Int J Clin Exp Med 2015;8(1):431-439
www.ijcem.com /ISSN:1940-5901/IJCEM0003929

Original Article

Neuroprotection of MAO-B inhibitor and dopamine agonist in Parkinson disease

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Received November 18, 2014; Accepted January 9, 2015; Epub January 15, 2015; Published January 30, 2015

Abstract: Parkinson disease is characterized by the death of dopaminergic neurons in the substantia nigra pars compacta. We explored the neuroprotective effect of Selegiline and Piribedil, the Monoamine Oxidase Type B (MAO-B) and dopamine agonist to Parkinson disease (PD). After embryonic Wistar rat were induced by cerebrospinal fluid (CSF) from PD patients, Selegiline and Piribedil were administered to Wistar rat. Immunohistochemical staining, RT-PCR and western blot were adopted to analyze the changes of morphology, lactate dehydrogenase activity, tyrosine hydroxylase positive neurons rate, and tyrosine hydroxylase (TH) expression in Wistar rat. The two drugs do not affect the normal growth of dopamine neurons. Selegiline and Piribedil both decreased the injury caused by CSF of PD patients in Wistar rat. We observed decreased lactate dehydrogenase (LDH) activity, increased TH (+)/total cells ratio and increased the TH expression in treated Wistar rat with dose-dependent effects. The morphological changes of cells are consistent with above observation. Selegiline and Piribedil have neuroprotective effects to induced PD Wistar rat with dose-dependent effect. Selegiline demonstrated stronger neuroprotective effect than Piribedil, and the two drugs have potential treatment effect in clinical for PD patients.

Keywords: Neuroprotection, parkinson's disease, selegiline, piribedil

Introduction

Parkinson disease (PD), an incurable and progressive neurodegenerative disorder of predominantly idiopathic origin, is characterized by the death of dopaminergic neurons in the substantia nigra pars compacta (SNc), a region of the brain which controls motor activity by projecting dopaminergic axons to the striatum [1, 2]. Early symptoms of PD are primarily movement related, which including shaking, rigidity, brady- and hypokinesia, tremor, and walking difficulty. More advanced stages of PD are associated with cognitive and behavioral problems including dementia [3]. Current treatment strategies for PD consist primarily of traditional levodopa replacement therapy, which could reduce clinical symptoms but cannot prevent disease progression. Researches have demonstrated that neuroprotective drugs could slow the progression and improve symptoms of PD [4, 5].

Tyrosine hydroxylase (TH) catalyses the formation of L-DOPA, which is the rate-limiting step in the biosynthesis of dopamine (DA) [6], compelling studies reported the aberrant activity of lactate dehydrogenase [7, 8]. These inspire us to explore the changing of the 2 targets in the neuroprotective effect.

Monoamine oxidase-B (MAO-B) is an enzyme found in astrocytes, MAO-B is involved in the neurodegenerative process associated with aging, and also involved in neurodegenerative diseases including Parkinson's and Alzheimer's disease [9]. MAO-B inhibitors, which has been demonstrated have neuroprotective effect, could prevent dopaminergic neuron degeneration [10] and decrease parkinsonian symptoms [11]. MAO-B inhibitors could inhibit metabolism and increase synthesis of dopamine (DA) selectively, and balance DA concentration of synaptic cleft in a relatively stable state in striatum.

DA agonist has directly effect on DA receptors in postsynaptic membrane without synthesizing enzyme system; DA agonists also produce stable stimulation to DA receptor with a longer half-life. DA agonists could selectively act on

the specific type of DA receptors, decrease DA turnover rate and free radicals generation and exact their potential neuroprotective effect on dopaminergic neurons in substantia nigra [12].

In this study, Selegiline and Piribedil, the MAO-B inhibitor and DA agonist drugs have neuroprotective activity were selected to explore their neuroprotective effect on Parkinson disease model, E14 Wistar rat induced by CSF from PD patient. There may provide useful information for the optimization of therapeutic agents for the treatment of Parkinson disease.

