# INVESTIGATION OF THE POSSIBLE NEUROPROTECTIVE EFFECT OF AN ESTROGEN RECEPTOR BETA AGONIST AGAINST ROTENONE-INDUCED PARKINSON'S DISEASE IN RATS

**BY**

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## ABSTRACT

Parkinson's disease (PD) is the second most common neurodegenerative disorder. Androst- 5-ene-3β, 17β-diol (ADIOL), an estrogen receptor (ER) β agonist, was found to induce a transrepressive mechanism, which selectively amend the extent of neuroinflammation and, in turn, neurodegeneration; nevertheless, its effect on PD has not yet been revealed. In consequence, our study was designed to examine the possible neuroprotective effect of ADIOL against a rotenone (ROT)-induced PD in rats. Reduction in the nuclear factor-kappa B (NF-κB) levels and the expression of down-stream inflammatory mediators was detected in the SN upon pre-treatment with ADIOL at the dose of 0.35 mg/kg/day. Likewise, light microscopy (LM) examination showed improvement in the number of viable neurons in the SN pars compacta (SNpc). In conclusion, the current study confirmed the ability of ADIOL to reduce neuroinflammation and, in turn, neurodegeneration process as well as motor impairment in PD.

## INTRODUCTION

Parkinson's disease (PD) is the second most common progressive neurodegenerative disease after Alzheimer's disease. PD results from the progressive loss of dopaminergic neurons in concert with the accumulation of α-synuclein aggregates in the substantia nigra pars compacta (SNpc) (Dauer and Przedborski, 2003). This loss of dopaminergic neurons causes decline in striatal dopamine (DA), resulting in motor symptoms like bradykinesia, rigidity, tremors and postural instability (Gaig and Tolosa, 2009).

Few data is available concerning the epidemiology and prevalence estimates of PD in Egypt. In the study of Khedr et al. (2012), the overall crude prevalence of PD in the city of Assiut was 557 per 100,000 for all ages and 2,748 per 100,000 for individuals aged of 50 or more, where these rates were higher than in other Arab countries and several European countries. Another survey was carried out in Qena

governorate which showed that the crude prevalence rate of PD was 436 in every 100,000 persons for all ages, and 2534 per 100,000 aged 50 or more (Khedr et al., 2015). Additionally, the prevalence rate of PD in the district of Al Kharga was found to be 213.15 per 100,000 individuals aged 40 or more; showing lower rate than Nile valley governorates of Assiut and Qena. However, the prevalence of PD in Al Kharga was significantly higher in rural compared to urban areas (El-Tallawy et al., 2013).

The complexity of PD pathogenesis is accredited to different pathways involved in the neurodegeneration process. Beside the role of oxidative stress, mitochondrial dysfunction and ubiquitin-proteasome system (UPS) failure (Abou-Sleiman et al., 2006), neuroinflammation appears as an important player in the progression of neurodegeneration (Hirsch et al., 2012). This was supported by the release of inflammatory cytokines upon microglial activation such as IL-1 β, IL-6 and TNF-α (Naik and Dixit, 2011; Pelletier et al., 2012), which in turn can activate microglia aggravating neuroinflammation. As a result, mediators released from the microglia and astrocytes act together stimulating neurotoxicity, and hence neurodegeneration (Glass et al., 2010).

Androst- 5-ene-3β, 17β-diol (ADIOL), a major metabolite of dehydroepiandrosterone (DHEA) (Simard et al., 2005), was found to mediate a transrepressive mechanism through estrogen receptor beta (ERβ); where this ADIOL-ERβ-C-terminal binding protein (CtBP) repression pathway might reduce the severity and duration of inflammation, and consequently, neurodegeneration (Saijo et al., 2011). This was illustrated through the inhibition of expression of IL-6 in the microglia, like, and B-cell activating factor (BAFF) and inducible nitric oxide synthase (iNOS) in the astrocytes (Gosselin and Rivest, 2011). This tempted us to investigate the possible neuroprotective effect of ADIOL against rotenone (ROT)-induced PD in rats.

## MATEIALS AND METHODS

### Ethics statement

The study was performed in accordance with the ethical procedures and policies approved by Research Ethical Committee of Faculty of Pharmacy, Ain Shams University (Cairo, Egypt).

