

Ellagic Acid Protects the Brain Against 6-Hydroxydopamine Induced Neuroinflammation in a Rat Model of Parkinson's Disease

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ABSTRACT

Introduction: Neuroinflammation may play as an important risk factor in progressive degeneration of dopaminergic cells. Antioxidants have protective effects against free radicals-induced neural damage in Parkinson's disease (PD). In the present study, we examined the effects of ellagic acid (EA) on locomotion and neuroinflammatory biomarkers in a rat model of PD induced by 6-hydroxydopamine (6-OHDA).

Methods: 6-OHDA (16 µg/2 µl) was injected into the right medial forebrain bundle (MFB) in MFB-lesioned rat's brain. Sham group received vehicle instead of 6-OHDA. PD-model was confirmed by rotational test using apomorphine injection. EA (50 mg/kg/2 ml, by gavages) was administered in PD+EA group. One group of MFB-lesioned rats received pramipexole (PPX; 2 mg/kg/2 ml, by gavages) as positive control group (PD+PPX group). Motor activity was assessed by stride length and cylinder tests. The levels of TNF-α and IL-1β were measured in both striatum and hippocampus tissues.

Results: MFB lesion caused significant reduction of stride-length (P<0.001) and also increased the contralateral rotations (P<0.001) and score of the cylinder test (P<0.001). Use of 6-OHDA to induce the PD significantly increased the levels of TNF-α (P<0.001) and IL-1β (P<0.001) in MFB-lesioned rats. EA significantly restored all of the above parameters.

Discussion: EA can improve the motor impairments in the MFB-lesioned rats via reducing the neuroinflammatory biomarkers and protect the brain against free radicals-induced neural damage. The results suggest that EA can be helpful in management of PD treatment.

1. Introduction

The main pathological feature of Parkinson's disease (PD) is the progressive loss of dopaminergic cells in the substantia nigra (SN) resulting in wide spectrum of motor impairments such as resting tremor, muscle rigidity, reduced motion and disturbed gait performance (Philippens, Hart, & Torres, 2010). The oxidation of dopamine

(DA) generates reactive oxygen species (ROS) and an imbalanced production of ROS, leading to oxidative stress and neuronal loss. These free radicals react with membrane lipids and cause lipid peroxidation (LPO) and cell death (Esposito et al., 2002). Also, it has been shown that deficiency of antioxidants such as vitamins (A, C, E and niacin) increase the risk of PD (Chaturvedi et al., 2006). Although the etiology of the PD is not fully understood, factors like genetic vulnerability, mitochon-

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drial dysfunction, free radicals production, environmental toxins and brain aging cause oxidative stress (OS) (Moreira et al., 2010).

Human clinical imaging, epidemiological and post-mortem investigations have revealed that chronic inflammation may play as an environmental risk factor to promote progressive degeneration of dopaminergic cells (Tansey & Goldberg, 2010). Thus, neuroinflammation has received raised attention for neuroprotective therapies in PD and two pro-inflammatory cytokines including interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF α) have been involved as chief effectors of the functional consequences of neuroinflammation in PD models (Leal, Casabona, Puntel & Pitossi, 2013).

In spite of the vast efforts of researchers, there is no definite treatment for PD. Present treatments are based on pharmacological strategies that pursue DA restoration in the striatum for example; administration of DA precursors or agonists (Obeso et al., 2010). There have been many efforts to develop valuable medical plants to achieve neuroprotection. Attention has been focused on a wide variety of natural antioxidants that can scavenge the free radicals and protect neurons from oxidative damage and inflammation. Various antioxidative supplements have been shown to play important role in neuroprotection (Hwang et al., 2011). Pomegranate (*Punica granatum* L.) which is a polyphenolic fruit, has been extensively used in traditional medicine. Pomegranate extracts protects neurons against the neurotoxic effects of 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). The possible mechanisms of this effect could be associated with their phenolic constituents and antioxidant activities. Pomegranates enriched by phytochemicals such as polyphenols (including phenolic acids and flavonoids), have shown antioxidant properties and can inhibit inflammation and other harmful processes involved in neurodegenerative diseases. Moreover, pericarp of pomegranate is enriched with tannins such as gallic acid and ellagic acid, other potent antioxidants (Braidly et al., 2013).

