248

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Journal of Nanjing Medical University, 2007, 21(4): 248-252

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Research Paper

Study of apoptosis pattern of dopaminergic neurons and neuroprotective effect of nicotine in MPTP mouse model☆

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Received 26 February 2007

Abstract

Objective: To investigate the apoptosis of dopaminergic neurons and the protective effect of nicotine in 1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine (MPTP)-induced mouse model of Parkinson's disease. **Methods**: The mouse model of Parkinson´s disease were formed by MPTP (30 mg/kg/d×7, i.p.); and the loss and apoptosis of dopaminergic neurons was observed by Tyrosine Hydroxylase(TH) and TUNEL stains.In "Nicotime plus MPTP" group, mice were pretreated with nicotine before MPTP injection. The putative protective effect of nicotine was analyzed. **Results**: The number of TH-positive cells decreased during MPTP treatment. Apoptotic neurons began to appear after three injections of MPTP and peaked on the 8th day. In the MPTP-intoxicated mice treated with nicotine, the loss of TH-positive cells was significantly less than that of MPTP-treated group (30 mg/kg/d×7) ($P < 0.05$). **Conclusion**: The chronic treatment of MPTP can induce the apoptosis of dopaminergic neurons in substantia nigra, and nicotine might have a neuroprotecitve effect on dopaminergic neurons against MPTP toxicity.

Keywords: Parkinson´s Disease; apoptosis; 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; Nicotine

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disease first described by an English physician, James Parkinson in 1817. The main clinical symptoms include resting tremor, rigidity, bradykinesia and postural instability. However, the etiology and pathogenesis of this disease are not yet completely understood. In pathology it is characterized by the degeneration and progressive loss of dopaminergic neurons in substantia nigra. Some researchers reported that the main form of dopaminergic neuronal death in PD is apoptosis based on studies in culture models, animal models and human PD patients' brains[1].

At present, the preferred therapeutic strategy for PD is dopamine replacement despite the emergence of several modern treatments. But all of these ap proaches have their shortcomings and some of them are still in the trial stage[2]. When PD symptoms appear, about 60% of the dopaminergic neurons in the substantia nigra pars compacta (SNpc) have been lost [3]. Herein, it is easy to understand the importance of neuroprotection in the early stage of the disease to delay or halt the neurodegenerative process of the dopaminergic neurons. Epidemiological studies have shown a negative association between cigarette

☆This project was supported by the National Natural Science Foundation of China (No. 30400516) and Youth Chenguang Project of Science and Technology of Wuhan City of China (No. 20045006071-2)

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smoking and PD [4]. Recently striking evidence from a twin patient study, demonstrating the reduced risk for PD associated with smoking also supported the protective effect of cigarette smoking [5]. Most of the relative basic researches focused on nicotine, the major component of cigarette, but the results of neuroprotective capacities of nicotine on dopaminergic neurons are still as yet controversial.

In order to acquire a better knowledge about the pathogenesis and effective therapeutic methods of PD, investigators successfully replicated numerous experimental models of PD, with 6-hydroxydopamine (6-OHDA) or 1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine (MPTP) being commonly used. In 6-OHDA animal models, dopaminergic neurons start to die via a non-apoptosis mechanism after 6-OHDA stereo injections into brains [6]. However, chronic MPTP treatment leads to apoptosis of dopaminergic neurons [7]. Therefore, MPTP is favored rather than 6-OHDA in order to simulate the pathogenesis of PD. In this study, we employed the MPTP mouse model for the study of the apoptosis pattern during chronic MPTP treatment and studied the putative protective effect of nicotine on dopaminergic neurons.

MATERIALS AND METHODS

Animals and reagents

C57BL/6 mice were purchased from Organ Transplantation Institute (Tongji Medical College, Huazhong University of Science and Technology). Reagents used in this study included:MPTP(Sigma-Aldrich, the United States of America(U.S.A.)), (-)-Nicotine(Sigma-Aldrich, U.S.A.), Rabbit Polyclonal anti-Tyrosine Hydroxylase (anti-TH, Chemicon, U.S.A.), Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling(TUNEL) Assay Kit(Boster, China), anti-Rabbit streptavidin-biotin-peroxidase (SP) Immunohistochemistry Kit and diaminobenzidine (DAB) Peroxidase Substrate Kit(Zhongshan,China).

Animal groups and drug treatment

8 to 10-week-old,25~30 g,male,C57BL/6 mice were used in this study. MPTP-treated mice(n = 28) received MPTP(30 mg/kg/d,i.p.) from day 1 to day 7 with an interval of 24h. The mice were subdivided into 7 groups and sacrificed at different times individually (4 mice per group). The same volume of saline was used in control group.

