Cinnamaldehyde attenuates dopaminergic neuronal loss in substantia nigra and induces midbrain catalase activity in a mouse model of Parkinson’s disease

Fereshteh Mehraein1,2*, Maryam Zamani1, Feraidon Negahdar1, Asieh Shojae3

1. Department of Anatomy, Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran
2. Minimally Invasive Surgery Research Center, Iran University of Medical Sciences, Tehran, Iran
3. Traditional Medicine Faculty, Iran University of Medical Sciences, Tehran, Iran

ABSTRACT

Background and Objective: Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease that affects 3% of the population. PD involves a progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc) and subsequent loss of dopamine. Dopamine depletion leads to movement dysfunction and is accompanied with tremor, rigid muscles and impaired balance. Mechanisms of the pathogenesis of PD include oxidative stress and inflammation. Cinnamaldehyde acts as a powerful antioxidant and anti-inflammatory agent. This research is focused on the effects of cinnamaldehyde on neurons of SNc of mouse model of PD.

Materials and Methods: Adult male mice with an average weight of 25-35 g were divided into 4 groups of 5 each: group 1: control PBS, group 2: MPTP, group 3: MPTP + cinnamaldehyde pretreatment (30 mg/kg), and group 4: MPTP + cinnamaldehyde treatment (30 mg/kg). Rotarod test was used to assess motor and balance of the mice. After behavioral studies, all the mice were anesthetized and perfused transcardially with 0.1 M PBS (pH=7.4) followed by 4% buffered paraformaldehyde fixative. The brains of the mice were removed and fixed in the paraformaldehyde and stained for TUNEL and IHC. Then the number of the apoptotic, TH+ and GFAP+ cells were counted. The level of MDA and catalase enzyme activity were also evaluated. Data was analyzed using SPSS software by one way of variance (ANOVA) and t-test.

Results: The results showed that groups 3 and 4 had significantly better locomotion than group 2 (p<0.05). Cinnamaldehyde also induced catalase enzyme activity and significantly decreased the number of apoptotic neurons in SNc in groups 3 and 4 as compared to group 2 (p<0.05).

Conclusion: This study showed that cinnamaldehyde attenuates dopaminergic neuronal loss in substantia nigra and induces midbrain catalase activity in a mouse model of Parkinson’s disease.

Key Words: Cinnamaldehyde Dopaminergic neurons Parkinson’s disease

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease that affects 3% of the population. PD involves a progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc) and subsequent loss of dopamine. Dopamine depletion leads to movement dysfunction and is accompanied with tremor, rigid muscles and impaired balance. Mechanisms of the pathogenesis of PD include oxidative stress and inflammation. Oxidative damage to lipids, proteins and nucleic acids of the dopaminergic neurons were observed in the SNc of PD patients (1,2).

*Corresponding Author: Fereshteh Mehraein
Department of Anatomy, Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran.
Email: femehra@yahoo.com
Oxidative stress results from an increase of free radical production and reduced antioxidant activity. Dopaminergic neurons are susceptible to oxidative stress due to high rate of oxygen metabolism. The antioxidants can defend body against oxidative stress. The current treatment for PD cannot stop the progression of the disease but relief the resulting symptoms (3-6), so in addition to current treatment, using antioxidants can be helpful. Recently, researchers have drawn their attentions toward natural antioxidants obtained from plants such as cinnamaldehyde, an essential oil of cinnamom, one of the oldest spices used in traditional medicine. Studies have shown the therapeutic effects of cinnamaldehyde in oxidative stress, inflammatory diseases, and diabetes (7-11). In the present study, the effect of cinnamaldehyde, on dopaminergic neurons of SNc, catalase enzyme activity in midbrain and movement of mice were investigated.

2. Materials and Methods

2.1. Chemicals

Cinnamaldehyde and MPTP (1 methyl-4-Phenyl-1, 2, 3-6 tetrahydro pyridine) were purchased from Sigma Aldrich (USA). MDA and CAT kits were obtained from Randox (UK). Mouse monoclonal antibody to tyrosine hydroxylase (primary antibody, dilution 1:80) and goat polyclonal secondary antibody to mouse IgG + IgM + IgA + horseradish peroxidase (dilution 1:100) were purchased from Abcam (USA) and 3,3'-diaminobenzidine from Dako (Denmark), Tunel assay kit from Roche (USA) and anti GFAP antibody from Abcam (USA).

2.2. Animals

Adult male mice with an average weight of 25-35 g were purchased from animal house of Iran University of Medical Sciences and housed in a temperature controlled room at 23±2°C. All animal works were approved by ethical guidelines for the care of laboratory animals of Cellular and Molecular Research Center of Iran University of Medical Sciences.