Ellagic acid (2,3,7,8-tetrahydroxybenzopyrano [5,4,3-cde]benzopyran-5-10-dione) is an excretion product of many plant species, particularly fruits and nuts (Wada & Ou, 2002) such as strawberries, cranberries, walnuts, pecans, and red raspberry seeds (Whitley, Stoner, Darby, & Walle, 2003). It has been reported that they have some different pharmacological anti-inflammatory and antioxidant properties (Lei et al., 2003). Therefore, it can be valuable approach in new researches and PD management.

In this study we have investigated the effects of ellagic acid on motor disturbances and neuroinflammatory biomarkers in a rat model of PD induced by 6-OHDA.

2. Methods

Drugs and assay kits: 6-hydroxidopamine (6-OHDA), desipramine, apomorphine, ellagic acid (EA) and pramipexole (PPX) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Ketamine and xylazine were obtained from Alfasan Company (Woerden, Holland). ELISA kits for IL-1beta (IL-1b) and TNF-alpha (TNF-a) were obtained from eBioscience Company (Vienna, Austria).

2.1. Experimental design

Forty adult male Wistar rats (250-300 g) were obtained from central animal Lab of Ahvaz Jundishapur University of Medical Sciences (AJUMS), Ahvaz, Iran. Rats were kept in animal house under constant temperature ($22 \pm 2^\circ\text{C}$) and humidity (55-60%) on a 12:12-h light-dark cycle with food pellets and water, ad libitum. All experiments were done during the light phase of the cycle (between 8:00 am and 5:00 pm) and were directed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and with approval from the AJUMS Animal Care and Use Committee (AJACUC). We tried to minimize animals stress and to reduce the number of rats used in this study. All animals were handled for 5 days (daily 5 min) before the tests. The animals were divided randomly into five groups ($n = 8$):

Sham operated; rats received vehicle of 6-OHDA (2 μl normal saline containing 0.01% ascorbic acid) into right MFB.

Sham+EA; sham-operated rats received EA.

PD+Veh; rats injected 6-OHDA (16 $\mu\text{g}/2 \mu\text{l}$ normal saline containing 0.01% ascorbic acid) into right MFB and vehicle of EA (normal saline containing DMSO).

PD+PPX (Positive control); MFB lesioned rats were treated with PPX (2 mg/kg/2 ml, by gavages for 10 consecutive days).

PD+EA; MFB lesioned rats were treated with EA (dissolved in 10% DMSO/normal saline).

In groups 2 and 5, EA (50 mg/kg, by gavages for 10 consecutive days) was administered 14 days after PD induction (Uzar et al., 2012).

2.2. Making the animal model of PD (MFB lesion)

The brain's right hemisphere MFB was lesioned using neurotoxin 6-OHDA. We used a slightly modified method of Tadaiesky et al., (2008). Briefly, rats were deeply anesthetized with combination of ketamine/xylazine (90/10 mg/kg, i.p.), and were placed in a stereotaxic apparatus (Narishige, Japan). 6-OHDA (16 μ g/2 μ l normal saline containing 0.01% ascorbic acid) or its vehicle (in sham groups) was infused into the right MFB using a 5 μ l Hamilton syringe according to the coordinates in Paxinos and Watson atlas: AP: -4.4 mm, ML: \pm 1.3 mm from bregma and DV: -8.4 mm from skull surface (Paxinos & Watson, 2006). Following injection, the cannula was left in the site for 5 min to allow complete diffusion of the drug. All animals received desipramine (25 mg/kg i.p., 30 min before surgery) to protect noradrenergic terminals depletion and consecutive depression by 6-OHDA. Sham-operated rats underwent the same protocol but vehicle was injected instead of 6-OHDA (Sarkaki, Norooz Zare, Farbood, & Pileverian, 2013).

