Sexually Dimorphic Effect of Zonisamide on Behavioral Locomotor Activity in a Rat Model of Parkinson's disease

Ardeshir Moayeri, Zynab Khalili, Marzieh Darvishi*

Department of Anatomy, Faculty of Medicine, Ilam University of Medical Sciences, Ilam, Iran.

ABSTRACT

Introduction: Zonisamide (ZA) is a new drug that has been shown to be effective against motor damage in Parkinson's disease. However, the behavioral effect of ZA has not been described in male and female of rats. The aim of this study was to evaluate sexually dimorphic effects of ZA on behavioral locomotor activity in a rat model of Parkinson's disease. Materials & Method: 48 adult Wistar rats (24 male and 24 female) were randomly divided into eight groups, consisting of four groups of each sex: 1) sham group 2) PD group (Parkinson's model) 3) ZA group (vehicle) and 4) PD plus ZA group (Parkinson's model with ZA treatment). Parkinson's disease was administered by an injection of 6-hydroxydopamine (6-HD) into medial forebrain bundles. The drug was orally administered at a dose of 50 mg / kg every 24 hours for 7 days after surgery. The behavioral effects of apomorphine-induced rotation and spontaneous motor tests (elevated body swing, cylinder, and footfault test) were investigated in rats of both sexes. Results: ZA decreased contralateral rotations in male and female rats compared to the negative control group ($p < 0.05$) that is correlated with cell loss. ZA improves spontaneous motor tests in both sexes, and this finding is statistically significant. In the PD plus ZA group, the male and female comparisons demonstrated that step through latency in female was higher than that of the male ($p < 0.001$). Conclusion: These findings suggest that ZA has positive effects not only on motor and memory function but also on histological symptoms such as cell death in rat PD models.

Key Words: *Zonisamide, 6-Hydroxydopamine, Parkinson's disease.*

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INTRODUCTION

Parkinson's disease (PD) is one of the most common and progressive diseases in the central nervous system (CNS), characterized by bradykinesia, muscle rigidity, tremor, and postural instability [1-3]. The primary motor symptoms are regulated via a nigrostriatal dopaminergic pathway (NDP) [4, 5]. The main mechanisms of the disease have not yet been identified, but some studies have shown that formation of free radicals [6], mitochondrial or proteasomal dysfunction [7], microglial inflammation [8], and abnormal protein structures under the name of Levi's bodies are involved in PD [9]. Dopaminergic transportation occurs after intra-neuronal dopaminergic metabolism, leading to creation of oxidative free radicals with a potentially damaging effect on the central nervous system [10, 11]. Production of reactive oxygen species (ROS) may be a cause of cell death and other clinical symptoms of PD [12, 13]. These radicals can lead to fragmentation of DNA, lipid peroxidation and generation of protein carbonyls [13, 14]. In addition, dopaminergic transportation might be regulated by estradiol [15, 16]. Dopaminergic neuron (DN) survival is mediated by estradiol through IGF-1 and activation of the path of phosphatidylinositol 3-kinase (PI3K)/Akt (PKB) [17, 18]. This path regulates apoptotic and anti-apoptotic proteins to increase cell survival [17-19]. Numbers and reactivity of microglia, similarly to astrocytes, modulate by gonadal steroids [20, 21]. It has been verified that activation of microglia through LPS can lead to the damage of DA-ergic neurons by the release of toxic factors, which can be prevented by use of estradiol for suppression of microglia [22, 23]. The gold standard of treatment for PD includes dopamine replacement with dopaminergic agonists (levodopa) and monoamine