Materials and methods

Ethnic consideration

CSF was taken from PD patient who were confirmed and was operated, this study was approved by an Institutional Review Board of Tianjin Medical University General Hospital and was conducted in accordance with good clinical practice, all applicable regulatory requirements and the guiding principles of the Declaration of Helsinki. Written informed consent was obtained from all subjects.

The diagnosis of PD was based on each patient's medical history, physical examination and laboratory examination. Normal CSF was drawn from normal surgical patients who confirmed without nervous system disease. The acquisition of CSF was performed in lumbar hemp, all collected CSF were checked by routine biochemical examination.

Embryonic Wistar rat were used in our study; all animal experiments were performed under approved protocols of the institutional animal use and care committee.

Preparation of dopaminergic neurons

Dopaminergic neurons, which were primarily cultured by the embryonic rostral mesencephalic tegmentum (RMT) of embryonic-day-14 Wistar rat, were induced by CSF from PD patients. Control group (CG) was made by dopaminergic neurons induced by CSF from healthy people.

Briefly, after hysterectomy was performed in chloral hydrate intraperitoneal injected rat, the uterus was taken out and placed in sterile cold dish contained Hank's Balanced Salt Solution (HBSS). Tear the uterus, then removed the embryo, cut fetal head and peel off the fetal scalp and skull, the sterile fetal brain was transferred into cold HBSS Petri dish. All these process were performed in the ice bath. The two sides of cerebral and cerebellar hemispheres were stripped to both sides, exposured and caudal colliculus colliculi rostralis, the diencephalon and mesencephalon were separated, then removed the brain meninges, cut off the tissue cover and ventral midbrain colliculi rostralis with 1.0 mm thick, finally left rostral mesencephalic tegmentum region (RMT) about 1.0 mm^3 (1 × 1.5 × 0.75) and cut into small pieces. After digested by trypsin (0.25%, Sigma-Aldrich, MO, USA) and terminated by fetal bovine serum (Gibco Invitrogen, Carlsbad, CA), cleaned by HBSS, cultured by DMEM/F-12 medium contains 0.04 mg/ml DNAse at 37°C for 8~10 min, the supernatant was discharged after centrifugation, and then DMEM/F-12 medium was added into cell for suspension.

Drug treatment procedure

Prepared dopaminergic neurons with concentration of 0.5-1 × 10^4 after incubated in ITSFn medium (DMEM/F-12 media containing, Insulin (5 μg/ml), Apotranferrin (50 μg/ml), Sodium Selenite (30 nM), Fibronectin (250 ng/ml, all from Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO) [13]. A total of 50 μl CSF from different group (control group, CSF of normal patients; treated group, CSF of PD group) were added into 24-well plate contained 250 μl of cell solution, a serial concentration of Selegiline (0.125 μM, 0.25 μM, 0.5 μM) and Piribedil (0.1 μM, 1 μM, 10 μM) were added in these wells, ITSFn medium served as blank control.

Morphology observation was performed under phase contrast microscope.

Lactate dehydrogenase (LDH) activity testing

LDH activity in cultured supernatants was tested by LDH kit (Zhongsheng Biotech, Beijing, CHN) after CSF was added 0, 24, 48 and 96 hours later.

Immunohistochemical staining

After cultured with Selegiline and Piribedil for 48, 96 hours, cells were fixed by 4% paraformaldehyde for 10 min, washed by 0.1 M PBS

