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Effect of WR-1065 on 6-hydroxydopamine-induced catalepsy and IL-6 level in rats

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ABSTRACT

Objective(s): Neuroinflammation and oxidative stress play a key role in pathogenesis of Parkinson's disease (PD). In the present study we investigated the effect of reactive oxygen species (ROS) scavenger WR-1065 on catalepsy and cerebrospinal fluid (CSF) level of interleukin 6(IL-6) and striatum superoxide dismutase (SOD) activity in 6-hydroxydopamine (6-OHDA) induced experimental model of PD.

Materials and Methods: Seventy two male Wistar rats were divided into 9 equal groups and 6-OHDA (8 µg/2 µl/rat) was infused unilaterally into substantia nigra pars copmacta (SNc) to induce PD. Catalepsy was measured by standard bar test, CSF level of IL-6 was assessed by enzyme-linked immunosorbent assay (ELISA) method and SOD activity measured by spectrophotometric method. In pre-treatment groups WR-1065 (20, 40 and 80 µg/2 µl/rat/day, for 3 days) was infused into the SNc before 6-OHDA administration and 21 days later, as a recovery period, behavioral and molecular assay tests were done.

Results: Our results showed that pre-treatment with WR-1065 improved (P<0.001) 6-OHDA-induced catalepsy in a dose dependent manner. In 6-OHDA-lesioned animals SOD activity in SNc and CSF level of IL-6 was decreased markedly (P<0.001) when compared with non-lesioned group, while pre-treatment with WR-1065(P<0.001) restored their levels up to the normal range.

Conclusion: Our study indicated that pre-treatment with WR-1065 could modulate catalepsy and IL-6 level in 6-OHDA-lesioned rats. Also WR1065 could increase SOD activity up to normal range. It can be regarded as an anti-oxidative drug in prevention or adjunctive therapy of PD.

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Introduction

Parkinson's disease (PD) is a movement disorder characterized by resting tremor, rigidity, bradykinesia, and postural instability and it often leads to non-movement dysfunction such as cognitive decline (1). Many investigations have been performed to detect the pathology and molecular mechanisms of PD (2). Progressive degeneration of dopaminergic neurons of substantia nigra pars compacta (SNc) is main cause of disease (3).

Reactive oxygen species (ROS) such as superoxide anions, nitric oxide and hydrogen peroxide that can be produced by mitochondrial respiration and other cellular processes are among the cellular toxic products that play crucial role in pathogenesis of PD (4). Over production of these ROS lead to neural cells damage or progressive neurodegeneration (2). Oxidative stress occurs due to impairment of balance between ROS and anti-oxidant enzyme activity such as SOD (5). It is well known that 1-methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine (MPTP) and 6-hydroxydopamine (6-OHDA) induce experimental model of PD by ROS production (6). Previous investigations have shown that many of substances with anti-oxidant effects such as vitamin E, C and A, co-enzyme Q10 (7), and silymarin (8) , have preventive effects in PD.

Oxidative stress stimulates immune response via receptors such as pattern recognition receptors (PRRs) and toll like receptors (TLRs) that lead to synthesis of pro and anti-inflammatory cytokines. These cytokines stimulates secretion of oxidative and nitrosative stress-inducing factors such as NO and superoxide. Uncontrolled and chronic activation of immune response implicated as a mechanisms of oxidative stress while short-term activation believed to be neuroprotective (6). Neuroinflammatory process