Corresponding author: Marzieh Darvishi
Address: Department of Anatomy, Faculty of Medicine, Ilam University of Medical Sciences, Ilam, Iran.
E-mail: ✉ Darvish-m @ medilam.ac.ir
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oxidase B inhibitors [24]. These drugs improve motor disturbances but do not affect degenerated dopaminergic neurons. However, these strategies are associated with an increased risk of side effects such as dyskinesia, vomiting, headache, fatigue, and tremors with disease progression and administration of daily levodopa dose [25-27] In recent years, new therapeutic approaches for pharmacological therapy of PD focus on decreasing daily levodopa dose administration. Zonisamide (ZA) is a benzisoxazole that is used clinically as an anti-epileptic agent and has been available as an anti-Parkinson's drug [28]. Although the mechanism of ZA has not been fully elucidated, ZA is proposed to act against PD through both dopaminergic and nondopaminergic pathways [29, 30]. Previous research has found that acute administration of ZA increased intracellular and extracellular levels of dopamine in the striatum by inhibiting monoamine oxidase type B (MAO-B) activity [31, 32]. MAO-B inhibitors are agents demonstrated for prolonged levodopa treatment. These agents promote synaptic dopamine levels and its decay and also increases expressed tyrosine hydroxylase (TH) that may improve motor impairments through dopamine synthesis [33-35]. This protective effect of ZA may reduce activation of T-type Ca2+ channels [36], voltage-dependent Na+ [37], and GABA-ergic transmission [38]. In addition, administration of low doses of ZA increases the cysteine transport system in astrocytes which induces glutathione production as an antioxidant agent [39]. In existing studies, there are no published data about the sexually dimorphic effect of ZA on behavioral activities in Parkinson's model of rats. The present study was conducted to clarify the actions of ZA in vivo to investigate the effects of the ZA on locomotor activity in male and female Parkinson's model of rat.

MATERIALS AND METHODS

Experimental animals

Male and female albino Wistar rats weighing 200- 250 g were used. Animals were housed under standard conditions of humidity, air quality and light (12 h light–dark cycle), and had free access to chow and water ad libitum. All procedures were done in accordance with the Ethical Committee for animal care use of laboratory animals at the Ilam University of Medical Science, Ilam, Iran.

Experimental design

Forty-eight adult rats of both sexes (twenty-four each) were divided into eight groups, four of each sex. These groups consisted of group one: sham group (treated with saline (oral) during the last 7 days); group two: PD group (negative control:Lesion in the medial forebrain bundle (MFB) with 6-HD; group three: ZA group (healthy rat that received 50 mg/kg doses of ZA (oral) for the last 7 days) and group four: PD plus ZA group (the rat of Parkinson's model with 6-HD treated with 50 mg/kg (oral) ZA during the last 7 days.

Surgical procedures

To achieve unilateral lesions of the nigrostriatal system (NS), the rats were anesthetized with an intraperitoneal injection of a mixture of ketamine: xylazine (80 mg/kg: 10 mg/kg) and placed on a stereotaxic frame (ear bars fixed symmetrically). The scalp was incised and the skull was exposed. A hole was drilled above the right MFB to inject a single dose of 6-HD solution (4µg 6-HD per µL in 0.01% ascorbic acid, pH = 5 (Sigma)). This toxin was injected into the right MFB using a Hamilton syringe at the following coordinates: AP: −4.0 mm; ML: ±1.5 mm; DV: −8.5 mm; tooth bar: ±0mm relative to bregma. The injection is done with a rate of 0.5 min and for prevents reflux the needle was left in place for another 5 min; finally, the skin was sutured [40].

Behavioral assessment

According to the previous study, the use of this 6-HD dose causes an effective massive DA depletion and behavioral deficits. However, the behavioral tests achieved two weeks after lesion over a period of eight weeks in order to identify the most suitable predictor of nigral cell loss.

I. Drug-free behavioral test

Elevated body swing test (EBST)

The EBST was done before the drug-induced rotation test. The test was performed every two weeks at weeks 4, 6 and 8 post-lesion. Borlongan and Sanberg described the test protocol in 1995: each rat is held 3 cm from the base of its tail and suspended 3 cm above the ground for 30 s. in the vertical axis. The number of left and right-biased swings is recorded. In the present study, the rat was lesioned in the right hemisphere, however, a biased swing to the left observed. Left biased swinging behavior was calculated as follows: L/ (L + R) (%) (L = number of left-biased swings, R = number of right-biased swings) [41].

Cylinder test

The cylinder test is used to evaluate the forelimb movements and akinesia following by unilateral neurological deficits. In this case, the ability of animals to bear weight on the inner wall of a glass cylinder (20 cm in diameter, 30 cm in height) was examined. The test was carried out at 4, 6 and 8 weeks post-lesion. The instruments used in the cylinder test include a glass cylinder, two mirrors, and a camera. Animals are placed individually in a clear glass cylinder to determine motor asymmetry. Forelimb movement is recorded through an experimenter who is blinded to the condition groups. The number of forelimb contacts on the cylinder wall are counted (R: the number of right forelimb touch; L: the number of left forelimb touch (L/R+L+(R+L) ×100) [42].