### Animals

Adult male Wistar rats (225 ± 20 g) were purchased from The Nile Company for Pharmaceuticals and Chemical Industries (Cairo, Egypt). Rats were allowed one week acclimatization period at the animal facility of Faculty of Pharmacy, Misr International University in standard polypropylene cages (four rats per cage). They were allowed free access to normal pellet diet (EL Nasr Pharmaceutical Chemicals Co., Cairo, Egypt) and tap water throughout the experimental period. All behavioral experiments were carried out in separate and isolated laboratories. The rats were kept under standard conditions of temperature (22 ± 2°C) and relative humidity (55 ± 5%) with 12-light/12-dark cycle.

### Experimental design

ADIOL (Steraloids Inc., Rhode Island, USA) dose (2.4 μmol/kg/day) was adopted from a previous study (Saijo et al., 2011), and converted to the equivalent dose in rat (0.35 mg/kg/day) (Freireich et al., 1966). ADIOL was suspended in a vehicle composed of 0.1% carboxymethyl cellulose, 2% polysorbate 80 and 0.1% metabisulfite

in phosphate buffered saline pH 7.4 (Nicoletti et al., 2010). ROT (Sigma-Aldrich, Missouri, USA) was emulsified in sunflower oil (Sigma-Aldrich, Missouri, USA) to a final concentration of 1.5 mg/ml (Bashkatova et al., 2004). Sixty rats were randomly divided into 3 groups, each containing 20 rats. The control group was injected the vehicle of ADIOL (1 ml/kg/day; s.c.), followed -one hour later-by sunflower oil (1 ml/kg/day; i.p.) for 30 days. The second group received ROT (1.5 mg/kg/day; i.p.) for 30 days (Bashkatova et al., 2004). The third group was pre-treated with ADIOL at the dose of 0.35 mg/kg/day; s.c., one hour before ROT injection (1.5 mg/kg/day; i.p.) for 30 days. The behavioral tests were performed for all rats in each group 24 hours after the last injections (Day 31) and were carried out between 9:00 AM and 2:00 PM.

At the end of the experiment, all animals were anesthetized with urethane (Thermo Fisher Scientific, New Jersey, USA) at the dose of 1.3 g/kg, i.p. (Guo et al., 2007) then each group of animals was divided into two subsets. Rats in the first subset were euthanized and their brains were quickly dissected and washed with ice cold saline, then tissue samples of SN were taken bilaterally, immediately weighed and stored at -80°C until assay of biochemical parameters. The second subset was euthanized and brain specimens were obtained after intra-cardiac perfusion of 20 ml of sterile physiological saline, followed by perfusion by 20 ml of 10% formalin. Each brain was coronally cut from the front to expose the striatum and also after removal of the cerebellum to expose the midbrain containing the SN, in accordance with Paxinos and Watson (2007). Then, brains were fixed in 10% formalin to be further processed to form paraffin blocks. Serial coronal sections of 5 µm thickness were obtained and subjected to histopathological examination.

**Methods**

Each rat was placed in the center of the open-field and was allowed to freely explore the area for 5 minutes, then observed for another 5 minutes. The test was done in a dark room and video recorded with Sony digital camera fixed to the open-field arena. The film was watched later and the following parameters were recorded: number of squares crossed (Zaitone et al., 2012), rearing frequency and inactive sitting (Castro et al., 2012).

**Determination of NF-κB**

Nigral levels of NF-κB were assayed using ELISA assay kit (EIAab Science Co. Ltd, Hubei, P. R. China). All procedures were done according to the manufacturers' instructions by means of Asys-Hitech GmbH (Eugendorf, Austria) microplate reader.

**Determination of iNOS and IL-6 by quantitative RT-PCR**

Total RNA was extracted from nigral tissues using RNeasy Kit (Qiagen, Hilden, Germany), and the tissue lysate was centrifuged for 3 minutes at 10,000 x g then the supernatant was taken. cDNA was synthesized from total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, California, USA) according to the manufacturer's guidelines. Quantitative real-time PCR was performed using SYBR® Green PCR Master Mix (Applied Biosystems, California, USA) as described by the manufacturer. The relative expression of target genes was obtained using the $\Delta\Delta$ $C_T$ method as described previously by Livak and Schmittgen (2001) using GAPDH as a housekeeping gene.