2.3. Apomorphine-induced rotational behavior

PD-model was confirmed by rotational test using apomorphine injection. All rats were tested for rotational behavior 2 weeks after MFB lesion (before treatment) and 4 weeks after lesion (after treatment). Contralateral rotations of each animal were recorded after subcutaneously injection of apomorphine (0.5 mg/kg in normal saline containing 0.01% ascorbic acid) to confirm the dopamine depletion in nigrostriatal system (Rizelio et al., 2010). The rotations were counted over a period of 30 min.

2.4. Stride length test

Stride-length (walk-length) was measured in all animals using a woody box (20 \times 17 \times 10 cm), in which a runway (4.5 cm wide, 42 cm long with walls of 10 cm height) was designed to lead out into dark wooden box. Stride-length was measured by moistening the forepaws of rats with ink and letting them run on a paper strip (4.5 cm wide, 40 cm long). The mean distance between two forepaw prints was measured manually as the forelimb stride-lengths. Five strides in total were typically observed. The three middle stride-lengths were selected in order to perform statistical analysis, from each run. The paw prints made at the beginning (7 cm) and the end (7 cm) of the each run were excluded because of variations in velocity (Metz, Tse, Ballermann, Smith, & Fouad, 2005; A. Sarkaki et al., 2013).

2.5. Cylinder test

Cylinder test was performed in all groups (n=8) to evaluate the behavior of animals and degree of forepaws asymmetry. The rats were placed in a transparent cylinder (diameter: 20 cm, height: 30 cm) for 3 min and the number of forepaws contacts to the wall of cylinder were counted (Schallert, Fleming, Leasure, Tillerson, & Bland, 2000). The score of cylinder test in this study was calculated as a contralateral bias. [(the number of contacts with the contralateral limb) - (the number of contacts with the ipsilateral limb) / (the number of total contacts) \times 100] (Roof, Schielke, Ren, & Hall, 2001).

2.6. Measurement of brain IL-1beta (IL-1 β) and TNF-alpha (TNF- α)

The rats were deeply anesthetized and were perfused intracardially with phosphate buffered saline (pH= 7.4) for 1 min to remove vesicular blood, and then were decapitated. The striatum and hippocampus were removed quickly, rinsed with normal saline and frozen. All samples were kept at -80°C until further processing. For the determination of the levels of cytokines, each brain tissue was weighed and homogenized in T-PER™ tissue protein extraction reagent with 0.5% Triton-x100, 150 mM NaCl, 50 mM tris, and a protease inhibitor cocktail (500 mg tissue per 2ml of the reagent).

Following homogenization, samples were shaken (for 90 min) and then centrifuged (at 4°C, 4000 \times g, for 15 min) and the supernatant was collected. The protein content of the supernatant was estimated using a protein assay reagent kit to ensure an equal amount of protein from each sample was used for the assay (A. R. Sarkaki, Khaksari Haddad, Soltani, Shahrokhi, & Mahmoodi, 2013). ELISA kits for IL-1 β and TNF- α were purchased from the eBioscience company and the assays were performed according to the manufacturer's guidelines. The concentrations of the cytokines were quantified as picograms of antigen per milliliter of the supernatant.

2.7. Statistics

Results were expressed as means \pm SEM. Normality of data was assessed using Kolmogorov-Smirnov test. All data had normal distribution and the statistical analysis was performed by SPSS version 22 and one-way ANOVA followed by Tukey's post hoc test for inter groups comparisons. A P-value less than 0.05 were considered as a significant difference.

3. Results

3.1. Apomorphine-induced circling behavior

As shown in Figure 1, two weeks after surgery the number of apomorphine-induced contralateral rotations increased significantly in MFB-lesioned groups as compared to sham group ($P < 0.001$). Four weeks after surgery, the number of contralateral rotations in the treated groups (PD+PPX and PD+EA groups) decreased significantly versus PD+Veh group ($P < 0.001$).

3.2. Stride-Length

As shown in Figure 2, forepaws stride-length significantly decreased in PD+Veh group as compared to sham-operated rats ($P < 0.001$). It significantly increased in PD+PPX and PD+EA groups versus PD+Veh ($P < 0.001$).