Footfault test

Sensorimotor scoring was carried out pre and at week 4, 6 and 8 post- lesion using footfault test. The rats were observed walking on a grid. The test evaluates the ability of animals to bear weight on a grid, which measures and analyzes the paw fall or slip between the wire for each right and left forelimb and hindlimb. The finding was contralateral to the lesioned side [43].

Passive avoidance test (PAT)

The effects of ZA on learning memory in rats were investigated using a passive avoidance test. This protocol performed by the shuttle box which it has a light (200×250×200 mm) and a dark (200×150×200 mm) chamber and separated by a shutter door. In addition, the floor of the apparatus consists of a stillness grid with a stimulator for the production of electrical shock. The procedure carried out on two stages of habituation and training. In the first stage of habituation, the animals are placed in an illuminated chamber and after 20 seconds, they are transferred into a darkened area and then returned to the home cage. The second habituation is similar to the initial stage. In training, after the rats enter the dark chamber, the door is closed and a foot shock is given (0.4 mA for 1.5 sec). This step is repeated again after two minutes. On the second day, the latency to cross the door (Step-Through Latency=STL) and time spent in a dark compartment (TDC) was measured without foot shock [44].

II. Drug-induced rotational behavior

Apomorphine -induced rotation

This test was conducted at 4, 6 and 8 weeks post-lesion and after EBST. Apomorphine was injected subcutaneously at a dose of 0.5 mg/kg. Each rat was placed in a glass bowl with a diameter of 20 cm to perform the test assay, which has an automated rotometer. After 10 min habituation, their motor asymmetry was recorded over 60 min [45].

Tyrosine hydroxylase immunostaining

In the eighth week of the experiment, the animals were anesthetized by using ketamine/xylazine and then perfused with normal saline followed by 4% paraformaldehyde in PBS. The sections were dehydrated (twice for 30 min on 70, 95 and 100% ethanol) and cleared in xylene. The sections were embedded in paraffin and sectioned with 7µm thick. They were then immunostained with the primary antibodies rabbit anti-TH monoclonal antibody (1: 500; Abcam) and incubated overnight at $4C^0$. After washing in PBS, the sections were incubated with the goat anti-rabbit secondary antibody conjugated with FITC (60 min). The cross-sections were showed and counted at 200× magnification.

RESULT

The elevated body swing test (EBST)

Figure 1 shows the percentage of left biased swings 4, 6 and 8 weeks after the MFB lesion in male and female rats. This finding demonstrated the percentage of left-biased swings 4 weeks after the MFB-lesion was significantly higher than that of the PD group (88.11±2.03 % male; 86.7±2.7 female) compared to the group of PD plus ZA in both sexes (77±1.8% male; 75.5±2.1% female). In addition, 6 weeks after the lesion, the percentage of left-biased swings in PD plus ZA male and female rats was 74.43±0.56% and 69.6± 1.74% (n = 6) respectively. Finally, at week 8, the percentage of left-biased swings in PD plus ZA male and female rats declined compared to 6 week. This is also significantly lower compared to PD rats and compared between the two sexes; the data showed a significant decrease in female in comparison with males (Figure 1).

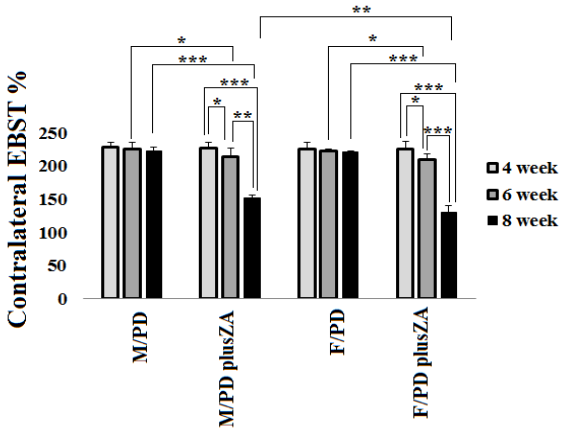


Figure 1. 6-HD induced left-biased swinging behavior in the elevated body swing test (EBST) after MFB lesioning. M/PD (male rat with MFB lesioning), F/PD (female rat with MFB lesioning), M/PD plus ZA (male rats with MFB lesioning and treat with 50 mg/kg ZA) and F/PD plus ZA (female rats with MFB lesioning and treat with 50 mg/kg ZA). Data are expressed as % mean ±SEM; n= 6 each group; *P < 0.05, ** P < 0.01, *** P < 0.001)