**Histopathology and Immunohistochemistry**

Five µm-thick paraffin sections were stained with hematoxylin and eosin (H&E) for histopathological examination. Morphometric studies were performed for the number of nerve cells in SNpc in H&E-stained sections. The measurements were performed using Leica QWin image analyzer software v2.8 installed on a Dell PC (Texas, USA) connected to the light microscope (Leica microsystems, Heerbrugg, Switzerland) in the Histology Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt.

**Statistical Analysis**

Parametric data were expressed as means ± SD and compared using the one-way ANOVA followed by Bonferroni's *post hoc* test. Non-parametric data were expressed as medians and interquartile ranges (IQR) and compared using the Kruskal-Wallis test followed by Dunn's *post hoc* test. Level of probability (*P* value) less than 0.05 was used as the criterion of significance. Normality was assessed by the Komogorov-Smirnov test. Statistical analysis was performed using the statistical software package GraphPad Prism®, Version 5.00 for Windows (California, USA).

**RESULTS**

**Behavioral test**

Compared to the control group, ROT caused 69.6% and 57.1 % decrease in the number of squares crossed ($P < 0.001$) and rearing frequency ($P < 0.01$), respectively, together with 11.8 folds elevation in inactive sitting duration ($P < 0.001$).

Compared to ROT-treated group, pre-treatment with ADIOL (0.35 mg/kg/day) caused 4.25 folds increase in number of squares crossed, with no significant effect upon rearing frequency. Concerning the inactive sitting duration, ADIOL showed significant decline from the ROT-treated group by 7.7 folds **(Figure 1)**.