3.3. Cylinder Test

As shown in Figure 3, the scores of the cylinder test in PD+Veh group significantly increased versus sham-operated rats ($P < 0.001$). On the other hand, it decreased significantly in PD+PPX and PD+EA groups as compared to PD+Veh ($P < 0.01$).

3.4. Changes in brain IL-1beta (IL-1 β) levels

IL-1 β levels (pg/ml) in the striatum (Figure 4A) and hippocampus (Figure 4B) significantly increased in PD+Veh group as compared to sham group ($P < 0.001$). But, this increase significantly restored in the rats which

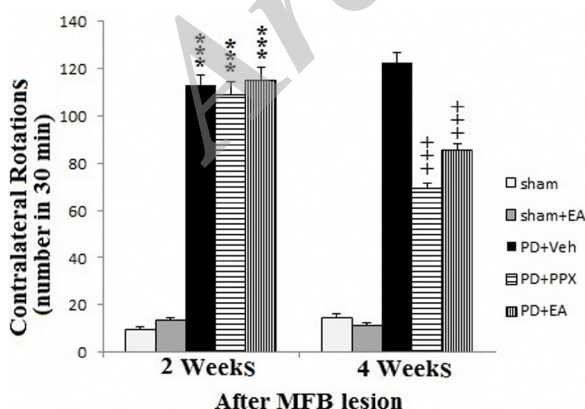
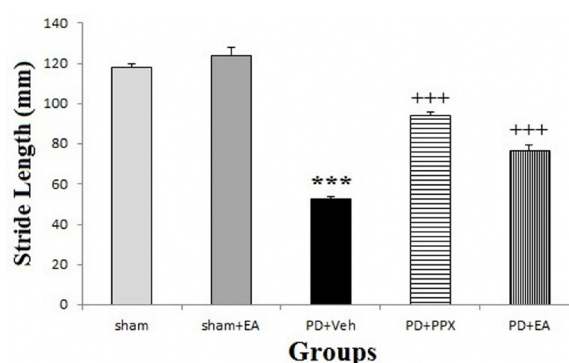


Figure 1. Effect of ellagic acid on apomorphine-induced rotational test. Values are as mean \pm SEM ($n = 8$). Data were analyzed by one-way ANOVA followed by Tukey's post hoc test. *** $P < 0.001$ vs. sham group 2 weeks after surgery, +++ $P < 0.001$ vs. PD+veh group 4 weeks after surgery and treatment.



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Figure 2. Effect of ellagic acid on stride-length. Values are as mean \pm SEM ($n=8$). Data were analyzed by one-way ANOVA followed by Tukey's post hoc test. *** $P < 0.001$ vs. sham group and +++ $P < 0.001$ vs. PD+Veh group.

treated with EA as compared to PD+Veh group in both striatum and hippocampus ($P < 0.001$).

3.5. Changes in brain TNF-alpha (TNF- α) levels

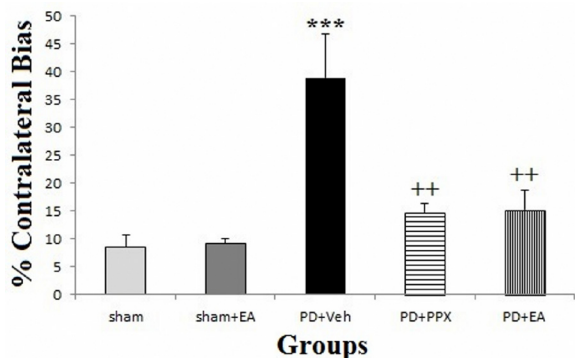
MFB-lesion significantly increased the levels of TNF- α (pg/ml) in the striatum (Figure 5A) and hippocampus (Figure 5B) versus sham-operated rats ($P < 0.001$). But, treatment of MFB-lesioned rats with EA significantly restored this increase in both striatum and hippocampus ($P < 0.001$).

4. Discussion

Several studies have shown that injection of 6-OHDA into the MFB enhanced contralateral apomorphine-induced rotation, suggesting 6-OHDA can exert neurotoxic effect on nigrostriatal pathway leads to enhance contralateral rotations (Kelly, Jenner & Marsden, 1984). Similarly, our results have shown that apomorphine-induced contralateral rotation increased significantly after MFB lesioning and confirm the PD-model preparation. Since the EA could restore these increased rotation (Figure 1), it may have a possible neuroprotective effect on nigrostriatal pathway.

