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# How do Chinese medicines that tonify the kidney inhibit dopaminergic neuron apoptosis?

Shaogang Lin<sup>1</sup>, Shuifen Ye<sup>2</sup>, Jinmu Huang<sup>1</sup>, Yun Tian<sup>3</sup>, Yihui Xu<sup>2</sup>, Mengqi Wu<sup>2</sup>, Jingxia Wang<sup>2</sup>, Songying Wu<sup>4</sup>, Jing Cai<sup>2</sup>

1 Hospital of Putian University, Putian 351100, Fujian Province, China

2 Academy of Integrative Medicine, Fujian University of Traditional Chinese Medicine, Fuzhou 350122, Fujian Province, China

3 College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, Zhejiang Province, China

4 The Second People's Hospital of Fujian University of Traditional Chinese Medicine, Fuzhou 350003, Fujian Province, China

## Abstract

Wistar rats were intragastrically perfused with Chinese medicines used for tonifying the kidney. These included 0.180 g/mL of *Herba Epimedii* (Epimedium), *Semen Cuscutae* (Dodder Seed), or *Herba Cistanches* (Desertliving Cistanche), 0.04 mg/mL monoamine oxidase-B inhibitor selegiline, or distilled water for 14 consecutive days to prepare drug-containing serum or blank serum. MES23.5 cells in the logarithmic phase were cultured in media supplemented with 15% drug-containing serum for 24 hours, followed by incubation in culture solution containing 100  $\mu\text{mol/L}$   $\text{H}_2\text{O}_2$  for 3 hours. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and flow cytometry results showed that all drug-containing serums improved the survival rate of  $\text{H}_2\text{O}_2$ -injured MES23.5 cells, inhibited pro-apoptotic FasL and caspase-3 expression, promoted anti-apoptotic Bcl-2 expression. However, drug-containing serums had little influence on Fas expression in  $\text{H}_2\text{O}_2$ -injured MES23.5 cells. Enzyme-linked immunosorbent assay results showed that serum containing *Herba Cistanches* or *Herba Epimedii* increased the expression of nerve growth factor, brain-derived neurotrophic factor, and glial cell line-derived neurotrophic factor in injured MES23.5 cells; serum containing *Semen Cuscutae* only increased brain-derived neurotrophic factor expression; while expression of the above neurotrophic factors remained the same in cells treated with serum containing selegiline. These findings indicate that Chinese medicines used to tonify the kidney can protect nerve cells by regulating the expression of apoptosis-related factors and neurotrophic factors in MES23.5 cells.

## Key Words

neural regeneration; traditional Chinese medicine; drug-containing serum; MES23.5 dopaminergic nerve cells; neurotrophic factors; apoptosis factors; Parkinson's disease; neuroprotection

## Research Highlights

- (1) This study monitored apoptosis-related factors and neurotrophic factors to reveal the inhibitory effects of Chinese medicines for tonifying the kidney on apoptosis at the molecular level.
- (2) Results showed that Chinese medicines for tonifying the kidney can protect nerve cells by regulating the expression of apoptosis-related factors and neurotrophic factors.

Shaogang Lin, Master.

Shaogang Lin and Shuifen Ye contributed equally to this work.

Corresponding author: Jing Cai, M.D., Ph.D., Professor, Doctoral supervisor, Fujian University of Traditional Chinese Medicine, Fuzhou 350122, Fujian Province, China, caij1@163.com.

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## INTRODUCTION

Parkinson's disease is characterized by degeneration and loss of dopaminergic neurons in the substantia nigra, a region of the midbrain, as well as Lewy body formation<sup>[1-2]</sup>. The loss of dopaminergic neurons and Parkinson's disease are highly correlated with activation of the apoptotic pathway<sup>[3-4]</sup>. A variety of cytokines and neurotrophic factors have been used for neuroprotection<sup>[5-6]</sup>. Compound preparations of Chinese medicines that can tonify the kidney have been reported to significantly improve symptoms of Parkinson's disease. However, the mechanism of action remains unclear<sup>[7-9]</sup>. We have previously shown that Chinese medicines that tonify the kidney can protect dopaminergic neurons by regulating the expression of apoptosis-related factors and increasing neurotrophic factor content in an animal model of Parkinson's disease<sup>[10]</sup>. However, evidence from *in vitro* experiments is lacking.

The MES23.5 cell line is hybridized from midbrain cells from the rat embryo and the mouse neuroblastoma glioma cell line N18TG2. This cell line has properties of dopaminergic neurons and is easier to culture than midbrain nerve cells. Therefore, these cells have been extensively used for studying neurodegenerative diseases<sup>[11]</sup>.