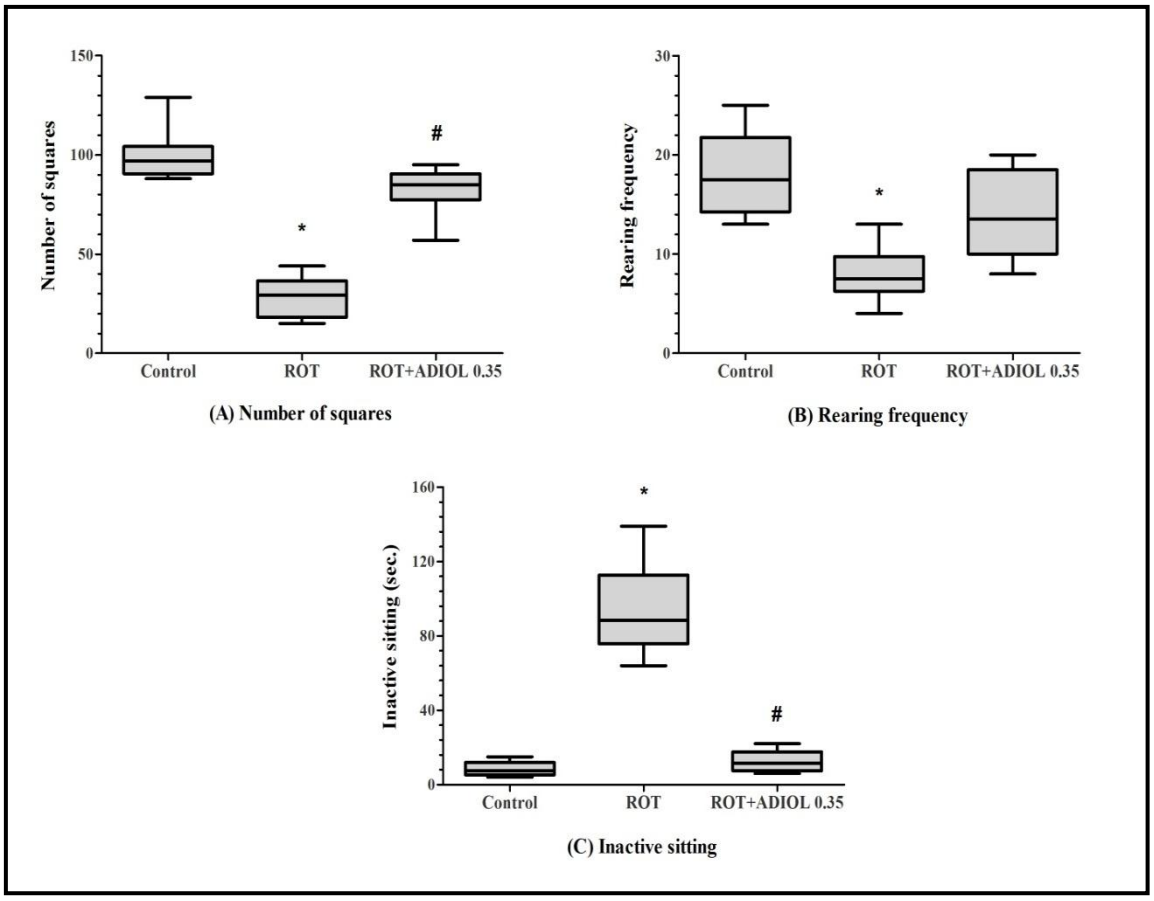


**Figure 1:** Box plots for open field test performed in groups of rats receiving vehicle (control), rotenone (ROT) (1.5 mg/kg/day; i.p.) and ROT + androst- 5-ene-3β, 17β-diol (ADIOL) 0.35 mg/kg/day; s.c., for 30 days. Values represent the median (IQR) of 20 rats. *, # vs. control and ROT using Kruskal-Wallis test followed by Dunn's multiple comparison test; $P < 0.05$.

## Biochemical parameters

### NF-κB concentration in the SN

As shown in **Figure 2**, the NF-κB was significantly elevated in the SN ($P < 0.001$) of ROT-treated group by 12.5 folds, in comparison to the control group.

Compared to the ROT-treated group, ADIOL significantly reduced the nigral NF-κB level by 58.4% **(Figure 2)**.

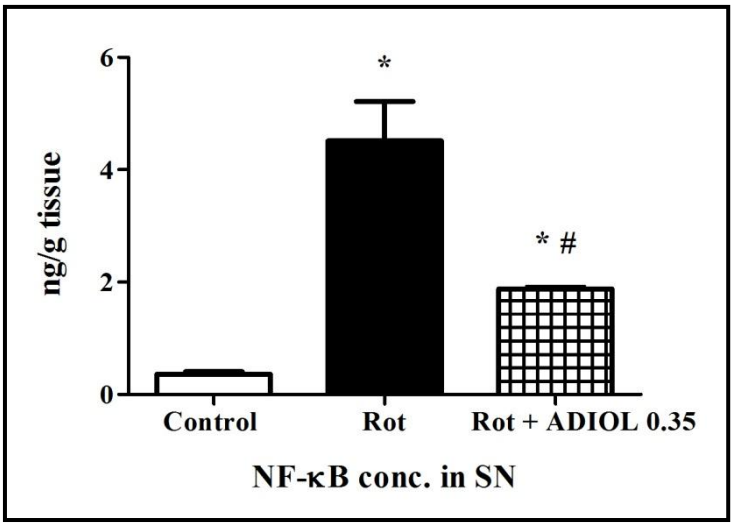


**Figure 2:** Effect of androst- 5-ene-3β, 17β-diol (ADIOL) (0.35 mg/kg/day; s.c.) on nuclear factor-kappa B (NF-κB) content in substantia nigra (SN) in rotenone (ROT)-induced (1.5 mg/kg/day; i.p.) Parkinson's disease (PD) in rats. Values represent the mean of 8 rats ± SD. *, # vs. control, ROT using one-way ANOVA followed by Bonferroni's multiple comparison test; $P < 0.05$.

*iNOS and IL-6 relative gene expression in the SN*

In the SN, ROT significantly increased iNOS ($P < 0.001$) and IL-6 ($P < 0.001$) gene expression by 9.4 and 7 folds, respectively, compared to the control group. These effects were significantly reduced via ADIOL as per the following percentages; iNOS (47%) and IL-6 (42.8%) **(Figure 3)**.