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begins with activation of glials and T-cells that leads to over expression of pro-inflammatory cytokines and other molecules involve in oxidative and inflammatory condition (9). Although release of these factors is typically intended to prevent further damage, over production of them causes toxic effects that mediates neuronal death and results in worsening of disease progression (10). The studies have confirmed that over production of pro-inflammatory cytokines occurs in the most cases of PD along with decrease of anti-inflammatory cytokines and anti-oxidant enzymes i.e. superoxide dismutase (SOD) and glutathione peroxidase (GPx) (11). So these two factors (inflammation and oxidative stress) have a relationship that every of them can causes other one initiate or increase (11). IL-6 is one of the cytokines identified as a B-cell differentiation factor capable of inducing the maturation of B-cell in to the antibody-producing cells. This cytokine involves not only in immune system but also in neurogenesis and neuroprotection in normal and injury models (12). It has been shown that level of IL-6 alters in Parkinson and other neurodegenerative disease and restoring its level to normal range displays protective effects (8, 12-14). It has been proposed that IL-6 has two different receptors that expressed in cell membrane. These two different types of receptors are responsible for Pro- and anti-inflammatory effects. It may be the reason that IL-6 exerts two different activities i.e. neurogenesis and neuroprotection beside pro-inflammatory effect (12).

Pharmacotherapy in PD is now generally based on dopamine providing to treat motor manifestations of disease (15). Most of these treatments are associated with serious complications such as levodopa-induced dyskinesia (LID) (16). It has proposed that one of the probable mechanisms in LID is converting dopamine to 6-hydroxy dopamine by oxidative mechanisms (17). Thus, the use of anti-oxidant drugs to prevent or treat PD is considered by most researchers. Since one of the main factors in PD etiology is ROS formation so it can be proposed that ROS scavengers will attenuate neuronal degeneration in PD (18).

WR-1065 is an aminothiol that acts as a free radical scavenger (19). WR-1065 is a cytoprotective drug with mechanism of ROS scavenging that first developed in US Army as a potential drug to protect against ionizing radiation damage (20). It has been shown that WR-1065 acts as an oxygen free radical scavenger in some oxidative stress situations which are consistent with impairment of SOD, GSH, and Catalase activity. Chronidou *et al* showed its beneficial effects on spinal cord ischemia in rabbit (19). They found that WR-1065 in rabbits with spinal cord induced ischemia significantly attenuated oxidative injury in their spinal cord (19). Other study showed that WR-1065 has protective effects on oxidative stress arising from radiation or chemotherapy inducing apoptosis in CNS (21). WR-1065 by scavenging ROS not only protects cells from DNA damaging but also stimulate DNA repair by mechanism of anoxia induction (20). However, its effect on motor complication associated with Parkinson's disease has not been studied up to now. The aim of present study is to investigate the effect of WR-1065 on 6-hydroxydopamine (6-OHDA)-induced catalepsy, CSF level of IL-6 as a marker of neuroinflammation and also SOD activity as the indicators of oxidative damage in brain of rat model of PD.

Material and Methods

Chemicals

6-OHDA, desipramine, WR-1065, protease inhibitor, Triton, EDTA, SDS and Tris HCl were purchased from Sigma Aldrich (St. Louis, MO, USA). Interlukin-6 ELISA rat kit was bought from abcam co. (France). The kit and drugs was stored in -20 ˚C until the day of examination. Solutions were made freshly on the days of experiment by dissolving drugs in normal saline (9%NaCl). 6-OHDA and WR-1065 were injected into the right SNc by infusion pump at the flow rate of 0.2 µl/min.

Animals and treatment protocol

Male Wistar rats (180-220 g) were used in this study. The animals were given food and water *ad libitum* and were housed in standard polypropylene cages, two per cage at an ambient temperature of 25± 2 °C under a 12 hr light/12 hr dark cycle. Animals were kept in laboratory for 5 days to habituate with environmental condition. Then training was done for each one by bar test to prepare for examination 2 days before behavioral test. Then healthy rats that their elapsed time in bar test was in normal range were included in our study. All the experiments described in this study were in accordance with the guide for the care and use of laboratory animals (National Institute of Health Publication No.80-23, revised 1996) and were approved by the Research and Ethics Committee of Tabriz University of Medical Sciences.