Also, we have shown that MFB lesion can induce motor deficiencies such as, significant decrease in the rats' forepaws stride-length (Figure 2), while score of cylinder test was increased significantly (Figure 3). Oral administration of EA could restore these motor deficiencies in MFB-lesioned rats. This confirms that, EA can exert neuroprotective activity against 6-OHDA-induced damage in the MFB.

There are many evidences for the involvement of oxidative stress (OS) in 6-OHDA-induced neuronal dam-



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Figure 3. Effect of ellagic acid on the scores of cylinder test. Values are as mean ± SEM (n = 8). Data were analyzed by one-way ANOVA followed by Tukey's post hoc test. ***P<0.001 vs. sham group and ++P<0.01 vs. PD+Veh.

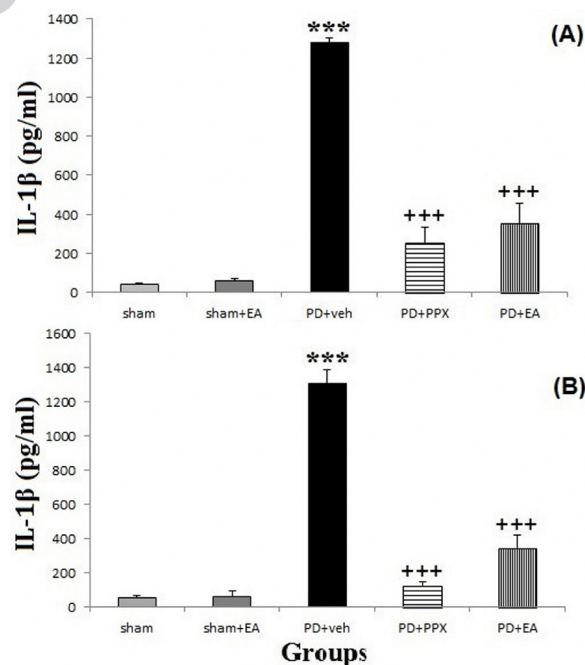
age. The neurotoxicity of 6-OHDA is due to its oxidation by molecular oxygen or monoamine oxidase in the brain. This leads to production of intracellular H_2O_2 which can be transformed into highly reactive hydroxyl radicals and more generation of hydrogen peroxide, reduction in glutathione (GSH) and SOD activity, increase in MDA levels and production of superoxide free radicals cause cell damage. (Hritcu, Ciobica & Artenie, 2008). OS contributes to the injury of lipids, proteins, DNA and the cascade of events leads to the death of dopaminergic neurons in PD (Fahn & Sulzer, 2004). The brain shows a high vulnerability to reactive oxygen species (ROS). Free radicals and OS can play important role in the PD (Dauer & Przedborski, 2003). In many of these processes, oxidative stress is a hall mark factor where the oxidation of dopamine (DA) produces ROS and an unbalanced production of ROS cause neural damage and death (Huang, de la Fuente-Fernandez, & Stoessl, 2003). It should be indicated that MFB lesion can increase the brain free radicals and cause motor impairments. It can be concluded that EA which can enhance the cerebral antioxidant defense leads to reduction of oxidative stress. Thus, EA can show neuroprotective effect against 6-OHDA induced neural oxidative damage.

A wide variety of neurodegenerative diseases such as PD, are associated with chronic inflammation (Block & Hong, 2005) and anti-inflammatory medication leads to a reduction in the risk of PD (Chen et al., 2005).

In this regard, our results showed that MFB lesion significantly increased the levels of IL-1 β (Figure 4) and TNF- α (Figure 5) in the striatum and hippocampus. It suggests that 6-OHDA can increase these inflammation biomarkers in the nigrostriatal pathway and plays a pos-

sible role in dopaminergic neurons degeneration. Instead, EA significantly decreased the levels of these biomarkers; it may exert anti-inflammatory and neuroprotective effects against 6-OHDA induced neuroinflammatory damage in the MFB-lesioned rats.