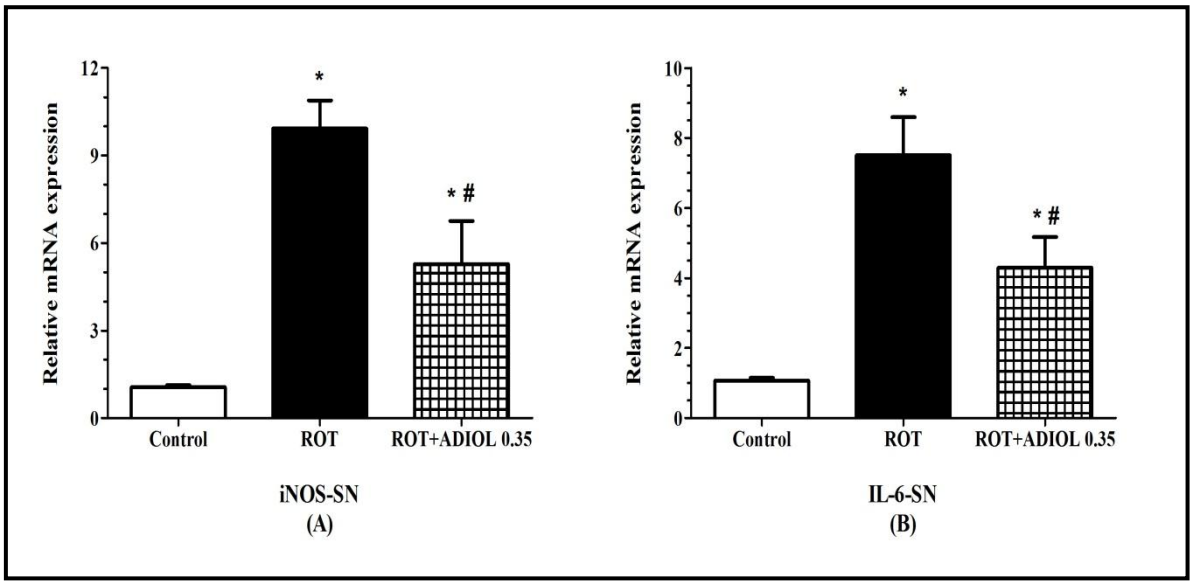


**Figure 3:** Effect of androst- 5-ene-3β, 17β-diol (ADIOL) (0.35 mg/kg/day; s.c.) on nigral gene expression of (A) inducible nitric oxide synthase (iNOS) and (B) interleukin-6 (IL-6) in rotenone (ROT)-induced (1.5 mg/kg/day; i.p.) Parkinson's disease (PD) in rats. Values represent the mean of 8 rats ± SD. *, # vs. control, ROT using one-way ANOVA followed by Bonferroni's multiple comparison test; $P < 0.05$.

**Histopathological results**

**Light microscopy examination of H&E-stained sections of SNpc**

Sections of the control group showed the SN divided into two parts. The dorsal part is the SNpc, a cell-rich region composed of medium and large cells of different shapes. The second part is the SN pars reticulata, a cell-poor region located ventrally close to the crus cerebri **(Figure 4A)**. Mean number of viable neurons was measured and illustrated in **Figure 5** ($P < 0.001$).

ROT-treated group showed degeneration of most neurons of the SNpc, which looked shrunken, with intensely stained cytoplasm and pyknotic nuclei **(Figure 4B)**. A Significant decrease in the number of nigral neurons by 79.3% was detected, as compared to the control group **(Figure 5)**.

Sections of the group pre-treated with ADIOL showed that many neurons were degenerated. However, some other cells were seen comparable to the control group **(Figure 4C)**. A 58.1% significant increase in neurons' number was detected, as compared to the ROT-treated group **(Figure 5)**.