In the beginning of study only the rats that showed normal immobilization in bar test were subjected to further experimentation. Seventy two Wistar rats were divided randomly into 9 equal groups: 1) Control group received no injection and were left untreated for the entire period of the experiment as intact animals; 2) Sham operated group that were subjected only to surgical procedure; 3) Vehicle (saline)-treated group that were received 2 µl saline (intra-SNc); 4) lesioned group that were received 6-hydroxydopamine (8 µg/2 µl/rat, intra-SNc); 5) Vehicle + 6OHDA group that rats were received saline as a vehicle 3 days once daily (2 µl/rat) before 6-OHDA injection; 6to 8) rats in these groups were pretreated with intra-SNc injection of WR-1065 (20, 40 and 80 µg/2 µl/rat) 3 days before 6-OHDA injection; 9) non-lesioned animals which were received intra-SNc injection of WR-1065 (80 µg/2 µl/rat) for three days.

Surgical procedures

The animals were anesthetized by intraperitoneal (IP) injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). After the rats were deeply anaesthetized (loss of corneal and toe pad reflexes), they were fixed in a stereotaxic frame (Stoelting, Wood Dale, IL, USA) in the flat position and stereotaxicaly injected with 6-OHDA into SNc through a 23 gauge sterile stainless steel guide cannula. The coordinates for this position were based on the rat brain atlas (22): anteroposterior from bregma (AP) = -5.0 mm, mediolateral from the midline (ML) = 2.1 mm and dorsoventral from the skull (DV) = -7.7 mm. Desipramine (25 mg/kg, IP) was injected 30 min before intra-SNc injection of 6-OHDA to avoid degeneration of noradrenergic neurons. We fixed cannula in right SNc with dental cement and kept rats in their cage for 4 days to recover the injury and inflammation raised from surgery. Then 6-OHDA (8 µg per rat in 2 µl saline with 0.2% ascorbic acid) was infused by infusion pump at the flow rate of 0.2 µl/min in to the right SNc.

Cannula verification

For confirmation of placement of cannula in the SNc, at the end of experiments all rats with guide cannula were euthanized by a high dose of ether and decapitated. The brains with the injecting tube in situ were removed and placed in a formaldehyde (10%) solution. After 1 week, the tissues were embedded in paraffin. Then serial sections (3 µm) were cut with a microtome (Leitz, Germany), and the placement of the tip of the cannula in the SNc was microscopically controlled. Data from rats with an incorrect placement of the cannula were excluded from the analysis.

Behavioral study

Three weeks (21 days) after the 6-OHDA injection catalepsy was evaluated by standard bar test. Anterior limbs of rat gently extended on 9 cm high bar (0.9 cm in diameter) and the duration of retention of rats in this imposed posture was considered as the bar test elapsed time. The end point of catalepsy was designated to occur when both front paws were removed from the bar or if the animal moved its head in an exploratory manner. The cut off time of the test was 720 sec and the test was done in four consecutive times with one hr interval (time 5, 60, 120 and 180 min). All tests and observations were performed between 9 AM and 4 PM.

CSF sampling

Animals were anesthetized by IP injection of ketamine and xylazine as explained before and mounted in a stoelting stereotaxic frame and skull was kept in 45 ° position after shaving the surface of the neck region and swabbing with ethanol (70%). Then the colorless CSF sample was slowly extracted in a volume of 100 µl by a needle (Sterile scalp vein-23) was put horizontally and centrally into the cisterna magna for CSF collection. The CSF samples were stored frozen at –70 °C until the day of assessment by enzyme-linked immunosorbent assay (ELISA) method.

