

Protective effect and mechanism of *Ginkgo biloba* leaf extracts for Parkinson disease induced by 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine¹

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KEY WORDS *Ginkgo biloba*; 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine; 1-methyl-4-phenylpyridinium; malondialdehyde; superoxide dismutase; dopamine; apoptosis; PC12 cells

ABSTRACT

AIM: To observe the effects of extracts of *Ginkgo biloba* leaves (*EGb*) on the Parkinson disease (PD) models induced by 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) and its ion 1-methyl-4-phenylpyridinium (MPP⁺). **METHODS:** MPTP was microinjected into substantia nigra of rats to induce a behavior change of rotation. *EGb* (ip, 50 or 100 mg·kg⁻¹·d⁻¹) was pretreated consecutively for 19 d before MPTP administered and 1 d after MPTP administered. The contents of malondialdehyde (MDA), superoxide dismutase (SOD), and dopamine (DA) in substantia nigra of model rats were determined. Apoptosis of PC12 cells was induced by MPP⁺, and the protective effect of *EGb* (25, 50, and 100 mg/L) was also observed. The cells of apoptosis were observed under a microscope and counted under a fluoroscope after stained with AO/EB. **RESULTS:** *EGb* (100 mg·kg⁻¹·d⁻¹) decreased the duration and frequency of the rotation of rats ($P < 0.05$, $n = 10$) while *EGb* (50 or 100 mg/L) inhibited the decreases of DA and SOD and the increase of MDA induced by MPTP, ($P < 0.05$ or $P < 0.01$, $n = 10$). MPP⁺ (10 μmol/L) induced the apoptosis of PC12 cells, and *EGb* (50 or 100 mg/L) prevented cells from apoptosis at 6 h, 12 h, and 24 h ($P < 0.05$ or $P < 0.01$, $n = 3$). **CONCLUSION:** *EGb* possesses

protective effect on the PD models *in vivo* and *in vitro*. The anti-oxidation and anti-apoptosis may be one of the mechanisms underlying the neuroprotective effect of *EGb*.

INTRODUCTION

Ginkgo biloba extracts is known to have beneficial effects on the pathology of cardiovascular and cerebrovascular disease^[1]. Several recent investigations have shown that *EGb* has anti-oxidative properties, as it is observed to inhibit the formation of malondialdehyde (MDA)^[2-4]. According to the current insight, Parkinson disease (PD) is a neurodegenerative disease of dopamine (DA) neurons in the substantia nigra based on the oxygen free radicals and lipid peroxidation (LPO) induced by the oxidative stress^[5,6]. At the same time, more and more evidence suggest that apoptosis of the neurons plays an important role in the PD process^[7,8]. Thereby, it is important for the treatment of PD that preventing the apoptosis of neurons. Increasing evidence suggest that the free radicals both of endogenous and exogenous can result in the apoptosis^[9,10]. Efforts have been directed to find pharmacological agents that could scavenge free radicals and decrease the apoptosis. Recently, there is growing research interest in the extracts of *Ginkgo biloba* leaves (*EGb*) for its antioxidation. It has been reported that bilobalide has protective effects against nitric oxide or amyloid beta-peptide 25-35 induced neurotoxicity in PC12 cells^[11,12], and that *EGb* could prevent the dopaminergic neurotoxicity of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)^[13]. But whether the *EGb* has antioxidation in the PD model and has antiapoptotic effect on the PC12 cells have not been reported. Thereby the present study, we observed the antioxidant effect of *EGb* on the PD model *in vitro* induced by MPTP and on the PD model *in vivo* (PC12 cells)^[14] induced by 1-methyl-4-phenylpyridinium.

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MATERIALS AND METHODS

Materials 1-Methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) and its ion 1-methyl-4-phenylpyridinium (MPP⁺) (Research Biochemical International Company, USA) were dissolved in artificial cerebral spinal fluid (ACSF) and Dulbecco's modified Eagle's medium (DMEM) respectively before experiments. EGb (major components: flavonoids: 30 %, terpenes: 8 %; Guiyang Xintang Pharmaceutical Company, Guiyang City, China) was dissolved in 0.9 % sterile saline prior to the *in vitro* experiment and in DMEM medium prior to the *in vivo* experiment respectively.