Cylinder test

Figure.2 shows the effect of ZA on forelimb asymmetry at 4, 6 and 8 weeks post-lesion. The finding demonstrated that ZA significantly increased the activity and movement of the forelimb (**p >0.01 and *** p >0.001) at 4 weeks after lesion compared with the non-treatment group (PD). These results were observed in both male and female rats. However, the percentage of contralateral touches in female rats was significantly higher than in males. Similar results were showed at 6 and 8 weeks; from the 4th to the 8th week, recovery increased. The control group of ZA (non- injury that received 50 mg/kg dose of ZA) did not significantly affect the forelimb movement compared to the sham group (Figure 2).

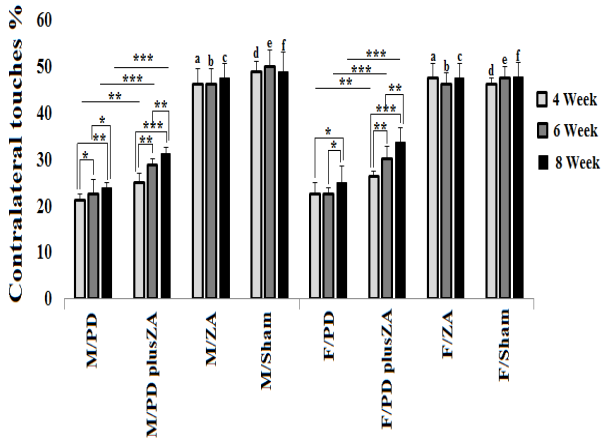


Figure 2. Histogram chart representing the total number of right forepaw contacts as a percentage of the total number of forepaw uses in the cylinder test at 4, 6 and 8 weeks post- lesion. M/PD (male rat with MFB lesioning), F/PD (female rat with MFB lesioning), M/PD plus ZA (male rats with MFB lesioning and treat with 50 mg/kg ZA), F/PD plus ZA (female rats with MFB lesioning and treat with 50 mg/kg ZA), M/ZA (non-injury that received 50 mg/kg dose of ZA in male rats), F/ZA (non- injury that received 50 mg/kg dose of ZA in female rats), M/Sham (sham group of male rats) and F/Sham (sham group of female rats). a-f indicate non significanty percentage contralateral touches on ZA group comparison with sham group (P>0.05). Data are expressed as % mean ±SEM; n= 6 each group; *P < 0.05, ** P < 0.01, *** P < 0.001).

Footfault test

Figure 3 presents the effect of ZA on forelimb and hindlimb footfault pre and post- lesion by 6-HD. The result demonstrated that PD rats significantly increased the occurrence of forelimb (p <0.05) and hindlimb (p < 0.05) footfaults at 4, 6 and 8 weeks post-lesion compared with intact rats. These results were similar for both sexes. Administration of ZA significantly reduced the number of forelimb and hindlimb footfaults on 4, 6 and 8 weeks compared to the PD group (p <0.05). In addition, comparison between the results of both sexes revealed that the number of forelimb and hindlimb footfaults of female rats was less than that of the males, and this difference was statistically significantly. In addition, the sham group did not show a significant effect in forelimb and hindlimb footfaults compared to the ZA group (p >0.05).