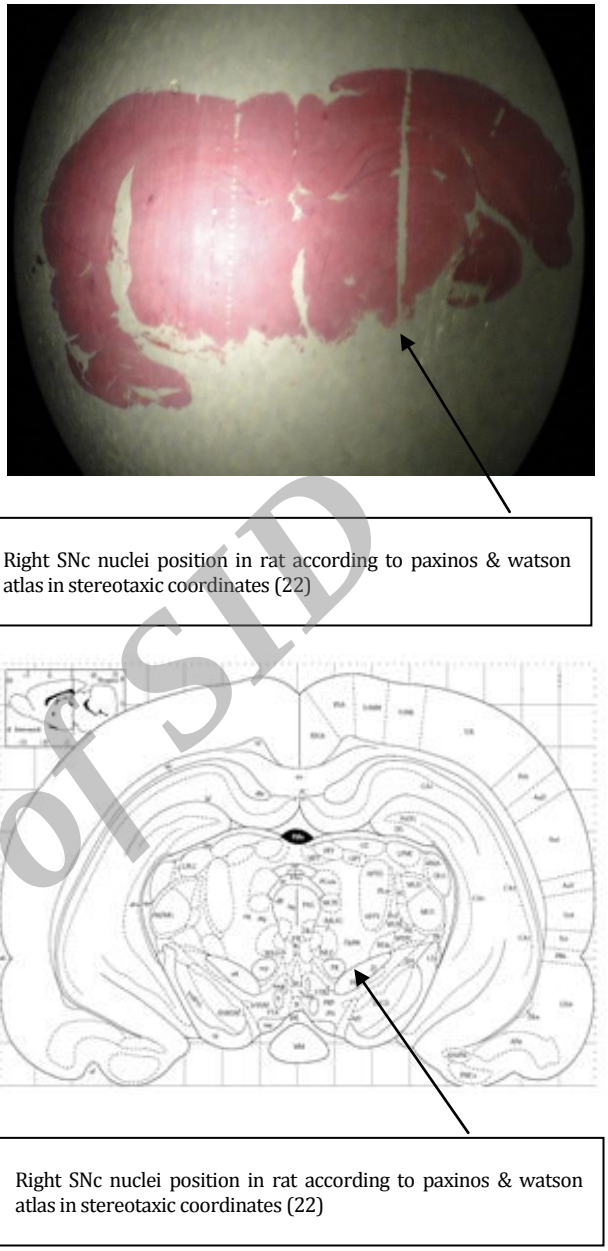

Figure 1. Photomicrograph section of rat brain. Site of the cannula is represented according to the paxinos & watson atlas in stereotaxic coordinates (22). Anteroposterior from bregma (AP)=-5.0 mm

SNc sampling

After CSF sampling all rats were euthanized by a high dose of ether and decapitated. The brain extracted and desired brain regions (midbrain, SNc) were rapidly removed, separated, cleaned and immediately frozen in liquid nitrogen and then were kept frozen at -70 °C until the day of SOD assessment (8).

IL-6 assay

To assay IL-6 level we used commercial ELISA rat kit (Abcam CO. France). Briefly, the frozen CSF samples were diluted, added into the wells and incubated at room temperature for 150 min on a microplate shaker.

After washing, diluted streptavidin-horseradish peroxidase-conjugated anti-mouse IL-6 were reacted for 60 min at room temperature on microplate shaker. After washing again, the wells were developed with tetramethyl benzidine (TMB) for 30 min and the optical densities were read at 450 nm with an ELISA reader. IL-6 concentration (pg/ml) was measured according to standard curve.

SOD assay

Activity of SOD was estimated according to Kakkar *et al* (1984) method (23). In this regard the reduction in amino blue tetrazolium formazan formation is measured in the mixture containing nicotinamide adenine dinucleotide (NAD) H, phenazinemethosulfate (PMS) and nitrobluetetrazolium (NBT). Intensity of the color was measured spectrophometrically at 560 nm (23, 24).

Statistical analysis

Statistical analysis of each data set was calculated by use of Prism software. Data were expressed as the mean±SEM, and were analyzed by one-way ANOVA in behavioral and biochemical experiments. In the case of significant variation (P<0.05), the values were compared by Tukey test.

Results

6-OHDA-induced catalepsy

Five groups of rats were scheduled as: Control (normal), sham operated, vehicle (saline), WR1065 (80 µg/2 µl/rat) and 6-OHDA treated groups. Drugs or saline were injected into the SNc through the implanted guide cannula. As it has been shown in Figure 2, 6-OHDA was able to induce significant (P<0.001) catalepsy in comparison with normal and sham-operated, WR1065 and vehicle-treated rats (Figure 2).