Animals and treatment Wistar rats (Grade II, Certificate No 24301050) of either sex weighing 230 g ± 20 g, were provided by the Experimental Animal Center of Third Military Medical University. The 40 rats were randomly divided into 4 groups: group I and group II were intraperitoneally injected normal saline 5 mL·kg⁻¹·d⁻¹ for 20 d, group III and group IV were administered intraperitoneally EGb 50 and 100 mg·kg⁻¹·d⁻¹ for 20 d, respectively. On d 20, the rats were anesthetized by aether, and group I was microinjected ACSF 20 μL/kg on the substantia nigra. The other 3 groups were microinjected MPTP 40 μg/kg (20 μL/kg) on same brain area. The rotation of the rats was then observed during 24 h after the microinjection, then the rats were decapitated and dissected. The contents of MDA in substantia nigra were measured with the thiobarbituric acid-reaction to indicate the lipid peroxidation, superoxide dismutase (SOD) (in substantia nigra) assay was based on the ability of SOD to inhibit the oxidation of epinephrine by the xanthine-xanthine oxidase system, and DA in striatum were determined with spectrofluorometer (λ_{ex} = 310 nm, λ_{em} = 390 nm, RF-5000, Japan).

Cells culture and treatment PC12 cells was cultured at 37 °C in a humidified under CO₂ (5 %) incubator in DMEM supplemented with fetal calf serum (10 %), benzylpenicillin (100 IU/L), and streptomycin (100 mg/L). Cells (1 × 10⁴ - 2 × 10⁴/L) were planted in 6 wells plate, in which a cover slide was preplaced, and cultured for 48 h. MPP⁺ (10 μmol/L) was then added in well 2 - 6 (group II), well 1 as control (group I), and Well 4 - 6 were pretreated with EGb at 25, 50, and 100 mg/L for 30 min before the cells exposed to MPP⁺ (group III, IV, and V, respectively). The covers were taken out from the wells at

6 h, 12 h, and 24 h, respectively. The PC12 cells on the cover were fixated as usual and stained with HE, then apoptotic cells were observed under the microscope. And the apoptotic cells were counted under a fluoroscope after stained by AO/EB (acridine orange/ethidium bromide, which purchased from Huamei Company, Shanghai).

Statistical analysis The data was expressed as $x \pm s$, and analyzed by *t*-test.

RESULTS

Effect of EGb on the rotation of rats A significant circling behavior contralateral to the lesioned side was observed in MPTP group, but not in ACSF group. The frequency and duration of the rotation in MPTP group were (39.4 ± 2.8) circles/min and (22.7 ± 1.7)h, respectively. In groups of EGb administration (50 mg/kg and 100 mg/kg), however, the frequency and duration of the rotation induced by MPTP were (37.7 ± 4.1, *P* > 0.05, *n* = 10), (36.1 ± 3.7, *P* < 0.05, *n* = 10) circles/min and 21.3 h ± 1.6h (*P* > 0.05, *n* = 10), 20.9 h ± 1.3 h (*P* < 0.05, *n* = 10). These results indicated that EGB could improve the circling symptoms of PD model to certain extent.

Effects of EGb on the contents of SOD, MDA, and DA in the model rat MPTP decreased markedly the levels of SOD and DA and increased the content of MDA in the substantia nigra of the model rats, and EGb (50 or 100 mg/kg) lowered the MDA while relatively increased the SOD and DA in PD models (*P* < 0.05 or *P* < 0.01). In this study, EGb could scavenge the free radicals and protected the dopaminergic neurons of PD model induced by MPTP (Tab 1).

Effect of EGb on the apoptosis of PC12 cells When PC12 cells were treated with MPP⁺ (10 μmol/L) for 6 h, 12 h, and 24 h, obvious apoptotic cells stained by HE were observed at the microscope; the apoptotic cells showed dimension of cell shrank and decreased, the connection between the synapse of the cells disappeared, the nuclei condensed and dark stained, and the cytoplasm showed strong acidophilous and dark red. The apoptotic body was observed in some apoptotic cells (Fig 1). The apoptotic cells were counted under a fluoroscope after stained by AO/EB. Under the fluoroscope; the cells appearing condensed or ball were apoptotic ones; the nuclear stained green were early apoptotic cells; the

Tab 1. Effects of EGb on the concentration of MDA, SOD, and DA in the substantia nigra of PD model induced by MPTP. $n=10$. $\bar{x} \pm s$. * $P < 0.01$ vs Group I. ^a $P < 0.05$, ^b $P < 0.01$ vs Group II.