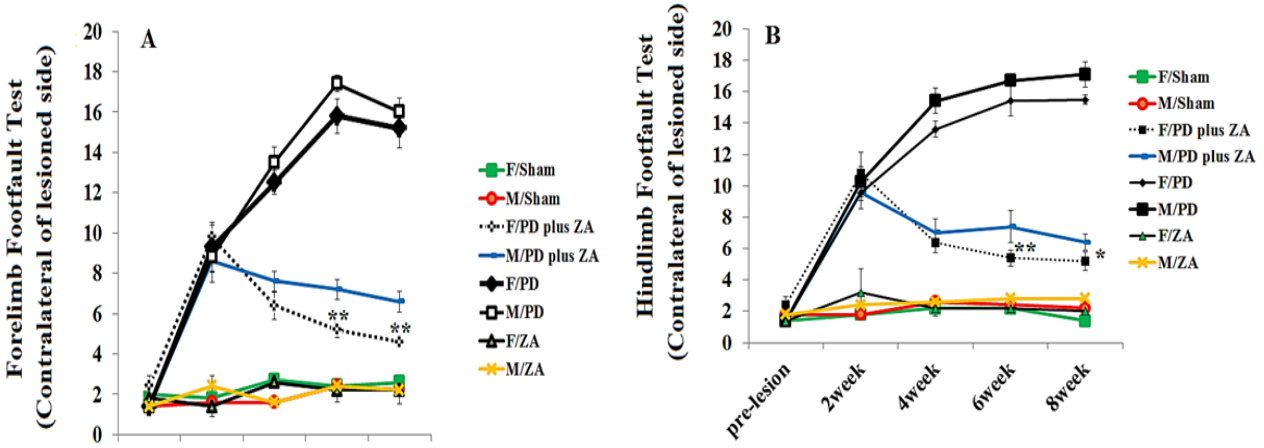


Figure 3. 6-HD administration increased the hindlimb and forelimb footfult on contralateral lesion side. On the contralateral side, the 50 mg/kg ZA exhibited less footfult than the PD group. The ZA groups also determined no significant different on hindlimb and forelimb footfult from the sham group on the contralateral side. *P_0.05, **P_0.01 compared to the PD group.

Apomorphine -induced rotation

Four weeks after lesioning, the male and female rats exhibited about 228±1.5 and 225±0.4 contralateral rotations in 60 min, respectively. In addition, the number of these rotations increased in weeks 6 and 8, and was not significantly different in males and females. Treatment with ZA significantly reduced the number of contralateral rotations at 6 and 8 weeks post-lesion compared to the PD group (p <0.05). However, there was no significant different between the treatment (PD plus ZA) and non-treatment (PD) groups at week 4 (p >0.05). In addition, there was a significant difference in the number of rotations in the 8th week in the male and female rats (p >0.01) (Figure 4).

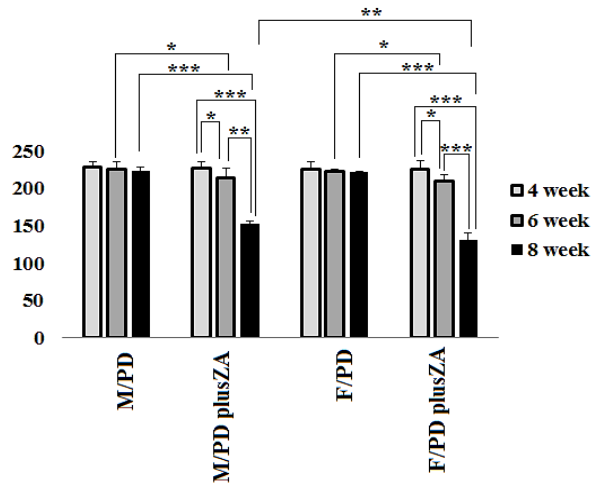


Figure 4. Effects of treatment with ZA on 6-HD induced loss of nigrostriatal neurons on apomorphine-induced rotation. M/PD (male rat with MFB lesioning), F/PD (female rat with MFB lesioning), M/PD plus ZA (male rats with MFB lesioning and treat with 50 mg/kg ZA) and F/PD plus ZA (female rats with MFB lesioning and treat with 50 mg/kg ZA). Data are expressed as mean ±SEM; n= 6 each group; *P < 0.05, ** P < 0.01, *** P < 0.001).