Effects of WR-1065 on 6-OHDA induced catalepsy

Four groups of 6-OHDA-lesioned rats received vehicle or one of the three different doses of WR1065 (20, 40 and 80 µg/2 µl/rat), respectively for 3 days before 6-OHDA administration. The result showed that WR1065 attenuated the severity of 6-OHDA-induced catalepsy (P<0.001) when compared with 6-OHDA-lesioned rats. Also it has been observed that WR1065 improved catalepsy in dose dependent manner (P<0.001) (Figure3). No significant data was found between each doses of WR1065 in different times (Figure 4).

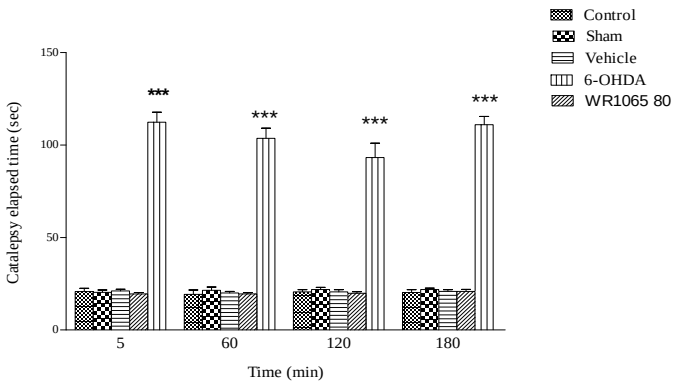

Figure 2. The result of bar test in control, sham-operated, vehicle injected, 6-OHDA(8 µg/2 µl/rat)-lesioned and WR1065 (80 µg/2 µl/rat) treated rats . Each bar represents the mean±SEM of catalepsy elapsed time (sec); n=8 rats in each group; ***P<0.001 when compared with control, vehicle injected and sham-operated groups

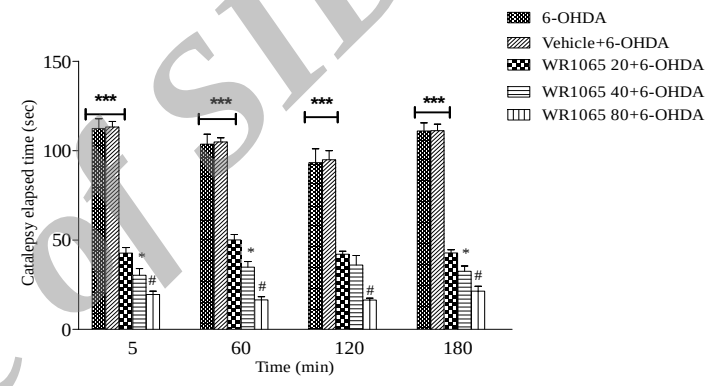

Figure 3.. The result of bar test in 6-OHDA (8 µg/2 µl/rat)-lesioned rats pre-treated with vehicle and WR1065 (20, 40 and 80 µg/2 µl/rat intra-SNc for 3 days). Each bar represents the mean±SEM of catalepsy elapsed time (sec); n=8 rats in each group; ***P<0.001 when compared with WR1065 treated groups; *P<0.001 when compared with WR1065 20 treated group; #P<0.001 when compared with WR1065 40 treated group

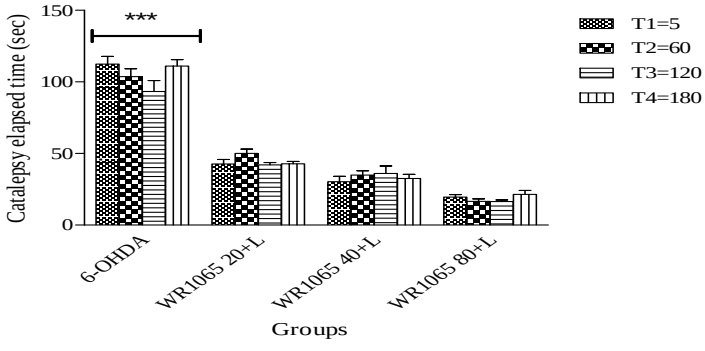

Figure 4. The result of bar test in 6-OHDA (8 µg/2 µl/rat)-lesioned rats pre-treated with WR1065 (20,40and 80 µg/2 µl/rat, intra-SNc for 3 days). Each bar represents the mean±SEM of catalepsy elapsed time (sec); n=8 rats in each group; ***P<0.001 when compared with WR1065 treated groups. (L means 6-OHDA)