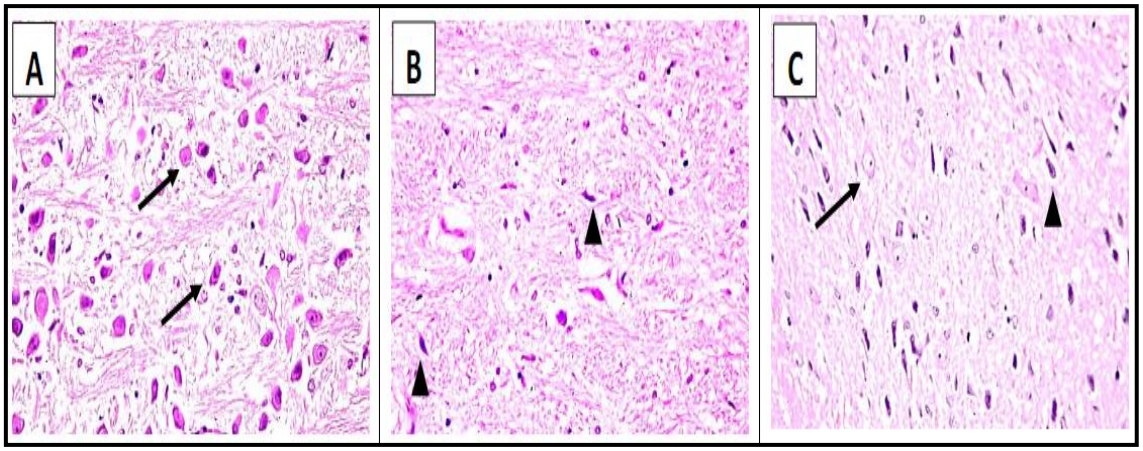


**Figure 4:** Micrograph showing hematoxylin and eosin (H&E) stained-sections of nigral neurons in rats. (A) Neurons of different shapes appear with their eosinophilic cytoplasm and vesicular nuclei (↑) in sections of the control (vehicle). (B) Obvious loss of neurons is evident in rotenone (ROT)-treated group (1.5 mg/kg/day; i.p.), with degenerated cells (▲). (C) Degenerated cells (▲) and apparently viable neurons (↑) is evident after ROT+ADIOL treatment (0.35 mg/kg/day; s.c.). *(H&E x 640)*

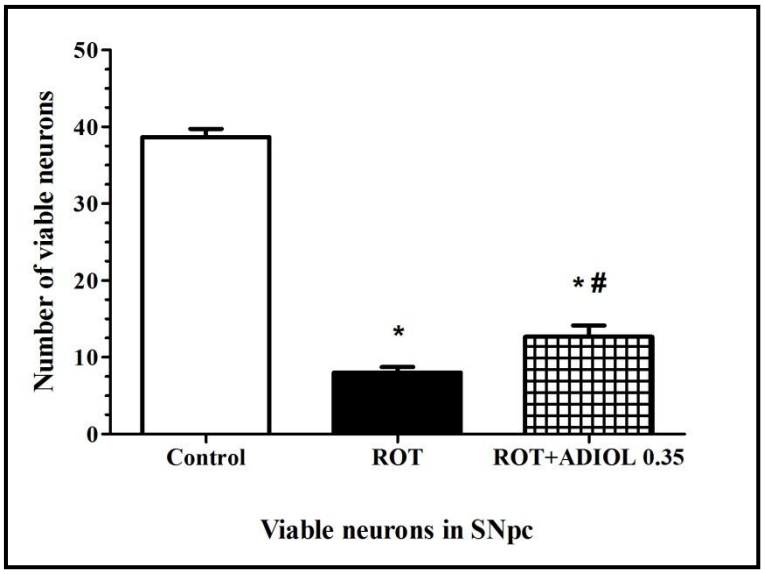


**Figure 5:** Histogram showing the mean number of viable neurons in the substantia nigra pars compacta (SNpc) of groups of rats receiving vehicle (control), rotenone (ROT) (1.5 mg/kg/day; i.p.) and ROT+ADIOL 0.35 mg/kg/day; s.c., for 30 days. Values represent the mean of 4 rats ± SD. *, # vs. control, ROT using one-way ANOVA followed by Bonferroni's multiple comparison test; $P < 0.05$.

## DISCUSSION

In order to detect the changes in neurodegeneration process in PD, histopathological examination was carried out to expose the effect of ADIOL upon the DA neurons in the SNpc. Upon LM examination of the SNpc of ROT-treated rats in the current study, degeneration of most neurons was noticed which was accentuated by the severe deterioration in the number of viable neurons. In agreement, LM examination of H & E stained sections of SN of rats receiving ROT showed prominent neurodegeneration with distorted, rod-shaped neurons (Angeline et al., 2012). Also, LM examination of H&E stained SN of rats injected with different doses of ROT in the striatum showed bigger number of degenerated neurons accompanied by attenuation of pigment granules from nigral cells especially with the high ROT dose (Abdel-Salam et al., 2014). In the current study, ADIOL managed to lessen the neurodegeneration in the SN that was detected in the ROT-treated rats.

Noticeable motor deficits become apparent upon progression of PD caused by the degeneration of DA neurons in the SNpc and exposed in the form of rigidity, tremors and postural instability. This was evident in the current study in the ROT-induced bradykinesia in the open field test, manifested through the decline in the number of squares crossed and rearing frequency and through the increase in the inactive sitting time. This was reinforced by the initial results obtained in the study of Bassani et al. (2014), when open field test was performed following injection of rats with ROT; where decrease in locomotor frequency and rearing frequency and prolonged immobility time was observed. Remarkably, this study showed contrasting results upon performance of open field test on day 38 of ROT administration; where locomotor frequency, rearing frequency and immobility time returned back to normal levels in the ROT-treated rats. The authors accredited these results to a possible compensatory neurochemical effect and neuroplasticity events trying to balance neuronal death in the

ROT-treated rats. Likewise, immobility time was increased with reduced rearing frequency upon injection of rats with ROT in a recent study (Abdelsalam and Safar, 2015). In our study, ADIOL was capable of increasing number of squares crossed as well as decreasing the time spent inactively sitting but failed to show change in rearing frequency. Thus, such behavioral results can be considered a sort of improvement following administration of ADIOL.