Neuroinflammation has received considerable attention for neuroprotective therapies in PD and two pro-inflammatory cytokines (IL-1 β and TNF α) have been involved as main effectors of the neuroinflammation in PD. High expression of inflammatory biomarkers at the site of neural injury indicates that these cytokines are mediators of neural damage and a possible target for the management of PD. In the healthy SN, the acute administration or expression of both cytokines have no dramatic effect on dopaminergic neurons, unless expressed at supra-physiological levels (Leal et al., 2013). IL-1 β and TNF- α are toxic for the dopaminergic cells. Contrasting this view, Ferrari et al., (2006) showed neuroprotective activities of IL-1B and TNF-alpha. The net effect of these cytokines on dopaminergic cell viability depends on many factors such as concentration, duration of expression and neighboring neurons. For example, the sustained expression of IL-1 β in the SN at pro-inflammatory levels causes irreversible and noticeable dopaminergic cell death (Ferrari et al., 2006).



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Figure 4. Effects of ellagic acid on IL-1beta (IL-1 β) levels in the striatum (A) and hippocampus (B). Values are expressed as mean±SEM (n=8). Data were analyzed by one-way ANOVA followed by Tukey's post hoc test. ***P<0.001 vs. sham group, +++P<0.001 vs. PD+Veh group.

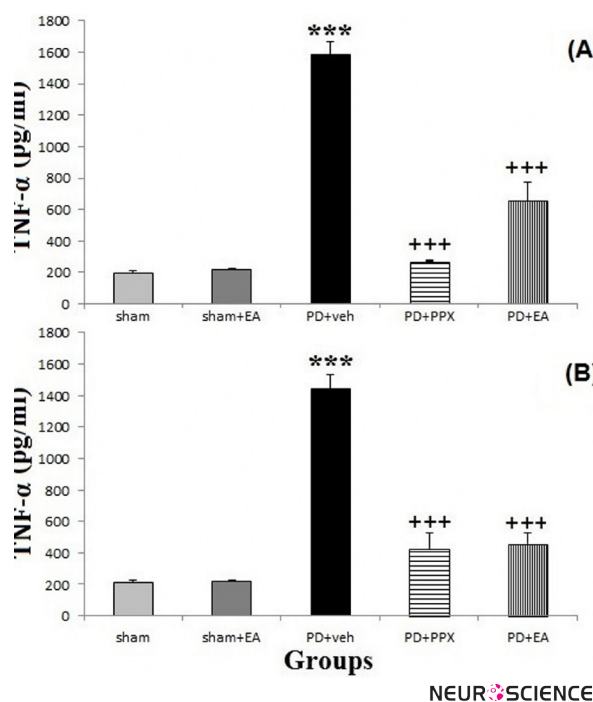


Figure 5. Effects of ellagic acid on TNF-alpha (TNF- α) levels in the striatum (A) and hippocampus (B). Values are as mean \pm SEM (n = 8). Data were analyzed by one-way ANOVA followed by Tukey's post hoc test. ***P<0.001 vs. sham group, +++P<0.001 vs. PD+Veh group.

Polyphenols and flavonoids prevent oxidative stress and are valuable for the prevention of cardiovascular, inflammatory and other diseases (Miguel, Dandlen, Antunes, Neves, & Martins, 2004). Dietary supplements such as grape seed extract (GSE) enriched in proanthocyanidin (PA) have been proposed to have several health profits, because of antioxidant and other useful properties of the PA (Chis et al., 2009).

In this study, we investigated the possible effects of ellagic acid on motor disturbances and neuroinflammatory biomarkers in a rat model of PD induced by 6-OHDA. Our results confirm that MFB lesion can impair the motor performances which are associated with increase in the levels of TNF- α and IL-1 β . On the other hand, EA can restore these impairments and decrease the inflammatory biomarkers which leads to improving motor disturbances. Taken together, these findings may provide experimental basis for the use of EA in the treatment and prevention of free radicals induced neural damage in PD. Further studies are necessary to clarify neuroprotective mechanisms of EA.

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Conflict of interest

The authors announce that there is no conflict of interest.

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