Effects of WR1065 on CSF level of IL-6 in 6-OHDA-induced hemi-parkinsonism

As it has been shown in Figure 5, 6-OHDA markedly decreased (P<0.001) IL-6 level in 6-OHDA lesioned rats when compared with control, sham operated and vehicle (saline) groups. No significant difference was found in comparison between vehicle (saline), sham and control groups. In 6-OHDA-lesioned rats which were pre-treated with WR1065 (20, 40 and 80 µg/2 µl/rat intra-SNc for 3 days before 6-OHDA) the CSF level of IL-6 was more (P<0.001) than 6-OHDA (L) group.

Effect of WR1065 on SOD activity in SNc of 6-OHDA-induced hemi-parkinsonism

According to the results (Figure 6) SOD activity were decreased markedly (P<0.001) in SNc of 6-OHDA-lesioned rats compared with control, sham and vehicle groups. Pretreatment with three different doses of WR1065 (20, 40 and 80 µg/2 µl/rat) for 3 days before 6-OHDA administration, significantly (P<0.001) elevated SOD activity and restore it to normal ranges compared with 6-OHDA lesioned rats.

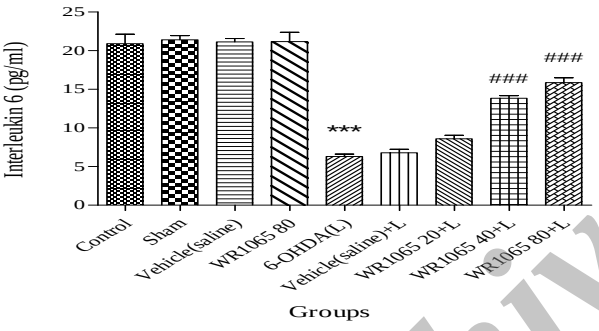

Figure 5. The CSF level of cytokines IL-6 in the control, sham, vehicle, WR1065 (80 µg/2 µl/rat), 6-OHDA and 6-OHDA-lesioned groups pre-treated with WR1065 (20, 40 and 80 µg/2 µl/rat intra SNc) for 3 days before 6-OHDA. Each bar represents the mean±SEM of cytokines concentration in the CSF (pg/ml) n=4 rats in each group;***P<0.001 when compared with control, sham and vehicle (saline) groups; ###P<0.001 when compared with 6-OHDA rats. (L means 6-OHDA)

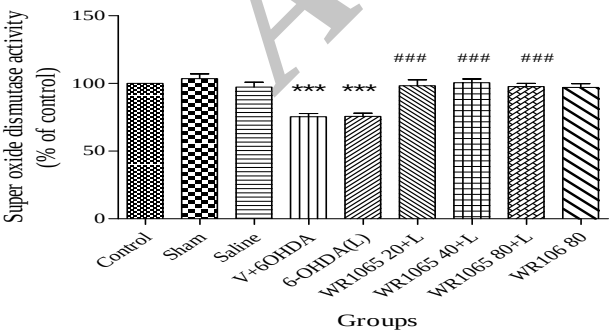

Figure 6. Effect of intra-SNc administration of WR1065 (20, 40 and 80 µg/2 µl/rat), for 3 days before 6-OHDA administration on SOD activity in SNc, compared with 6-OHDA lesioned groups. Each value represents the mean±SEM. ***P<0.001 when 6-OHDA lesioned group compared with control, sham and vehicle group. ###P<0.001 when treated groups compared with lesioned group using one way ANOVA with tukey *post hoc* test (L means 6-OHDA)(n=4)

Discussion

In the present study, we investigated the effects of pre-treatment with WR1065 (free radical scavenger) on 6-OHDA-induced catalepsy, CSF level of IL-6 and striatum Superoxide dismutase (SOD) activity in hemi-parkinsonian rats. Several line of evidences show that oxidative stress and neuroinflammation have a fundamental roles in PD pathogenesis (3, 6). Oxidative stress promotes inflammation and reversely, inflammation increases oxidative effects (11). Previous researches indicate that administration of agents with anti-oxidative properties such as vitamins (E, C, A) (7), silymarin (25), co-enzyme Q10 (7) prevent motor impairment and molecular changes in PD.