"Nicotine group"(n = 4) received (-)-nicotine (2 mg/kg,i.p.), five times a day at 2-h intervals for 17 days. "Nicotine plus MPTP group"(n = 4) received (-)-nicotine with the same regimen. During the last 7 days, MPTP (30 mg/kg/day, i.p.) was daily co-administered with the last injection of nicotine.

Tissue preparation

Mice were anaesthetized with 1% pentobarbitone (6 mg/kg) and transcardially perfused with 20ml saline followed by 50 ml 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). The brains were promptly removed and the substantia nigra were postfixed in the same fixative overnight at 4℃. The fixed samples were dehydrated and embedded in paraffin. Then paraffin-embedded tissues were cut at 5 μm consecutively.

Immunohistochemical analysis

The fixed sections were deparaffined and incubated with 3% H_2O_2 for 10min to remove the endogenous peroxidase. After blocked with 10% normal goat serum and washed at least twice with 0.01M phosphate buffered saline, sections were immunoreacted with rabbit polyclonal anti-TH(1:1000) for 12~18 h at 4℃,then with biotinylated goat anti-rabbit IgG for 1h at 37℃ and horseradish peroxidase-labeled streptoavidin for 1 h at 37℃. Following rinsing, the peroxidase in sections were revealed by incubation with reagents from DAB Peroxidase Substrate Kit. The sections were dehydrated in xylene and coverslipped. Negative controls were conducted by replacing the primary antibody with 0.01M PBS.

TUNEL analysis

Before TUNEL assay, the sections were deparaffined and incubated with 3% acetic acid(pH 2.5) for 10min. After rinsing in distilled water, sections were treated with Proteinase K for 15 min at 37℃, then reacted with TdT and DIG-d-UTP for 2 h at 37℃ and biotinylated anti-digoxin for 30 min at 37℃. The specimens were incubated with Strept Avidin-Biotin Complex-Alkaline Phosphatase(SABC-AP) for 30 min at 37℃. Following repeated rinsing with 0.01 M Tris-buffered saline (TBS), sections were treated with 5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt/ Nitro-Blue Tetrazolium Chloride (BCIP/NBT) to reveal apoptotic cells. Negative controls were processed in parallel but without TdT.

Cell counting and statistical analysis

According to the illustrative plates atlas, TH-positive and TUNEL-positive cells in substantia nigra were counted respectively under microscope. Three sections were selected from each brain sample and three different fields of view were checked for each

section (400 ×). The data was analyzed with SPSS10.0 software package. Cell numbers were compared between groups using the *t* test. In all cases, a *P*-value of less than 0.05 was considered to be statistically significant.

RESULTS

Behavioral changes induced by MPTP and nicotine

In MPTP-treated groups, acute symptoms appeared within 3 minutes after MPTP injection and vanished within 2 to 3 hours. These symptoms included tremor, straub tail, piloerection, sialorrhea, hyperpnea and teeth chatter.

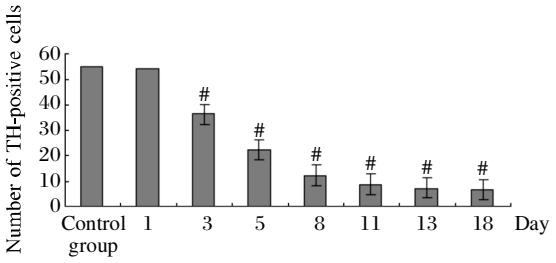
In nicotine-treated groups, mice got an increased heart rate and loss in locomotor activity after nicotine injection. The symptoms lasted for 5 to 10 minutes. However, these effects gradually disappeared after 8 injections of nicotine.

Effect of MPTP treatment on dopaminergic neurons

In MPTP-treated mice (untreated with nicotine), the number of TH-positive cells began to decrease on day 3 in compared with control group (***Fig 1 and 5***). TUNEL-positive cells started to be detected on day 3, and the number of apoptotic cells in substantia nigra reached peak on day 8 and then decreased gradually(***Fig 2 and 6***).

Effect of nicotine treatment

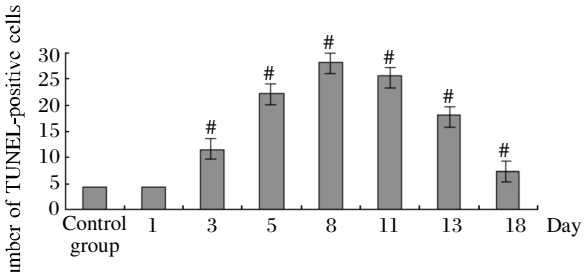
In the "Nicotine plus MPTP" group, the loss of TH-positive cells was significantly less severe and fewer TUNEL-positive cells were detected when compared with the MPTP-treated group (30 mg/kg/d×7)($P< 0.05$)(***Fig 3-6***). However, no statistically significant difference was found in TH-positive cells or TUNEL-positive cells between control group and "Nicotine group"($P > 0.05$).