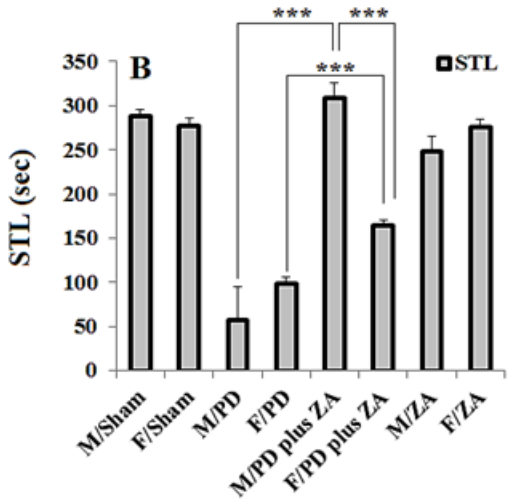
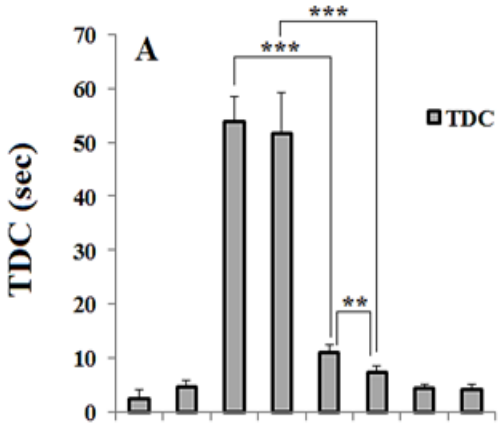


Figure 5. Indicated step through latency (STL) and time spent in dark compartment (TDC). Data are expressed as mean ±SEM; n= 6 each group; ** P < 0.01, *** P < 0.001.

Passive avoidance test (PAT)

We found a significant difference in STL and TDC between the ZA and PD groups. In the sham group, results showed that STL had the highest rate. In contrast, TDC had the lowest value compared to other experimental groups. In addition, there was no significant difference between the two sexes. In the treatment group (PD plus ZA group), the male and female comparisons showed that STL in females was higher than that of the males (p <0.001), and this difference was significant compared to sham and PD groups (p >0.05). These findings are in contrast to the results of TDC and the low level of this index is seen in the female group (p >0.01) (Figure 5).



After TH immunostaining, counting of the dopaminergic neuron in the substantia nigra (SN) and ventral tegmental area (VTA) was performed on five sections of experimental groups. The number of immunopositive cells on SN/VTA was counted in these sections (Figures 6 and 8). This finding demonstrates a correlation between apomorphine-induced rotation and TH-cell loss in male and female rats (Figure 7).

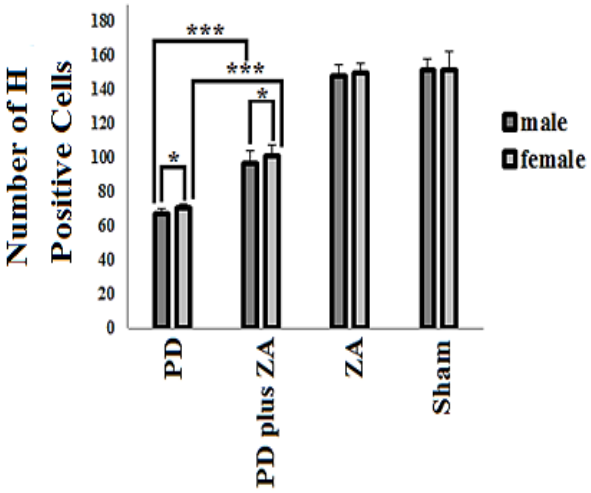


Figure 6. Average number of TH stained cells in substantia nigra (SN) and ventral tegmental area (VTA). PD (male and female rat with MFB lesioning), PD plus ZA (male and female rats with MFB lesioning and treat with 50 mg/kg ZA), ZA (male and female rats treat with 50 mg/kg ZA only) and sham. Data indicated as mean ±SEM; *P < 0.05, and *** P < 0.001.