To investigate whether Chinese medicines that tonify the kidney can protect *in vitro* cultures of dopaminergic neurons, we induced oxidative damage in MES23.5 cells using H<sub>2</sub>O<sub>2</sub>, and treated the injured cells with serum containing *Herba Epimedii* (Epimedium), *Semen Cuscutae* (Dodder Seed), or *Herba Cistanches* (Desertliving Cistanche). Flow cytometry and enzyme-linked immunosorbent assay (ELISA) were utilized to detect the expression of apoptosis-related factors and neurotrophic factors. We aimed to explore the neuroprotective mechanism of Chinese medicines that can tonify the kidney for Parkinson's disease prevention. Selegiline and monoamine oxidase-B inhibitor were used as controls.

## RESULTS

### Influence of different concentrations of H<sub>2</sub>O<sub>2</sub> on MES23.5 cell growth

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) results showed that the survival rate of MES23.5 cells remained after exposure to 50, 100, 150, 200, 300 and 400 μmol/L H<sub>2</sub>O<sub>2</sub> for 1 hour ( $P > 0.05$ ), but significantly reduced after 3 hours, except the cells exposed to 50 μmol/L H<sub>2</sub>O<sub>2</sub> ( $P < 0.01$ ). Moreover, there was no significant difference in the survival rate among different H<sub>2</sub>O<sub>2</sub> concentration groups, except 50 μmol/L H<sub>2</sub>O<sub>2</sub>, at each time point ( $P > 0.05$ ). The survival rate of MES23.5 cells was not reduced until 50 μmol/L H<sub>2</sub>O<sub>2</sub> treatment for 24 hours ( $P < 0.05$ ). Thus, we treated MES23.5 cells with 100 μmol/L H<sub>2</sub>O<sub>2</sub> for 3 hours (Table 1).

### Influence of different concentrations of drug-containing serum on the growth of H<sub>2</sub>O<sub>2</sub>-induced MES23.5 cells

MTT results showed that no alteration was found in H<sub>2</sub>O<sub>2</sub>-induced MES23.5 cells pretreated with 5% (v/v) and 10% (v/v) drug-containing serum for 24 hours ( $P > 0.05$ ). However, pretreatment with 15% (v/v), 20% (v/v), 25% (v/v) and 30% (v/v) drug-containing serum for 24 hours significantly improved the survival rate of H<sub>2</sub>O<sub>2</sub>-induced MES23.5 cells ( $P < 0.05$  or  $P < 0.01$ ), but no significant difference was observed among the concentrations ( $P > 0.05$ ). Thus, 15% (v/v) drug-containing serum was used in subsequent experiments (Table 2).

### Influence of drug-containing serum on the expression of apoptosis-related factors in H<sub>2</sub>O<sub>2</sub>-induced MES23.5 cells

Flow cytometry showed that FasL and caspase-3 content significantly increased in the model group compared with the blank serum-treated group; ( $P < 0.05$ ). FasL and caspase-3 content significantly reduced in the *Herba Epimedii*, *Semen Cuscutae*, *Herba Cistanches* and selegiline groups when compared with the model group ( $P < 0.05$ ). However, FasL and caspase-3 content in the *Herba Epimedii* group, as well as

FasL content in the *Herba Cistanches* group, remained higher when compared with blank serum group ( $P < 0.05$ ; Table 3).

Changes in Bcl-2 content in cells from each group were opposite to FasL and caspase-3 levels. Bcl-2 content was significantly reduced in the model group when compared with blank serum group ( $P < 0.05$ ); Bcl-2 content was significantly higher in the *Herba Epimedii*, *Herba Cistanches* and selegiline groups when compared with the model group ( $P < 0.05$ ), but the increase in Bcl-2 content was not obvious in the *Semen Cuscutae* group (Table 3). No significant difference was found in Fas content among the groups ( $P > 0.05$ ; Table 3).

### Influence of drug-containing serum on the expression of neurotrophic factors in H<sub>2</sub>O<sub>2</sub>-induced MES23.5 cells

ELISA results revealed that nerve growth factor content significantly reduced in the model and selegiline groups

when compared with the blank serum group ( $P < 0.05$ ). However, nerve growth factor levels were greater in the *Herba Epimedii*, *Semen Cuscutae*, and *Herba Cistanches* groups when compared with the model group, but significant differences were only found between the *Herba Cistanches* and model groups ( $P < 0.05$ ; Table 4).

Brain-derived neurotrophic factor content was significantly reduced in the model and selegiline groups when compared with the blank serum group ( $P < 0.05$ ), and was significantly greater in the *Herba Epimedii*, *Semen Cuscutae*, and *Herba Cistanches* groups when compared with the blank serum group ( $P < 0.05$ ; Table 4).

Glial cell line-derived neurotrophic factor content was significantly greater in the *Herba Cistanches* group when compared with the blank serum, model, *Semen Cuscutae* and selegiline groups ( $P < 0.05$ ), but no significant difference were found between the *Herba Epimedii* and *Herba Cistanches* groups (Table 4).