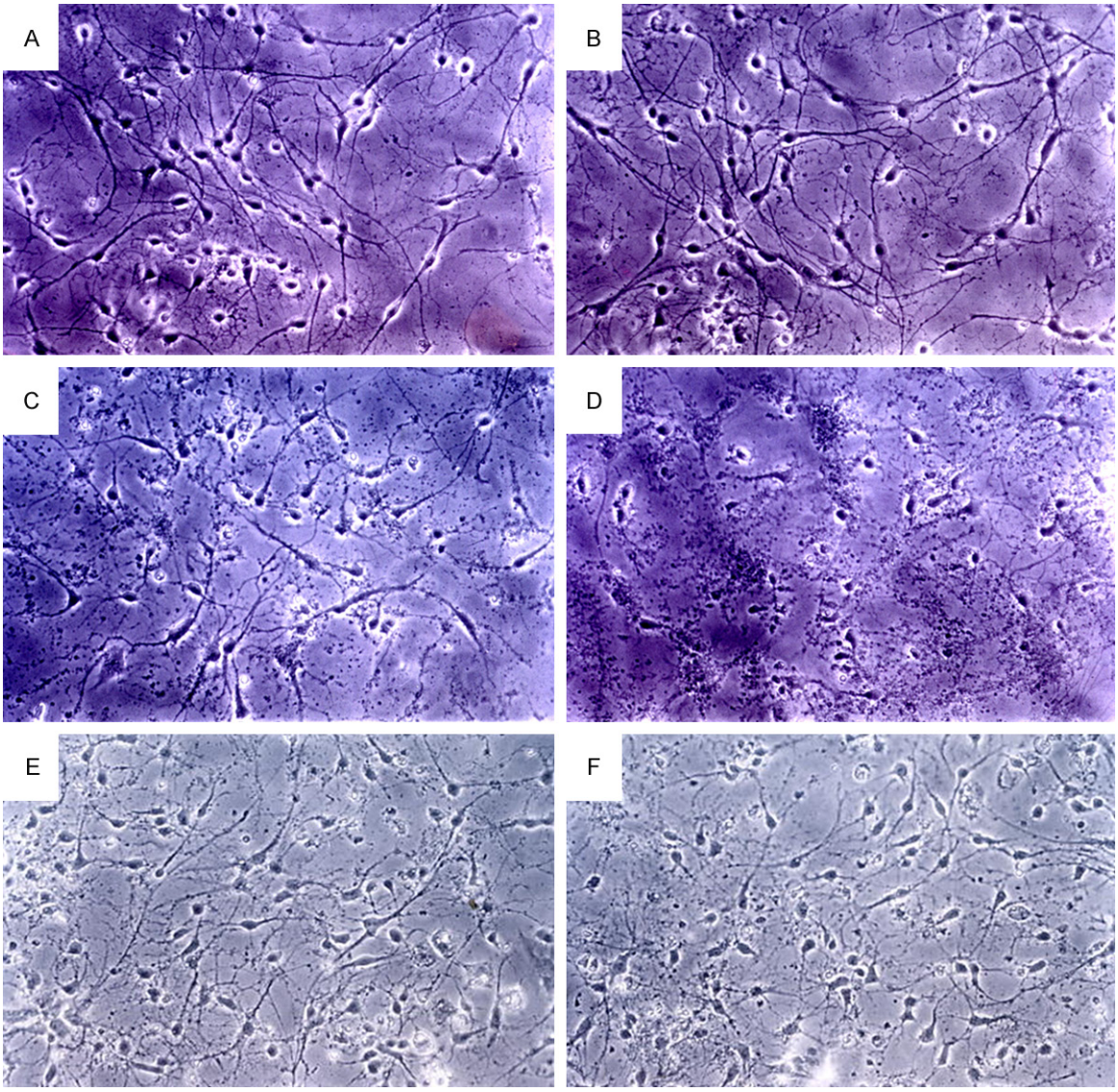

Figure 1. Morphological changes of dopaminergic neurons cells after adding CSF and drugs (400×). Control group cultured for 48 hr (A) and 96 hr (B); CSF of PD patients added and cultured for 48 hr (C) and 96 hr (D); (E) Treated group with Selegiline 0.5 μM; (F) Treated group with Piribedil 10 μM.

for 5 min 3 times; the endogenous catalase was blocked by 0.3% H_2O_2-10% - methanol PBS for 30 min, washed by 0.1 M PBS for 5 min 3 times, and then blocked by 10% goat serum (Gibco Invitrogen, Carlsbad, CA). After discarded the goat serum, anti-tyrosine hydroxylase antibody (ab41528, Abcam, UK) was used, DAB- hematoxylin staining was performed.

