibrinolytic effect of metformin by decreasing the activity of plasminogen activator inhibitor 1 [51]. Although bleeding as a side effect of metformin is rare, some cases suffered epistaxis and fatal gastrointestinal bleeding early in the course, especially with a high dose, more in patients aged more than 50-years [52].

This was potentiated by the use of selegiline as stated by Hayashi & Park [53] who demonstrated that monoamine oxidase (MAO) inhibitors are able to enhance the action of indirect sympathomimetics because they blocked the metabolic inactivation of the free cytoplasmic NE by that enzyme, which permitted greater build-up of NE increasing the level of circulating NE. This was in accordance to our results as treatment with high dose metformin together with selegiline caused cerebral hemorrhage seen in our pathological samples.

5. Conclusion
Metformin showed greater efficacy than selegiline and could be used in neuroprotection against Parkinson’s disease where it can be used early in the course of PD to retard the disease pathology, attenuate worsening in early PD and delay the onset of disability necessitating levodopa therapy. Also, metformin can serve as an add-on therapy later in the course of PD with selegiline especially in low dose. In addition, it was found to decrease the incidence of PD in diabetic patients receiving metformin as their anti-diabetic treatment compared to those receiving other anti-diabetic medications.

List of Abbreviations
Parkinsonian disease (PD), Monoamine oxidase inhibitors (MAOIs)
Research Involving Animals

All applicable guidelines for the care and use of animals were conducted with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines.

Conflict of Interest

The authors declare no competing interests.

Authors Contribution

Dr Ghada Hashem revised all the study, Dr Ghada Farouk supervised the practical work, shared in writing. Dr Walaa Ibrahim carried out the biochemical part and finally Monica did the practical work, helped in writing.

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