2.3. Experimental design

The animals were divided into 4 groups of 5 each as follows: group 1: control PBS, group 2: MPTP, group 3: MPTP + cinnamaldehyde pretreatment (30 mg/kg), group 4: MPTP + cinnamaldehyde treatment (30 mg/kg). Groups 2, 3 and 4 were injected intraperitoneally with a single daily dose of 20 mg/kg of MPTP (12) for four days. Single daily dose of 30 mg/kg of cinnamaldehyde dissolved in PBS was injected intraperitoneally to group 3 for two weeks before the injection of MPTP. One hour after PD induction of group 4, a single dose of 30 mg/kg of cinnamaldehyde dissolved in PBS was injected. Group 1 received only PBS.

2.4. Measurement of motor coordination

Rota rod test was used to assess motor balance of the mice. In this test, the mouse has to keep its balance on a rotating rod (13). After placing the mouse on the rod, each mouse was trained to stay on the rotating rod. Once the trained mice were able to stay on the rod rotating at 4 rpm for 1 minute, they were proceeded to the test. Each mouse was evaluated for three trials separated by 15 minutes inter trial intervals each day at an accelerating speed at 20 rpm in 300 seconds. The periods that each mouse was able to maintain its balance on the rod was recorded before and after MPTP administration.

2.5. Immunohistochemical analyses

After behavioral studies, all the mice were anesthetized with ketamine (50 mg/kg) in combination with xylazine (5 mg/kg) and perfused transcardially with 0.1 M PBS (pH=7.4) followed by 4% buffered paraformaldehyde fixative. The brains of the mice were removed and fixed in the paraformaldehyde overnight, then the brains dehydrated in ascending alcohol series, cleared in xylene, infiltrated with paraffin and embedded in paraffin. The 5 µm coronal sections were collected and stained for immunohistochemistry (IHC). For IHC staining, the sections were incubated at 62°C for 20 minutes, rehydrated in descending alcohols, immersed in 10% H2O2/methanol to reduce endogenous peroxidase activity for 10 minutes. Then, the sections were washed in Tris buffer [pH 7.4] and kept in citrate buffer (pH 6) in autoclave to boil for 11 minutes. After cooling, the sections were washed in Tris and incubated in BSA for 10 minutes. Afterward, the sections were incubated in the primary antibody for 1 hour. The sections were washed again in Tris wash
buffered (pH 7.4), and then incubated in the secondary antibody for 1 hour. The sections were washed in Tris (pH 7.4). To visualize the bound antibody, the sections were reacted with DAB for 10 minutes, washed in Tris (pH 7.4), and counterstained with immersing in Hematoxylin for 10 minutes, then washed in tap water for 3 minutes and dehydrated in ascending alcohols, cleared in xylol and covered with cover slip. For negative control, the sections were processed as described above except that the primary antibody was not used. The number of the neurons, TH+ and GFAP+ cells, were counted in 5 fields of each section.

2.6. TUNEL assay

The sections were also stained for TUNEL assay using a detection kit, according to the manufacturer's instructions. Briefly, the sections were dehydrated, incubated in 3% H2O2 for 10 minutes and then incubated in proteinase K (20 μg/ml) in 10 mM Tris/HCL (pH 7.6) for 30 minutes. After incubation, TUNEL reaction mixture was added to the sections and incubated for 1 hour. Each 5 μm section was incubated further with antibody conjugated horseradish peroxidase for 30 minutes and developed with 0.05% 3,3 diaminobenzoidine (DAB) for 1-2 min and counterstained with Hematoxylin. The neurons with brown stained nuclei were counted at a magnification of 400x. For positive control, the 5 μm sections were incubated in DNAase (3000 U/ml in 50 mM Tris HCL, pH 7.5), (1 mg/ml BSA) for 10 minutes to induce DNA strand break prior to labeling procedure. For negative control, the sections were incubated with labeling buffer.