Control group and MPTP-treated groups(Day 1 to Day 18)

$^{\#}P < 0.05$ *vs.* control

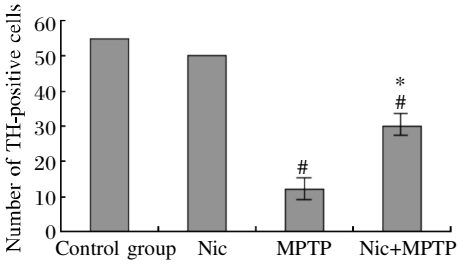
Fig 1 Comparisons in the number of TH-positive cells



Control group and MPTP-treated groups(Day 1 to Day 18)

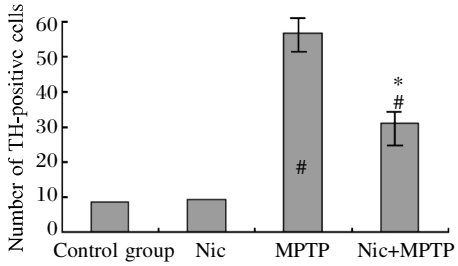
$^{\#}P < 0.05$ *vs.* control

Fig 2 Comparisons in the number of TUNEL-positive cells



$^{\#}P < 0.05$ *vs.* control; $^{*}P < 0.05$ *vs.* MPTP-treated group(Nic=Nicotine)

Fig 3 Comparisons in the number of TH-positive cells

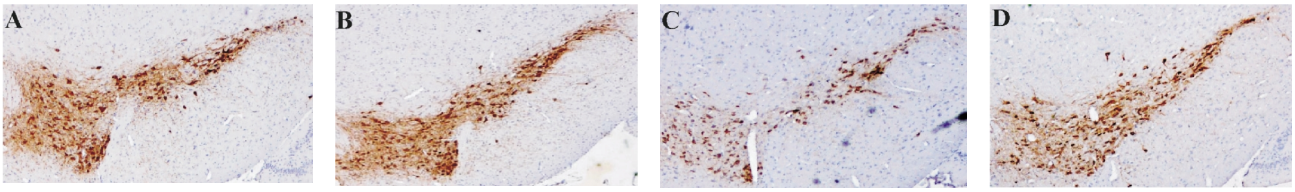


$^{\#}P<0.05$ vs. control; $^{*}P<0.05$ vs. MPTP-treated group(Nic=Nicotine)

Fig 4 Comparisons in the number of TUNEL-positive cells

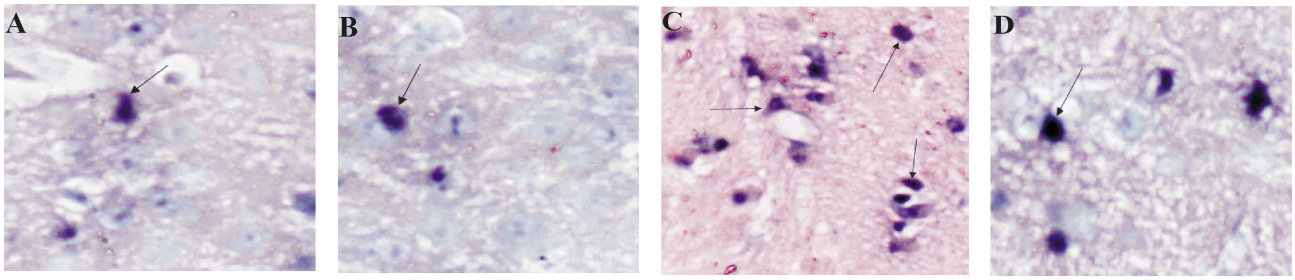
DISCUSSION

PD is a common neurodegenerative disease mainly affecting the middle-aged and the old. However, its etiology and pathogenesis remain unclear. To study its pathogenesis and effective therapy methods, investigators replicated multiple animal models of PD. And MPTP has been widely used in the formation of mouse model and monkey model of PD. MPTP, a neurotoxin, can rapidly enter the brain through the blood-brain barrier(BBB). In the brain, it's converted into 1-menthyl-4-phenylpyridinium (MPP^+) by monoamine oxidase B (MAO-B) in the astrocyte. Once MPP^+ is released into the extracellular space, MPP^+ is uptaken into dopaminergic neurons by the dopamine transporter (DAT). Cytosolic MPP^+ enters mitochondria and begins to exert its neurotoxic ef-



A:Control group; B:Nicotine-treated group; C:MPTP-treated group(30 mg/kg/d×7); D:MPTP-intoxicated group pretreated with nicotine.

