

Ginkgo Biloba Extract Protects Substantia Nigral Neurons from Apoptosis in PD Rat Model

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Abstract

Purpose: To investigate the apoptotic mechanism during the pathological process of Parkinson disease (PD) and explore the availability of Ginkgo biloba extract (EGb) to cure PD.

Methods: Followed 6-OHDA injection stereotaxically to right side of substantia nigra pars compacta (SNc), at the date of 1, 7, 14 and 21, we used biochemical, immunohistochemical, TUNEL and electron microscopic techniques to evaluate the level of apoptosis (TUNEL), monitor the degree of oxidative reaction (MDA.SOD), and to observe the change of ultrastructure of substantia nigra in the experimental PD models of rats (PD group), the EGb treatment group (EGb group) and the control group.

Results: Contrast to the Control group, the number of apoptotic neurons and free radical level increased in the PD group and the EGb group, but the PD group surpassed the EGb group ($p < 0.05$). In the PD group, the ultrastructure of substantia nigra exhibited the morphological characteristics of apoptosis from 1d to 21d.

Conclusions: Free radical insult contributes to the pathological process of PD in rats. EGb may ameliorate the injury of substantia nigra through expiring free radicals during the pathological process of PD in rats.

Keywords: Ginkgo Biloba Extract (EGb); Parkinson disease; Apoptosis; Free radical

Introduction

Parkinson disease is one of the most common diseases in senile. Recent research point out that PD is an episode induced by multiple factors, such as oxidation stress and apoptosis. But the concrete mechanism still should be elucidated. 6-hydroxydopamine (6-OHDA), the dopamine metabolite which is involved in the pathological process of PD, is often used as a chemical damage preparation to mimic dopaminergic nerve injury *in vivo*. Ginkgo Biloba extract (EGb), which was reported in Spirit of Ben Chao Ping Hui, was gymnosperm and has been used as drug for thousand of years in China. In this paper, we will explore the mechanism of EGb to protect the substantia nigra neurons and discuss the possibility of applying it to clinic work.

Material and Methods

All the following procedures have been approved by the Ethic Committee of Animal Care and Use of Tongji Medical College. Total 130 male Sprague Dawley rats (offered by Animal Center of Tongji Medical College), with body weight 180-200 g (aged 3 months), were used in this experiment.

EGb suspension preparation

EGb, with the commodity name of Taping, was offered by Kang Engbei Medical Conglomerate of Zhejiang province. The component of EGb included 24% omni-flavone and 6% ginkgo lactone, which were solved in the 0.5% carboxymethyl cellulose sodium. Concentration of EGb suspension was 20 mg/ml. It should be used as soon as dispensed and preserved in hypothermia, avoiding of light and enclosed. Before being used, EGb suspension should be agitated by BIM-8A ultrasound shaker.

PD rat models operation

The PD rat models were operated according to the improved Thomas's method with little alterations [1]. Before 6-OHDA injection to the right substantia nigra, generally speaking, all rats were perfused

consecutively through stomach with EGb suspension (Dosage was 100 mg/kg/d) for 7 days. After anesthesia with 10% chloral hydrate, the 6-OHDA was injected stereotaxically into the right substantia nigra. In the following days, the EGb group rats were undergone consecutively stomach perfusion with EGb suspension each day till the date point, while the Control group and the PD group were perfused with isometric normal saline.

Tunel

The apoptotic neurons were detected by the TUNEL method according to Seaton's method [2,3]. After anesthesia with 10% chloral hydrate, the rats were sacrificed. Then the midbrain was taken out and made into frozen preparation sections. Through HE staining, the sections with clear-cut damage of substantia nigra were reserved at 4°C and used in the followed procedures. Then the apoptotic neurons were examined by TUNEL test kit according to the manufacturer provided by Nanking Jiancheng Corporation, China, and the apoptotic cells were counted.

Lipid peroxidate products and free radical expiring ability examination

After anesthesia with 10% chloral hydrate, the rats were sacrificed. Then the tissue of right substantia nigra was fetched. Lipid peroxidate products and free radical expiring ability were examined by malondialdehyde (MDA) and superoxide dismutase (SOD) biological

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product test kit according to the manufacturer provided by Nanking Jiancheng Corporation, China.

Observation of electron microscope

0.5 mm³ substantia nigra was picked out and double fixed with 2.5% glutaral, 1% osmium acid lead, and then desiccation and embed in Epon812. Ultrathin sections were made by LKB-III microtome, double stained by acetic acid U and lead nitrate, and then observed by transmission electron microscope.

Statistical analysis

All data were presented as mean ± standard deviation (SD), and analyzed by one-way ANOVA. A value of *p*<0.05 was taken as significant.

Results

Counting apoptotic cells of substantia nigra

The number of apoptotic cells in the PD group and EGb group was increased gradually compared with the Control group, but the EGb group was fewer than the PD group; Compared with the PD group, the EGb group decreased 57%, 54%, 48% and 50% respectively at each time point (Table 1).