2.7. MDA level and CAT enzyme activity assays

The midbrains of mice were separated and homogenized in ice 10 nmol/Tris-HCL. The homogenates were centrifuged at 12000× g at 4°C for 20 minutes. The supernatants were collected for CAT enzyme assay. Lipid peroxidation level was measured by thiobarbituric acid (TBA) method. This method was used to measure spectrophotometrically the color produced by the reaction of TBA with malondialdehyde. For this purpose, the supernatant proteins were precipitated with trichloroacetic acid and the mixture was heated with 3 ml TBA in 2 M sodium sulfate in boiling water for 30 minutes. The colored product was extracted with n butyl alcohol, and the absorbance of the sample was measured at 530 nm. MDA levels were expressed as nanomol per milligram of protein (nM/mg protein). CAT enzyme activity was measured based on the ability of the enzyme to break down H2O2. For this purpose, the tissues were homogenized and centrifuged at 1000 ×g for 10 minutes. A volume of 20 µl of 100 fold diluted supernatant was added to 980 µl assay mixture, containing 10 mmol/l of H2O2 Tris-HCL buffer (50 µl, PH 8), and distilled water (30 µl ). The rate of decomposition of H2O2 was monitored spectrophotometrically at 240 nm.

2.8. Data analysis

Data was analyzed using SPSS software by one way analysis of variance (ANOVA) and t-test. The results were expressed as the mean ± SD.

3. Results

The impairment of behavioral activities among MPTP-induced mice was significant as compared to control group, group 4 had better locomotion than group 3 (Fig. 1). On the other hand, there was no significant difference between groups 4 and 1. Fig. 2 shows the level of CAT enzyme activity in the four groups. A significant decrease was observed in the group 2 in comparison with control group (p<0.05). Administration of cinnamaldehyde caused a significant increase of CAT activity in group 3 and 4 as compared to group 2 (p<0.05) but differences between groups 3 and 4 were not significant. A significant reduction of the level of MDA was observed in groups 3 and 4 as compared to group 2 (p<0.05) (Fig. 3). As shown in Fig. 3, the midbrain tissue of MPTP-induced mice had significantly higher MDA level than the other groups (p<0.05), while the level of MDA in groups 3 and 4 was similar. TUNel staining analysis revealed that the number of apoptotic neurons in group 3 and 4 were less than group 2 (p<0.05) (Fig. 4) and the number of TUNel-positive neurons in substantia nigra of the group 3 was similar to group 4 (Fig. 4). Counting of dopaminergic neurons in the SNc revealed that there was a significant decrease in group 2 as compared to the other groups (p<0.05) (Figures 5 and 6), the neurons of groups 3 and 4 were
significantly more than group 2 (p<0.05) (Figures 5 and 6). GFAP staining showed that group 2 had the highest number of GFAP-stained cells as compared to the other groups (p<0.05) (Fig. 7). Counting of GFAP-stained cells revealed that group 4 had similar number of GFAP-stained cells as group 3 (Fig. 8).

Fig. 1. Effect of cinnamaldehyde on the impairment locomotion of the MPTP-induced mice. As shown in the histogram, the impairment of behavioral activities among MPTP-induced mice was significant as compared to control (p<0.05). There was significant difference between MPTP group and MPTP + cinnamaldehyde treatment group (p<0.05). Values are expressed as mean ± SD.

Fig. 2. The activity of catalase (CAT) enzyme in four groups. MPTP group had the least activity of catalase enzyme as compared to the other groups (p<0.05). Differences between MPTP + cinnamaldehyde pretreatment and MPTP + cinnamaldehyde treatment were not significant. Values are expressed as mean ± SD.

Fig. 3. The levels of malondialdehyde (MDA) in four groups. The histogram shows that the level of MDA in MPTP group is higher than the other groups (p<0.05), while the level of MDA in MPTP + cinnamaldehyde pretreatment and MPTP+ cinnamaldehyde treatment groups was similar. Values are expressed as mean ± SD.

Fig. 4. Histogram and photomicrograph of Tunel staining of SNc in the four groups (400x). A: MPTP group, B: Control group, C: Pretreatment group, D: Treatment group. The number of apoptotic neurons in MPTP + cinnamaldehyde pretreatment and MPTP + cinnamaldehyde treatment groups was less than group 2 (p<0.05). Arrows show apoptotic cells.
Fig. 5. Photomicrograph of TH⁺ neurons in IHC staining. A: control PBS, B: MPTP, C: MPTP + cinnamaldehyde pretreatment (30 mg/kg), and D: MPTP + cinnamaldehyde treatment (30 mg/kg). Arrows show TH⁺ neurons.

Fig. 6. Columns represent the mean number of dopaminergic neurons (DA). As shown in the histogram, the number of dopaminergic neurons in MPTP group decreased as compared to the other groups (p<0.05), but the number of DA neurons in MPTP-induced mice which pretreated and treated with cinnamaldehyde was significantly higher than MPTP group (p<0.05).

Fig. 7. Photomicrograph of glial cells in GFAP staining: A: control PBS, B: MPTP, C: MPTP + cinnamaldehyde pretreatment (30 mg/kg), and D: MPTP + cinnamaldehyde treatment (30 mg/kg). Arrows show glial cells.