RT-PCR and western blot analysis for tyrosine hydroxylase

To test the expression of tyrosine hydroxylase (TH), reverse transcriptase polymerase chain reaction (RT-PCR) and Western blot methods were performed. The cDNA was generated from 5~10 × 10^6 cells total RNA isolated with Trizol reagent (Invitrogen, Carlsbad, CA) by the RT-PCR kit (MBI, Fermentas, Lithuania) employing oligo (dT) primers, β-actin served as control. The PCR primers used were: TH: 5'-tca gag cag gat gcc aag-3' (sense) and 5'-cac ctc gaa gcg cac aa-3' (anti-sense); β-actin: 5'-aga cgg ggt cac cca cac tgt gcc cat cta-3' (sense) and 5'-cta gaa gca ttt gcg gtg cac gat gga ggg-3' (anti-sense). Western blot analysis was carried out as described previously [14].

Table 1. Comparison of LDH activity between control and treated group with Selegiline

LDH activity	Concentration of Selegiline			
	0 μM	0.125 μM	0.25 μM	0.5 μM
Control group	12.27 ± 2.99	11.76 ± 2.38	11.51 ± 2.54	11.25 ± 2.67
Treated group	67.92 ± 6.64	63.12 ± 6.24	47.05 ± 4.68**	25.55 ± 3.62**

N = 32. ANOVA was done in TG and CG respectively. Comparison was performed by Tukeys honestly significant difference. $^{**}P < 0.01$ compared with treated group without drug.

Table 2. Comparison of LDH activity between control and treated group with Piribedil

LDH activity	Concentration of Piribedil			
	0 μM	0.1 μM	1 μM	10 μM
Control group	12.27 ± 2.99	12.01 ± 2.62	11.79 ± 2.79	11.63 ± 2.47
Treated group	67.92 ± 6.64	65.39 ± 6.59	49.71 ± 6.52**	26.94 ± 3.65**

n = 32. ANOVA was done in control and treated group respectively. Comparison was performed by Tukeys honestly significant difference. $^{**}P < 0.01$ compared with treated group without drug.

Results

Routine biochemical examinations of all samples of CSF were within the normal range (data not shown).

Morphology change of dopaminergic neurons cells after adding CSF and drugs

Morphological changes were recorded after CSF and drugs were added. Compared with control group (**Figure 1A**, **1B**), after cultured with CSF of PD patients 48 and 96 hr later (**Figure 1C**, **1D**), the numbers of cells were significantly reduced, the connected cells are sparse, and a small amount of cell debris was found around the growth of DA neurons. At the same time, no significant morphological changes were observed after 0.125 μM or 0.1 μM Piribedil were added. Along with increased concentration of Selegiline (0.25 μM, 0.5 μM (**Figure 1E**)) and Piribedil (1 μM, 10 μM (**Figure 1F**)), we observed their neuroprotective effect with the number of cells increased significantly, cell edge was clearly visible, enhanced halo of light appeared surrounding cells, and extensive connections were established between neuritis.

LDH activity changing

After adding Selegiline or Piribedil to control group, LDH activity in culture supernatants have no significant change. There was no significant change before or after drug added ($P > 0.05$), which means drug with different concentrations does not affect cell growth.

In treated group, LDH activity decreased gradually along with increasing concentration of Selegiline (**Table 1**). When concentration was 0.125 μM, LDH activity declined slightly with no statistically significance ($P > 0.05$). LDH activity reduced about 31% when concentration was 0.25 μM, whereas decreased by 62% ($P < 0.01$) at 0.5 μM. This showed a significant dose-dependent protective effect.