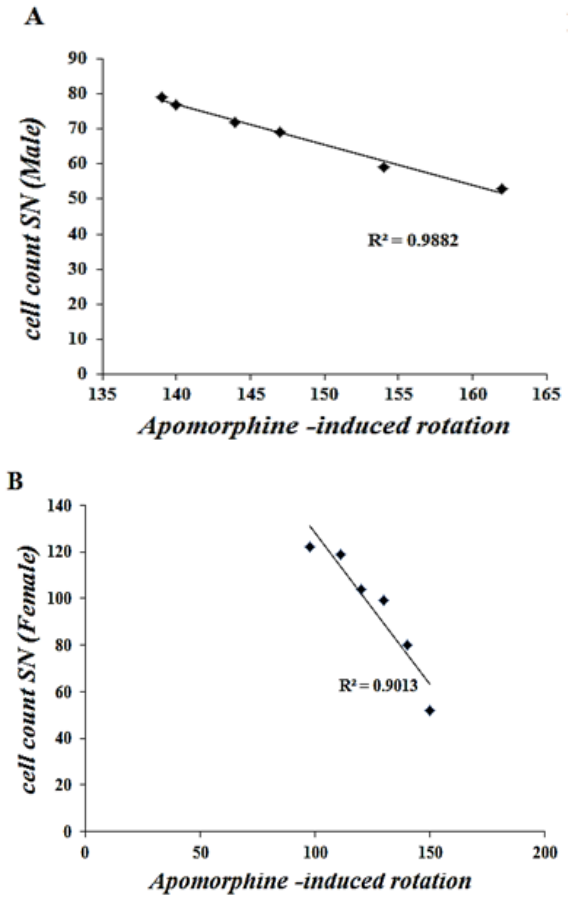
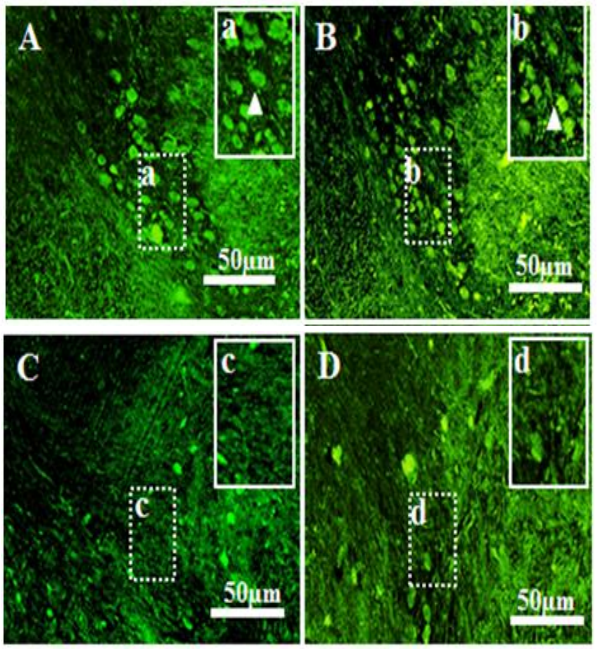


Figure 7. Scatter plots demonstrating the correlation between Apomorphine -induced rotation and the TH-cell loss in male (A) and female of rats (B). Apomorphine –induced rotation enhanced linearly with the loss of TH positive cells in male and female of rats ($P < 0.01$).



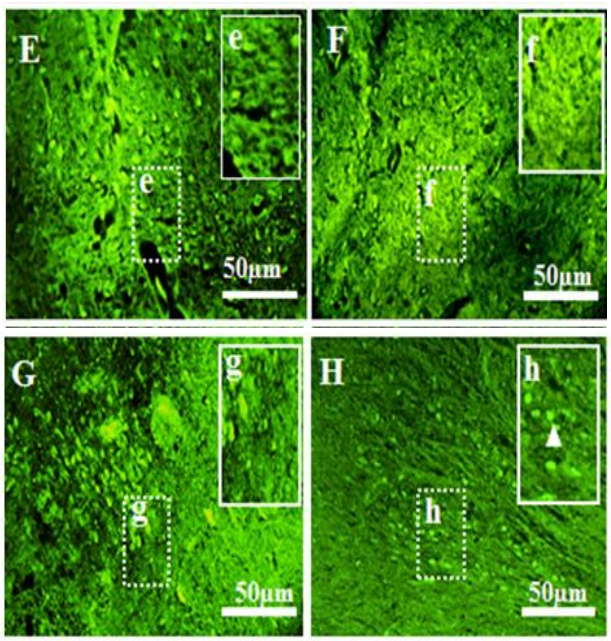


Figure 8. Effect of 6-HD and ZA administered in the MFB on the number of TH-positive cells in the substantia nigra (SN) and ventral tegmental area (VTA). F/Sham (A), M/Sham (B), F/PD (C), M/PD (D), F/ZA (E), M/ZA (F), F/PD plus ZA (G) and M/PD plus ZA (H). The lowercase represent a high magnification. The white triangle indicates TH-positive cells.