Fig 5 Macroscopic photographs showing TH-positive neurons in substantia nigra(40 ×)



A:Control group; B:Nicotine-treated group; C:MPTP-treated group (30 mg/kg/d×7); D:MPTP-intoxicated group pretreated with nicotine. Arrows indicated apoptotic nuclei.

Fig 6 Macroscopic photographs showing TUNEL-positive cells(i.e. apoptotic cells)(400×)

fects by inhibiting Complex I. The damage of dopaminergic neurons induced by MPTP is highly selective.

In the present study, the MPTP-treated mice appeared acute symptoms like straub tail and piloerection. These behavioral changes might be related with acute alterations in noradrenergic and serotonergic systems [8]. However, compared with MPTP-treated monkey, functional deficits(parkinsonian symptoms) in MPTP-treated mouse vanished quickly. Tremor appeared just in acute reaction to MPTP and existed only a few hours. This phenomenon implicates that MPTP-treated mice possess a strong capacity of dopaminergic function recovery. Here, we list two possible compensatory mechanisms involved in this. One is that dopamine turnover is elevated in remaining survival neurons. Besides, up-regulation of postsynaptic dopamine receptors might also play a role in the fast functional recovery of mice treated with MPTP.

TUNEL assay showed in MPTP-treated groups apoptotic cells began to appear on day 3 and reached peak on day 8.These indicate chronic MPTP treatment mode (30 mg/kg/d) can induce the apoptosis of dopaminergic neurons in substantia nigra. The mechanisms by which MPTP induce apoptosis possibly include oxidative stress, neuroinflammation, and disturbance of intracellular calcium, and so on [9]. A study showed MPTP couldn't be detected in cerebrospinal fluid within 20 to 30 minutes after systematical MPTP injection and the level of MPP^+ peaked within 30 to 40 minutes[10]. It suggests during chronic MPTP treatment, the damage to dopaminergic neurons is the accumulated neurotoxic effect of MPTP. Apoptosis begin to appear only when the toxic effect reaches a definite degree. With the increasing of MPTP dosage, the damage of dopaminergic system became severe gradually. After seven injections of MPTP, the number of apoptotic cells decreased and even on day 18, a few TUNEL-positive cells could still be detected. Another study showed the level of dopamine in the striatum decreased for up to one month after chronic treatment of MPTP(30 mg/kg/d) on mouse[11]. Therefore, the neurotoxic effect of MPTP still exists after treatment withdrawal. However, the mechanism in this prolonged effect remains unknown.

Nicotine is a naturally occurring alkaloid, which is the major constituent and exerts the major pharmacological effects in cigarette smoke [12]. In light of the results from epidemiological studies on the relationship of cigarette smoking and PD, putative neuroprotective properties of cigarette smoke have been focused on nicotine. However, conflicting reports exist. The discrepancies may be due to the different dosing and timing of nicotine administration. This study demonstrated that chronic intermittent nicotine treatment protected dopaminergic neurons against the chronic toxic insult of MPTP, which might help explain the epidemiological findings. The underlying mechanism might be related with nicotinic acetylcholine receptors (nAchRs)[13-15]. Here we presented several possible mechanisms involved in this protective effect. Nicotine could evoke dopamine release

by activating presynaptic or postsynaptic nAchRs on nigral dopaminergic neurons[16,17]. Then dopamine released into synaptic clefts competes with MPP^+ to bind dopamine transporter, which results in less neurotoxin entering dopaminergic terminals, and subsequently attemuates cell damage. Excitotoxicity is thought to play a role in MPTP-induced cell death. In an in vitro study, investigators found that nicotine treatment can protect PC12 cells against the excitoxicity of glutamate, by reducing the level of intracellular calcium, promoting the expression of bcl-2 protein and down-regulating the expression of bax protein, which can be prevented by the addition of nAchRs antagonist[13].

In conclusion, this study showed MPTP injuries dopaminergic neurons mainly by inducing apoptosis in MPTP mouse model. Furthermore the accumulated neurotoxic effect of MPTP needs to reach a definite degree to lead to an irreversible damage and then loss of dopaminergic neurons. Additionally, nicotine showed a protective effect on dopaminergic neurons against MPTP toxicity.

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