SOD and MDA in substantia nigra

There was no notable change of MDA and SOD level in the Control group at each time point. Contrast to the Control group, both of MDA and SOD in the PD and the EGb group was increasing with time, but the level of MDA and SOD in the EGb group was less than that of the PD group (Tables 2 and 3).

Electron microscopic observation of the ultrastructure of substantia nigra

1d PD group: The cellular membrane of substantia nigra neurons presented "blister phenomenon". Nuclear membrane was irregular, and cytoplasm was concentrated.

1d EGb group: The shape of substantia nigra neurons was normal, and the nuclear membrane was regular.

7d PD group: Neurons of substantia nigra were diminished. Concentrated chromatins were encircled around the edge of the nuclei like a crescent.

7d EGb group: The concentrated chromatins were encircled at the edge of the nucleus, and the nuclear membrane became irregular.

14d PD group: Neurons of substantia nigra were diminished. Concentrated chromatin presented like a mass and the glial cells were proliferated.

14d EGb group: The nuclei of substantia nigra neurons were concentrated, and divided into several leaves with focal edema.

21d PD group: Neurons of substantia nigra were shrunken. Nuclei were concentrated into a mass and the Satellitosis phenomenon could be seen. Moreover, the apoptotic neurons were phagocytosed.

21d EGb group: Neurons of substantia nigra were shrunken. Concentrated chromatins were encircled at the edge of nucleus. Phenomenon of blister could be seen.

Discussion

6-OHDA can lead to oxidative stress injury and produce free radicals, which reflected the degree of injury of cells indirectly [3,4]. While the vitality of SOD reflected the ability of body to clean off free radicals, and SOD vitality was increased in the experimental models, the increased SOD level cannot compensate the injury. Choi [5] thought that the degree of SOD increasing is less than that of free radical increasing by far in PD patients. In our study, we found that the level of MDA and vitality of SOD increased gradually with the injury time. This effect revealed that the oxidative stress injury of substantia nigra was getting even more serious gradually.

Apoptosis was proved to be the common pathway in the episode of PD. Recent study in vitro found that free radicals played a major role on cell apoptosis [6]. In our study, we found that the number of apoptotic cells of substantia nigra was increased gradually from 1 d to 21 d, and the ultrastructure of substantia nigra presented typical apoptosis alteration.

As a classic agent of Chinese traditional medicine, EGb can clean off free radicals. Some literature reported that EGb can prevent the granular cells of cerebellum, hippocampus neurons of rats from apoptosis [7,8]. In our study, we found that the level of MDA and SOD in the EGb group was less than the PD group notably, which mean oxidative stress injury in the EGb group was less serious than the PD group. Our study also found that EGb can prevent the cells of substantia nigra from apoptosis effectively. The apoptotic cells of substantia nigra in the EGb group decreased 57%, 54% 48%, 50% compared with the PD group at the date of 1, 7, 14, 21 respectively. The ultrastructural damage of the EGb group was less than the PD group either. EGb can protect neurons in many ways because of its multi-component and multi-valence pharmacologic effects. EGb can decrease not only membrane fluidity and protein conformation change evoked by free radicals, but also the concentration of peroxide anion, for instance, it can conjugate with peroxidase prosthetic group Fe³⁺ and relieve neuronal toxic effect of glutamate acid [9]. More over, it can reduce toxic effect of MPTP and prevent the injury of mitochondria and DNA.

Taking together, our study found that EGb protect neurons from

Groups	n	Days after 6-OHDA injection			
		1 d	7 d	14 d	21 d
Control	20	65 ± 8	55 ± 9	71 ± 11	56 ± 8
PD	20	358 ± 31*	388 ± 48*	413 ± 22*	456 ± 14*
EGb	20	154 ± 18*#	179 ± 21*#	212 ± 21*#	228 ± 28*#

**p*<0.05 vs. Control group, #*p*<0.05 vs. EGb group

Table 1: Number of apoptotic cells of substantia nigra (/mm²).

Groups	n	Days after 6-OHDA injection			
		1 d	7 d	14 d	21 d
Control	20	107 ± 18	109 ± 17	113 ± 11	104 ± 17
PD	20	135 ± 11*	165 ± 15*	181 ± 20*	197 ± 22*
EGb	20	104 ± 19*#	112 ± 20*#	137 ± 19*#	148 ± 12*#

**p*<0.05 vs. control group, #*p*<0.05 vs. EGb group

Table 2: MDA in substantia nigra (NU/mg).

Groups	n	Days after 6-OHDA injection			
		1d	7d	14d	21d
Control	20	350 ± 36	355 ± 27	357 ± 25	362 ± 30
PD	20	398 ± 31*	409 ± 39*	435 ± 29*	472 ± 41*
EGb	20	227 ± 30*#	258 ± 33*#	265 ± 32*#	288 ± 36*#

**p*<0.05 vs. Control group, #*p*<0.05 vs. EGb group

Table 3: SOD in substantia nigra (nmol/g).

free radicals and decreased the apoptosis ratio and the ultrastructure damage of substantia nigra in PD rat model. EGb might alleviate the symptom of PD and become a potential drug for PD patients.

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