Group	Treatment	MDA/ $\mu\text{mol} \cdot \text{g}^{-1}$ wet tissue	SOD/ $\text{IU} \cdot \text{g}^{-1}$ wet tissue	DA/ $\mu\text{g} \cdot \text{g}^{-1}$ wet tissue
I	ACSF + NS	8.8 ± 0.7	$39\ 650 \pm 1\ 934$	10.0 ± 0.4
II	MPTP + NS	17.9 ± 1.0^a	$9\ 230 \pm 713^a$	1.91 ± 0.11^c
III	MPTP + EGb (50 mg/kg)	16.1 ± 1.0^a	$10\ 020 \pm 742^a$	2.02 ± 0.00^c
IV	MPTP + EGb (100 mg/kg)	13.1 ± 1.5^b	$15\ 630 \pm 2\ 304^b$	3.5 ± 0.4^d

Tab 2. The effects of EGb on the apoptotic rate of PC12 cells induced by MPP⁺ (10 $\mu\text{mol/L}$). $n=3$. $\bar{x} \pm s$. * $P < 0.01$ vs Group I. ^a $P < 0.05$, ^b $P < 0.01$ vs Group II.

Group	Treatment	Apoptosis rate/%		
		6 h	12 h	24 h
I	DMEM/F12	0.50 ± 0.03	1.00 ± 0.04	2.50 ± 0.02
II	MPP ⁺	7.50 ± 0.13^c	26 ± 5^c	42 ± 10^c
III	MPP ⁺ + EGb (25 mg/L)	6.73 ± 0.09	25 ± 7	40 ± 7
IV	MPP ⁺ + EGb (50 mg/L)	5.00 ± 0.07^a	22 ± 4^c	33 ± 6^c
V	MPP ⁺ + EGb (100 mg/L)	2.22 ± 0.36^b	7.2 ± 1.0^d	15 ± 4^d

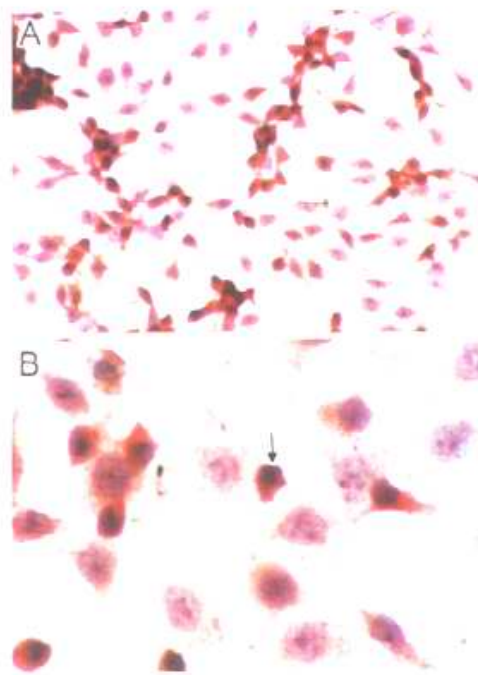


Fig 1. HE staining of the apoptotic PC12 cells induced by MPP⁺ (10 $\mu\text{mol/L}$) for 6 h. A) $\times 40$. B) $\times 100$.

results suggested that MPP⁺ induced the apoptosis of PC12 cells, and EGb could lower the apoptotic rate of PC12 cells induced by MPP⁺.

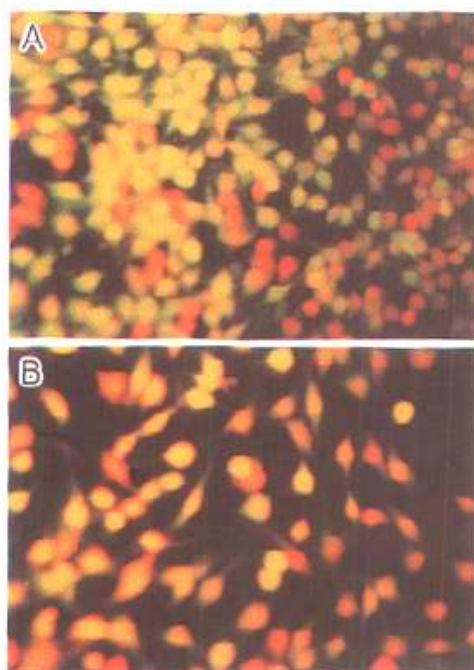


Fig 2. AO/EB staining of the apoptotic PC12 cells induced by MPP⁺ (10 $\mu\text{mol/L}$) for 6 h (A) and 24 h (B). $\times 40$.