The ROT-induced neuroinflammation, a recognized contributor to the pathogenesis of PD, was revealed through the increase of NF-κB level which in-turn activated the signaling cascade of the downstream pro-inflammatory mediators, leading to enhanced expression of the iNOS and IL-6 in the SN of ROT-treated rats. The influence of these current results is that inflammatory response became evident; which in a positive feedback mechanism led to more activation of microglia. As a result, products released from both the microglia and astrocytes acted in a combined way ending up with neurotoxicity and thus neurodegeneration (Glass et al., 2010), which was formerly confirmed in our earlier results. Additionally, the study of Tapias et al. (2014) showed enhanced nigral iNOS expression upon i.p. injection of rats with ROT till motor impairment became established, while the study of Thakur and Nehru (2015) exposed elevated levels of NF-κB, and IL-6 in the midbrain upon s.c. injection of rats with ROT.

Overall, the findings of the current study offer an evidence for the neuroprotective effect of ADIOL, by preventing ROT-induced neurotoxicity and thus presenting a new viewpoint to reduce the neuroinflammatory cascade in the ROT-treated rats. This was revealed through the ability of ADIOL to decrease the level of NF-κB as well as the expression of the downstream inflammatory mediators; iNOS and IL-6 in the SN. These results confirm the previous hypothesis concerning the molecular mechanism of ADIOL; which stated that ADIOL could act through an ERβ-CtBP repression pathway leading to inflammatory response modulation**,** established through the inhibition of inflammatory genes expression like IL-6 in the microglia, and iNOS in the astrocytes (Saijo et al., 2011).

In conclusion, the current study proved the proposed hypothesis that ADIOL is capable of alleviating neuroinflammation and, in turn, neurodegeneration and the motor manifestations in PD. Therefore, ADIOL can be regarded as a novel promising therapy targeting PD. Still, further investigation is recommended before proceeding to clinical trials. Moreover, additional research using ADIOL could be directed towards other neurodegenerative diseases, in which neuroinflammation is an important player.

## FUNDING AND DISCLOSURE

The authors declare no conflict of interest.

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# التحقق من التأثير الواقي المحتمل للخلايا العصبية عن طريق ناهض مستقبل هرمون الاستروجين ضد مرض الشلل الرعاش المحدث تجريبيا بالروتينون في الجرذان

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مرض الشلل الرعاش هو ثاني أمراض التنكس العصبي الأكثر شيوعا. تم اكتشاف قدرة الأندروستينديول (أديول)، وهو ناهض لمستقبل هرمون الاستروجين بيتا، من تحفيز آلية تتمكن من تثبيط إلتهاب الخلايا العصبية، و بالتالي التنكس العصبي. و مع ذلك لم يتم دراسة تأثيره علي مرض الشلل الرعاش، مما أدي الي القيام بهذه الدراسة للتحقق من التأثير الواقي المحتمل للخلايا العصبية لدواء الأديول ضد مرض الشلل الرعاش المحدث تجريبيا بالروتينون في الجرذان. و قد أظهرت هذه الدراسة انخفاض مستوي العامل النووي-كابا بي وكذلك التعبير الجيني لوسطاء الإلتهاب المترتبة على العامل النووي-كابا بي في الجسم المخطط عند إعطاء الأديول بجرعة ٠,٣٥ ملج/ كج/ يوم. بالإضافة الي ذلك، أظهر الفحص المجهري للجسم المخطط زيادة في عدد الخلايا العصبية الحيّة عند إعطاء الأديول بنفس الجرعة. إجمالا، لقد تمكنت الدراسة الحالية من إثبات قدرة الأديول علي تخفيف إلتهاب الخلايا العصبية و بالتالي عملية التنكس العصبي و الإعاقة الحركية التي تحدث في مرض الشلل الرعاش.