Piribedil demonstrated similar pharmacological effect (**Table 2**). LDH activity declined slightly with concentration of 0.1 μM. When concentration increased to 1 μM and 10 μM, LDH activity reduced about 27% and 60% respectively. Piribedil also has a significant dose-dependent neuroprotective effect.

Semi-quantification of TH-positive neurons by immunohistochemical staining

In this study, TH-positive neurons rate was used as an index to characterize the effect of drugs. When compared with blank control (**Figure 2A**), we observed about 90% TH-positive neurons rate in control group after adding normal CSF to cell culture for 48 and 96 hrs (**Figure 2B**, **2C**); whereas in treated group (**Figure 2D**, **2E**), TH-positive neurons rate increased gradually along with increased concentration of Selegiline, TH-positive neurons rate increased slightly when Selegiline concentration each to 0.125 μM (**Figure 3A**), and when concentration of Selegiline reached to 0.25 μM (**Figure 3B**) and 0.5 μM (**Figure 3C**), there was significant difference of TH-positive neurons rate as compared with cells without drug. At the meantime, we observed consistent results in Piribedil treated cells, TH-positive neurons increased slightly when added 0.1 μM of Piribedil to culture (**Figure 3D**), while the TH-positive neurons rate

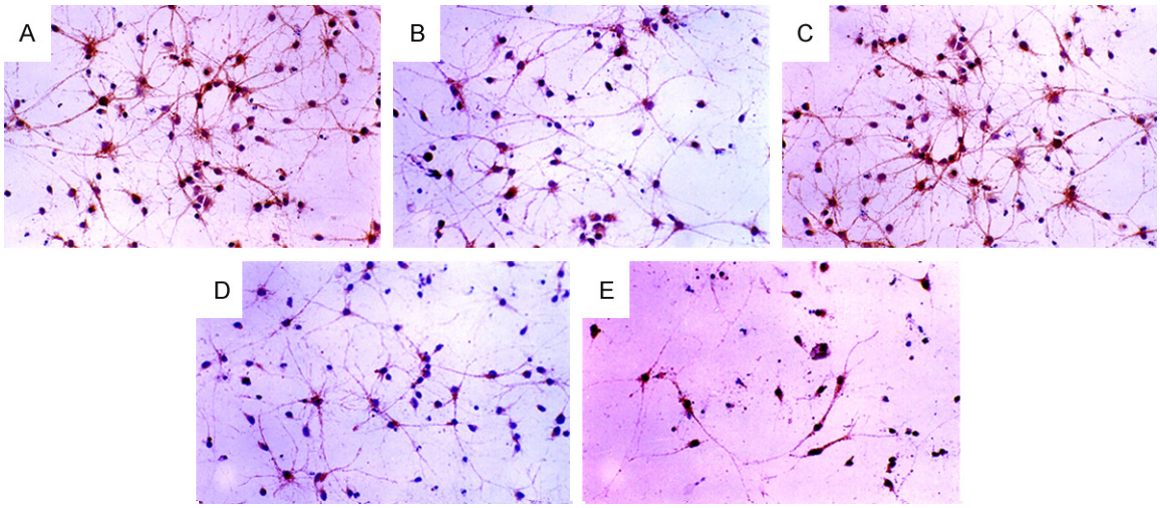

Figure 2. Immunohistochemical staining results of TH-positive neurons. (A) Cell culture without CSF or drug; (B) Control group after adding normal CSF to cell culture for 48, and 96 hrs (C); (D) Treated group after adding CSF of PD patients to cell culture for 48 hrs, and 96 hrs (E).

reach 56.78% and 79.47% when concentration of Piribedil reached to 1 μM (**Figure 3E**) and 10 μM (**Figure 3F**) ($P < 0.01$). Piribedil also showed dose-dependent neuroprotective effect but the effect was less than Selegiline.