nuclear stained orange were late apoptotic cells (Fig 2). In the culture pretreated with EGb, the apoptotic rate of PC12 cells was significantly decreased (Tab 2). These

DISCUSSION

It is known that MPTP administered intraperitoneally induced Primates PD model, but not rat or mice, which resulted in the expensive experiment. There is few reports that MPTP was infused into the substantia nigra inducing rat or mice PD model^[15]. In our study, first time we successfully microinjected MPTP into the substantia nigra inducing rat PD model. In this study, our results showed that MPTP really had dopaminergic neurotoxicity leading to the rotation of rats, in agreement with previous studies. Moreover, our results further confirmed that the neurotoxicity of MPTP is much relative with the free radicals, for MDA being increased markedly in MPTP group, but not in ACSF group. Above all, our result showed that MDA was reduced, and SOD and DA were increased while treatment with *EGB*, and this suggested that *EGB* could improve the ability of scavenging the free radicals and have antioxidative effect on PD model induced by MPTP.

In vitro experiment of our study, MPP^+ induced the apoptosis of PC12 cells, and the *EGB* could lower the apoptotic rate of PC12 cells induced by MPP^+ , this result implies that the neurotoxicity of MPTP is much relative with its ion MPP^+ which induced the apoptosis of dopaminergic neuron *in vitro* PD model, and that the protective effects of *EGB* result from its both antioxidation and antiapoptotic effect.

It has been shown that the apoptosis of neuron plays an important role in PD^[7,8], an oxidation has much connection with the apoptosis^[9,10]. In our study, we observed that *EGB* had antioxidation in PD model *in vivo* induced by MPTP and could prevent the apoptosis of PC12 cells induced by MPP^+ . These results suggest that *EGB* has protective effects on PD, and the anti-oxidation and anti-apoptosis may serve as one of the mechanisms underlying the neuroprotective effect of *EGB*. Thereby *EGB* may have a bright future in the therapy of PD, although whether the *EGB* has antiapoptotic effect or not on the PD model *in vivo* need to be further explored.

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银杏叶提取物对 1-甲基-4-苯基-1,2,3,6-四氢吡啶所致帕金森症的保护作用及机制¹

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关键词 银杏; 1-甲基-4-苯基-1,2,3,6-四氢吡啶; 1-甲基-4-苯基吡啶; 丙二醛; 超氧化物歧化酶; 多巴胺; 细胞凋亡; PC12 细胞

目的: 观察银杏叶提取物(EGb)对 1-甲基-4-苯基-1,2,3,6-四氢吡啶(MPTP)及其离子 1-甲基-4-苯基吡啶(MPP⁺)诱导的帕金森症(PD)模型的保护作用。 **方法:** 用脑立体定位仪向黑质(AP - 5.4 mm, -2.2 mm, H 8.3 mm)内注射 MPTP 诱导大鼠旋转。 在注

射 MPTP 24 h 后将大鼠处死, 硫代巴比妥法测定黑质中丙二醛(MDA), 羟胺法(即改进的黄嘌呤氧化酶法)测定黑质中超氧化物歧化酶(SOD), 荧光分光光度法(激发波长 310 nm, 发射波长 390 nm)测定纹状体中多巴胺(DA)的含量。 MPP⁺ 诱导 PC12 细胞凋亡, HE 染色, 光镜下观察凋亡细胞; 吖啶橙/溴乙锭(AO/EB)染色, 荧光显微镜计数凋亡细胞, 观察不同浓度 EGb (25, 50, 100 mg/L)在 6 h, 12 h, 24 h 对细胞凋亡率的影响。 **结果:** EGb 100 mg/kg 组可减少模型鼠的旋转次数及旋转持续时间($n = 10$, $P < 0.05$); 与 MPTP 组比较, EGb 50 mg/kg 和 100 mg/kg 组 MDA 相对降低, SOD 及 DA 相对增高($n = 10$, $P < 0.05$ 和 $P < 0.01$)。 MPP⁺ 10 $\mu\text{mol/L}$ 可诱导 PC12 细胞凋亡, EGb 50 和 100 mg/L 组在 6 h, 12 h, 24 h 可降低细胞凋亡率($P < 0.05$ 和 $P < 0.01$, $n = 3$)。 **结论:** EGb 对 MPTP 诱导的 PD 动物模型及其离子 MPP⁺ 诱导的 PD 细胞模型均有保护作用, 其保护机制与清除自由基及抑制神经元凋亡有关。

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