DISCUSSION

In this study, we investigated the ZA declined loss of DN in the substantia nigra (SN) and ventral tegmental area (VTA) that this result is an agreement with the behavioral assessment. Previous studies reported ZA enhanced expression of TH and activated dopamine synthesis in MPTP-treated mice [30]; our findings showed that ZA increases dopaminergic neurons after injecting 6-HD unilaterally into the MFB. The finding of this study showed that ZA administration in both genders significantly decreased the intensity of apomorphine-induced rotation, and improved in EBST, cylinder, footfault, and passive avoidance tests. In addition, the results showed improvement in females was higher than that seen in males, and this difference was statistically significant. However, several studies have shown that free radical release by 6-HD leads to impairment in the brain motor area [46, 47]. It appears ZA reduces motor disturbance through free radical removal. Akinesia is being developed to reduce dopamine [48]. In a study by Choudhury et al., behavioral assessment in animals found that ZA enhanced motor function and significantly inhibited MAO in the neuron and astrocyte [49]. Through MAO inhibition, ZA suppresses dopamine circulation and increases motor activity [50]. Apomorphine-induced rotation tests in PD models can be used to characterize the extent of the discharge of dopamine and to detect

therapeutic effects of ZA [51]. The ZA activates dopamine synthesis and prevents dopamine quinone formation [52]. Also, this drug reduced loss of dopaminergic neurons and neurotoxicity of α-synuclein in animal models of PD, thus leading to motor improvement in rats [29, 30]. We found a relationship between apomorphine-induced rotation and nigral cell counts. Furthermore, rats with the highest mean percentage of nigral TH-immunopositive neurons recorded the lowest rotation scores over repeated testing. This finding is in agreement with other studies [53, 54].

ZA through its antioxidant activity has a neuroprotective effect as well as an effect on GABA receptors in improving the motor function in the PD models [55]. ZA via RNA expression of brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and tropomyosin receptor kinase can protect spinal motor neurons in motor neuron disease and muscle atrophy [56]. The *in vitro* findings confirm the effect of ZA on mouse embryo primary culture [57]. We also found that the test of the cylinder can be a sign of cellular loss. It is used to evaluate forelimb asymmetries in animals by investigating their rearing and leaning their forehand against the wall of a cylinder. We analyzed the weight-bearing forelimb use and simultaneous use of both limbs for landing. In addition, we found this test predicted dopaminergic neuron loss. In this study, posture bias in unilaterally lesioned rats was determined by EBST. The result found a correlation with other behavioral tests such as cylinder and drug-induced rotation. We found that ZA in male and female tended to decrease swing contralateral to the side of the lesion. In agreement with previous reports higher EBST has been seen in female rats compared to male animals [58]. The estrogen hormone seems to be effective in different baseline on EBST. Lindgren et al. have shown that 6-HD induced memory impairment, executive dysfunction, and visuospatial deficits [59]. Mura et al. found that a bilateral injection of 6-HD on MFB can damage the learning in Morris water maze, as well as disturbance of spatial memory, examined in an open field hole board test [60]. Results from the present study showed that ZA cause improvement of non-spatial memory assessed by the passive avoidance test since the TDC decreased significantly in male and female rats compared to the PD group. Similarly, in agreement with this finding, results showed that ZA can lead to enhanced STL in treated groups. However, significant improvement in memory functions was observed neither in short-term nor in long-term memory.

CONCLUSION

In conclusion, the administration of ZA improved motor and memory function in male and female rats in PD models. Our present results showed neuroprotective effects of ZA on the viability of dopaminergic neuron and correlation with behavioral disorder induced by 6-HD. These findings suggest that ZA has therapeutic effects on motor symptoms and cellular death in PD.

Ethics

This study is approved by the Ilam University of Medical Sciences Ethics Committee (IR.MEDILAM.REC.1395.106).

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