Expression of TH

After cultured for 96 hrs, relative gray value of TH/β-actin was used to evaluate the expression of TH mRNA and TH protein (**Tables 3** and **4**). For control group, different concentrations of Selegiline or Piribedil have no effect on the expression of TH mRNA (**Figure 4**) and TH protein (**Figure 5**). While treated by Selegiline or Piribedil, the expression of TH both increased gradually along with increased concentrations. Compared with drug untreated cells, the expressions of Selegiline were significant ($P < 0.05$) at concentration of 0.25 μM and 0.5 μM. Consistent with Selegiline, we observed similar results in Piribedil at concentration of 1 μM and 10 μM ($P < 0.05$).

Discussion

Parkinson's disease (PD) is a degenerative disorder of the central nervous system, which has been widespread around the world with a high incidence recent years. The current treatment for PD is drugs such as levodopa. Although levodopa could improve some symptoms, the progression cannot be stopped [15]. Thus the research of neurotoxicity of CSF from PD patients, and further understanding of occurrence and development of DA neurons injury are important. Exploring neuroprotective effect of some drugs could enrich the treatment of PD, which have both theoretical significance and clinical value.

Selegiline is a dextral compounds isolated from deprenyl phcnylisopropyl-N-methylproinylsmine (E-250) [16]. Pharmacological studies demonstrated that E-250 is a non-reversible inhibitor of MAO-B. As a represent of MAO-B inhibitor, Selegiline is reversible and competitive inhibition at the initial stage, then followed by irreversible inhibition maintained [17]. A large number of clinical trials indicated that Selegiline has therapeutic effect by increasing DA concentration in the synaptic cleft [18, 19]. The possible mechanisms are: (1) inhibiting activity of MAO-B and decreasing degradation of physiological synthesis and exogenous obtaining of DA [20]; (2) amphetamine as one metabolite of Selegiline can inhibit reuptake of DA in the synaptic cleft by dopamine transporter [21]; (3) increasing the synthesis of DA through promoting the synthesis of tyrosine hydroxylase [22].

In addition, Selegiline could promote gene expression and protein synthesis of a variety of nerve growth factor [23], including ciliary neuronotrophic factor (CNTF) and basic fibroblast growth factor (bFGF) in reactive astrocytes. Selegiline could also promote the gene expression and protein synthesis of glial fibrillary acid-