Fig. 8. The histogram shows the mean number of GFAP-stained cells in different groups. MPTP group had significantly more cells compared to the other groups (p<0.05).
4. Discussion

In this study, injection of MPTP caused rapid onset of Parkinson’s disease. MPTP is a lipophilic compound that can cross the blood brain barrier. Inside the brain, MPTP is metabolized into the toxic cation 1 methyl 1-4 phenylpyridinium by the enzymes of glial cells. MPTP destroys dopaminergic neurons in pars compacta of the substantia nigra because it interferes with the electron transport chain of mitochondria metabolism, which leads to cell death and causes the buildup of free radicals, toxic molecules that contribute further to cell destruction (1-4). It is hypothesized that PD is the result of excess production of free radicals which contributes to neuronal damage and cell death (4,5). Mechanism of protection from oxidative free radical damage include antioxidants which detoxify the free radicals (5,6). The efforts are focused on the natural antioxidants for protection of dopaminergic neurons against free radicals. Since cinnamaldehyde is a strong antioxidant agent (14,15), the current study was carried out to investigate its effects on the locomotion recovery, CAT enzyme activity as an antioxidant and dopaminergic neuron (DA) death of SNC in PD mouse model. According to the results of this study, cinnamaldehyde had strong effect on protection of DA neurons of SNC in MPTP-induced mice. Neuroprotective effects of cinnamaldehyde is due to inhibition of up-regulation of inducible nitric oxide synthase and cyclooxygenase-2 which maintain the number of DA cells of SN region (14,15). Cinnamaldehyde has also a potential neuroprotective effect against the ischemic stroke, which may be via inhibition of neuroinflamation through attenuating iNos, Cox2 expression and NF-kB signaling (15). Several studies have also shown that expression of BDNF is significantly reduced in the SNC of patients with PD and that BDNF protects DA neurons from 6-hydroxydopamine induced toxicity (16). Cinnamaldehyde increases the production of BDNF in cultured astrocytes and neurons (16-19) and also can upregulate DJ-1 and parkin that are known to support the survival of DA neurons (18). The major product of lipid peroxidation is MDA. In this study MDA was significantly increased in MPTP mouse model (group2) as compared to control group. Administration of cinnamaldehyde to groups 3 and 4 significantly lowered the level of MDA. Cinnamaldehyde has been reported to possess anti-oxidative effects and decreases malondialdehyde (MDA) content in myocardial tissue (19). A significant decrease in MDA level and increased CAT activities were also observed in testicular tissue of rats consuming cinnamaldehyde that resulted in decreasing the abnormal sperm rate and apoptotic germ cell count (20). CAT is an enzyme that breaks down H2O2 and detoxifies free radicals. Decreasing of CAT activity results in increasing of oxidative stress. SNC of Parkinson's patients has increased level of iron which catalyzes the conversion of H2O2 to free radicals to damage neurons and cell death (21,22). In the present study, CAT enzyme activity was significantly decreased in group 2, while the administration of cinnamaldehyde significantly increased activity of CAT in groups 3 and 4. Interestingly, the number of GFAP-stained cells was the most in MPTP-induced mice when compared to the other groups. Glial cell activation play an important role in the pathogenesis of neurodegenerative diseases, so that reduced glial activity protects neuron from apoptosis (23,24). Cinnamaldehyde is an agent against neuroinflamation in neurodegenerative diseases which inhibits microglial activation and improves neuronal inflammation and protect neuronal damages (25).

MPTP administration caused hypolocomotion in mice while high doses of cinnamaldehyde treatment improved behavioral activities of the mice. In this study evaluation of locomotor activities of the mice by rotarod showed that the latency to fall increased in groups 3 and 4 significantly (26). This result is in agreement with Khasanavis et al that reported cinnamaldehyde treatment decreases functional impairment in MPTP intoxication in mice (18).

The results of a study indicated that the rats which were treated with cinnamaldehyde of the dose level of 73.5 mg/kg showed imbalance in the antioxidant status and this may affect the neuromuscular coordination in the rats which result in changes of behavioral parameters, therefore the effects of cinnamaldehyde is time and dose dependent (18, 26).

These findings suggest that cinnamaldehyde as a natural antioxidant may protect dopaminergic neurons against Parkinson's disease.

Acknowledgement

This research was supported by Iran University of Medical Sciences (Tehran, Iran).

References


22. Panickar KS, Polansky MM, Graves DJ, Urban JF Jr, Anderson RA. A procyanidin type A trimer from cinnamon extract attenuates glial cell swelling and the