In this study we used WR-1065 as a strong free radical scavenger to test its beneficial effects in parkinsonian rats. Previous studies confirmed that WR-1065 as an anti-oxidant agent reduces oxidative stress and inflammation in central nervous system but none have shown its effects on experimental model of PD (19-21). According to our results unilateral injection of 6-OHDA was able to create catalepsy. This result is in agreement with our previous report showing catalepsy inducing effect for 6-OHDA (26). 6-OHDA is a neurotoxin which is used commonly to induce animal model of PD by producing ROS. It cannot cross Blood brain barrier (B.B.B) so it must be injected to directly into the SNc (6). Catalepsy, as a manifestation of PD, is measured by standard bar test technique. Our results showed that pre-treatment with WR-1065 improved 6-OHDA-induced catalepsy in a dose dependent manner. It has been shown that WR1065 through hypoxia up- regulates the expression of proteins involve with DNA repair and inhibition of apoptosis and results in biosynthesis of anti-oxidant enzymes (20). According to this study WR-1065 protects dopaminergic neurons in SNC from damaging and apoptosis by neurotoxin (6-OHDA) so we could observe reduction in catalepsy elapsed time when compared with no-treated hemi-parkinsonian rats.

We assessed level of IL-6 in the CSF of parkinsonian rats pre-treated with different doses of WR1065. Results showed that CSF level of IL-6 decreased significantly in 6-OHDA induced hemi-parkinsonism in compared with control group. This is in agreement with the findings of Sharifi *et al* showed that IL-6 level decreased in 6-OHDA hemi-parkinsonian rats (13). On the other hand, IL-6 level increased up to normal rang by pre-treatment with WR-1065. Cytokines are the part of immune system that mediate inflammatory process. But in the case of IL-6, it has been recognized that IL-6 also has anti-inflammatory, neurogenesis and neuroprotection effects. These two effects depend on underlying mechanism of IL-6. It has two signaling pathway, one is classic signaling by which it stimulates target cells via a membrane bond bound receptors leading to anti-inflammatory activity; and second

pathway named trans-signaling that needs soluble form of IL-6 to act via gp130 on the cell surface that is responsible for pro-inflammatory effects. Target cells differ in IL-6 receptor on their cell surface, so some kinds of cells express IL-6 receptor while others display gp130 on their surface. These two different pathway leads pro-inflammatory and anti-inflammatory effects of IL-6 (12).

Previous data demonstrate that WR1065 can elevate the expression of mnSOD (27). Other studies showed that balance impairment between ROS generation by mitochondrial respiration and anti-oxidant enzyme activity including SOD and GSH leads to increment of oxidative stress (11). In this study we observed that 6-OHDA injection to SNc decreased SOD activity and pre-treatment with WR1065 increased SOD activity to its normal range. According to the results potent anti-oxidant activity can be considered as a possible treatment for PD. It can postulate that the anti-neuroinflammatory effects of WR1065 in 6-OHDA induced hemi-Parkinsonian rats, partially belongs to its oxidative stress suppression by the drug.

Conclusion

We can suggest that pre-treatment with WR-1065 could improve catalepsy as motor complications of PD through modulation of SOD activity and IL-6 level near to their normal physiological level as one of the possible protective properties beside other mechanisms that should be investigated. It may be used as adjunctive therapy along with routinely used anti-parkinsonian drugs to delay progression of disease. However, further molecular and clinical researches are needed to prove this hypothesis.

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