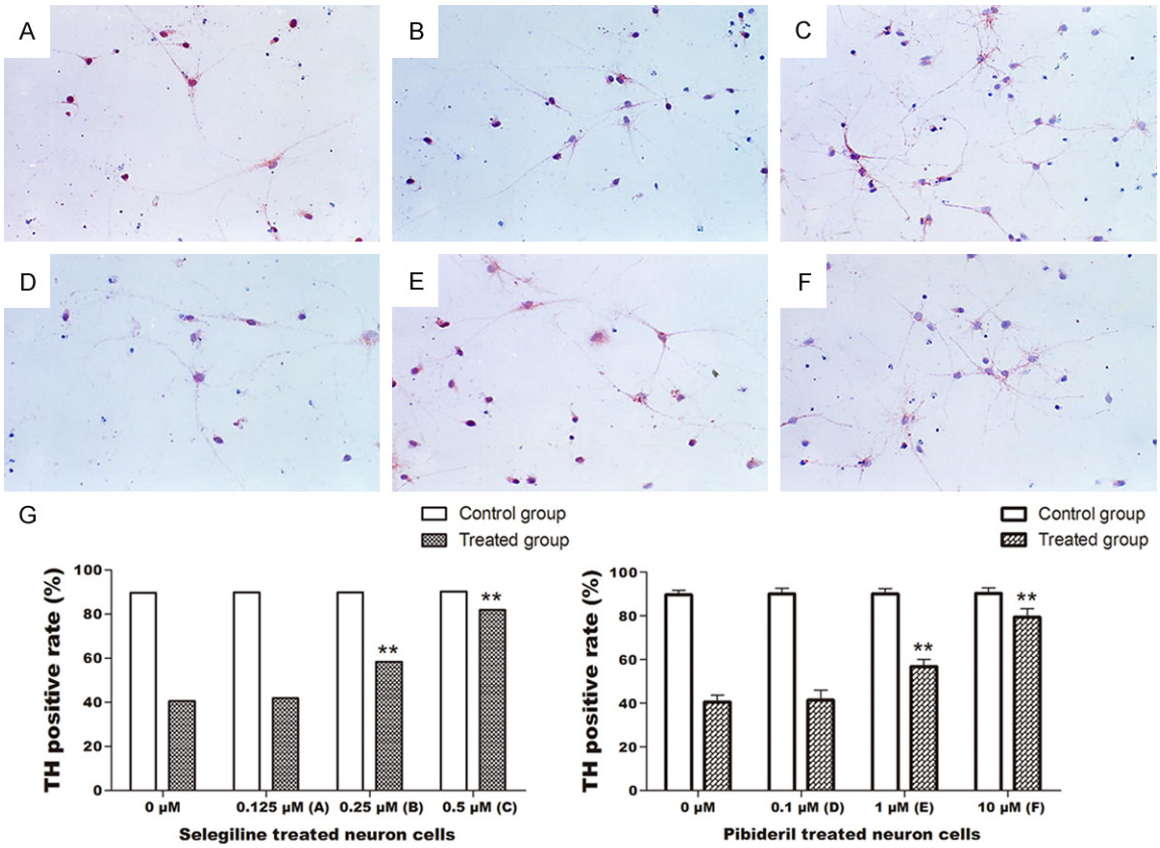

Figure 3. Immunohistochemical staining results of TH-positive neurons after treated by drugs. A. Treated by 0.125 μM Selegiline; B. Treated by 0.25 μM Selegiline; C. Treated by 0.5 μM Selegiline; D. Treated by 0.1 μM Piribedil; E. Treated by 1 μM Piribedil; F. Treated by 10 μM Piribedil; G. Comparison column chart of TH-positive neurons rate treated by different concentration. $^{**}P < 0.01$, compared with drug untreated cells with CSF of PD patients.

Table 3. Comparison of relative gray value in control and treated group (TH protein)

relative gray value	Concentration of Selegiline			
	0 μM	0.125 μM	0.25 μM	0.5 μM
Control group	0.9640 ± 0.0532	0.9730 ± 0.0455	0.9760 ± 0.0744	0.9960 ± 0.0493
Treated group	0.678 ± 0.0497	0.7360 ± 0.0532	0.7980 ± 0.0342*	0.9100 ± 0.0965**

*$P < 0.05$, **$P < 0.01$, compared with treated group without drug.

Table 4. Comparison of relative gray value in control and treated group (TH protein)

relative gray value	Concentration of Piribedil			
	0 μM	0.1 μM	1 μM	10 μM
Control group	0.9560 ± 0.0493	0.9720 ± 0.0363	0.9860 ± 0.0611	0.9940 ± 0.0751
Treated group	0.6760 ± 0.0358	0.6860 ± 0.0503	0.7560 ± 0.0416*	0.8500 ± 0.0292**

*$P < 0.05$, **$P < 0.01$, compared with treated group without drug.

ic protein (GFAP) in astrocytes, and promote the expression of messenger ribonucleic acid (Mrna) in growth factor receptor [24]. CNTF and bFGF have demonstrated could improve the level of antioxidant enzymes, regulate the intracellular Ca^{2+} concentration, and reduce the neurotoxicity of excitatory amino acids.

In our study, we analyzed neuroprotective effect of Selegiline, the results showed that with the

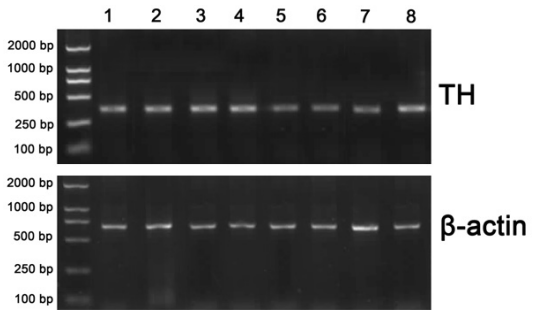

Figure 4. Result of TH mRNA expression after treated by Selegiline. Line1: control group + 0 μM Selegiline; 2; Control group + 0.125 μM Selegiline; 3: control group + 0.25 μM Selegiline; 4: control group + 0.5 μM Selegiline; 5: Treated group + 0 μM Selegiline; 6: Treated group + 0.125 μM Selegiline; 7: + 0.25 μM Selegiline; 8: Treated group + 0.5 μM Selegiline.

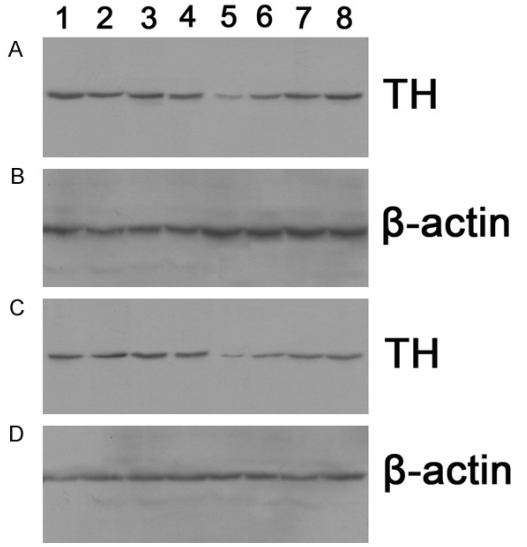

Figure 5. Result of TH protein expression treated by drugs. A, B. Treated by Selegiline; C, D. Treated by Piribedil; Line 1: CG + 0 μM Selegiline/Piribedil; 2: CG + 0.125 μM Selegiline/0.1 μM Piribedil; 3: CG + 0.25 μM Selegiline/1 μM Piribedil; 4: CG + 0.25 μM Selegiline/10 M Piribedil; 5: TG + 0μM Selegiline/Piribedil; 6: TG + 0.125 μM Selegiline/0.1 μM Piribedil; 7: TG + 0.25 μM Selegiline/1 μM Piribedil; 8: TG + 0.25 μM Selegiline/10 M Piribedil.

dosage increased, neuroprotective effect also increased, these findings are conclusive proof that Selegiline could protect DA neurons from CSF toxic damage of PD patients.

Piribedil is adopamine receptor agonists of D2 and D3. By direct stimulating of D1 receptor in the substantia nigra-striatal pathway and D2 receptors in the postsynaptic, the number of DA could be increased [12]. Compared with levodopa, Piribedil has advantage of rarely causing movement complications, particularly dyskinesia [25]. In this experiment, Piribedil also has neuroprotective effect to DA dose-dependently. Application of dopamine agonist Piribedil could reduce the dosage of levodopa, thus reduced the metabolism of free radicals, which is the basis of neuroprotective effects of Piribedil for PD. Studies have confirmed that levodopa could cause the degeneration of dopaminergic neurons, and increase the generation of hydroxyl radicals, as a result led to lipid peroxidation, DNA damage, apoptosis and destruction of mitochondrial respiratory chain activity [26].

We used two different types of drugs, MAO-B inhibitor and dopamine agonist, to analyze their neuroprotective effects for DA. The Wistar rat model of Parkinson's disease had been established through primary culture of E14 RTM induced by CSF from PD patients. The results showed two drugs did not affect the normal growth of dopamine neurons. Selegiline demonstrated stronger neuroprotective effects than Piribedil, both which are two ideal drug candidates for the treatment of PD. New treatment options and medications will prevent or slow down the progress of PD, improve patient quality of life and prolong the patient's life expectancy, promoting and enhancing the overall prevention and treatment of PD.

As a representative of monoamine oxidase B and dopamine antagonist, Selegiline and Piribedil showed obviously dose-dependent neuroprotective effect, which we hope can provide valuable reference to clinical treatment.

Disclosure of conflict of interest

None.

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