Table 1 Survival rate (%) of MES23.5 cells treated with different concentrations of H<sub>2</sub>O<sub>2</sub> over time

Treatment time (hour)	H <sub>2</sub> O <sub>2</sub> concentration (μmol/L)					
	50	100	150	200	300	400
1	96.42±10.22	99.22±9.73	97.51±9.11	96.86±8.95	99.51±9.68	94.08±9.21
3	95.37±9.68	50.28±8.76 <sup>b</sup>	44.70±9.13 <sup>b</sup>	41.53±7.36 <sup>b</sup>	40.54±8.54 <sup>b</sup>	40.03±9.18 <sup>b</sup>
6	94.59±7.91	46.44±9.38 <sup>b</sup>	43.91±7.85 <sup>b</sup>	35.59±10.49 <sup>b</sup>	35.05±6.77 <sup>b</sup>	35.01±9.81 <sup>b</sup>
9	93.49±8.73	41.64±9.33 <sup>b</sup>	37.83±7.96 <sup>b</sup>	31.66±6.54 <sup>b</sup>	30.81±8.67 <sup>b</sup>	29.19±8.66 <sup>b</sup>
12	91.02±9.45	37.51±8.43 <sup>b</sup>	36.20±9.51 <sup>b</sup>	29.38±7.14 <sup>b</sup>	29.91±11.21 <sup>b</sup>	27.59±9.18 <sup>b</sup>
24	58.70±9.74 <sup>a</sup>	29.30±10.68 <sup>b</sup>	28.84±6.48 <sup>b</sup>	27.91±9.53 <sup>b</sup>	17.63±9.54 <sup>b</sup>	16.41±9.57 <sup>b</sup>

Results are expressed as mean ± SD. Two parallel wells were set in each group at each time point. The experiment was conducted in triplicate. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , vs. 0 μmol/L H<sub>2</sub>O<sub>2</sub> group using one-way analysis of variance followed by least significant difference test. Survival rate (%) = mean absorbance of experiment group/mean absorbance of negative control group × 100%<sup>[12]</sup>. 0 μmol/L H<sub>2</sub>O<sub>2</sub> group served as the negative control group, with the survival rate of cells = 100%.

Table 2 Survival rate (%) of H<sub>2</sub>O<sub>2</sub>-induced MES23.5 cells following treatment with drug-containing serum at different concentrations

Group	Concentration of drug-containing serum (%)					
	5	10	15	20	25	30
Negative control	100 <sup>b</sup>					
Blank serum	56.42±7.22	79.22±9.03 <sup>b</sup>	91.51±6.31 <sup>b</sup>	86.86±7.36 <sup>b</sup>	89.51±7.74 <sup>b</sup>	84.08±9.32 <sup>b</sup>
Model	55.03±6.73	58.11±7.18	56.71±9.04	57.12±8.11	56.53±7.87	57.21±8.23
<i>Herba Epimedii</i>	56.71±8.17	62.02±7.66	76.81±7.54 <sup>b</sup>	73.45±6.38 <sup>b</sup>	70.81±8.42 <sup>a</sup>	70.41±9.06 <sup>a</sup>
<i>Semen Cuscutae</i>	53.14±7.37	61.31±8.13	77.31±7.39 <sup>b</sup>	68.82±9.11 <sup>a</sup>	68.51±7.28 <sup>a</sup>	68.64±8.16 <sup>a</sup>
<i>Herba Cistanches</i>	52.97±9.31	61.22±6.18	80.66±8.57 <sup>b</sup>	76.31±7.66 <sup>b</sup>	78.14±6.83 <sup>b</sup>	75.43±7.94 <sup>b</sup>
Selegiline	53.86±8.15	64.26±5.36	82.72±7.63 <sup>b</sup>	80.12±6.89 <sup>b</sup>	81.22±7.46 <sup>b</sup>	81.64±8.04 <sup>b</sup>

Except the negative control and blank serum groups, all groups were treated with 100 μmol/L H<sub>2</sub>O<sub>2</sub>; the model group was treated with blank serum; *Herba Epimedii*, *Semen Cuscutae*, *Herba Cistanches* and selegiline groups were treated with corresponding drug-containing serum. Results are expressed as mean ± SD. Three parallel wells were set in each group at each time point. The experiment was conducted in triplicate. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , vs. model group using one-way analysis of variance followed by the least significant difference test. Survival rate (%) = mean absorbance of experiment group/mean absorbance of negative control group × 100%.

Table 3 FasL-, Fas-, caspase-3-, and Bcl-2-positive expression rate (%) in H<sub>2</sub>O<sub>2</sub>-induced MES23.5 cells following treatment with drug-containing serum

Group	FasL	Fas	Caspase-3	Bcl-2
Blank serum	1.440±0.277	1.999±0.435	1.512±0.317	1.829±0.401
Model	3.829±0.360 <sup>a</sup>	2.321±0.412 <sup>a</sup>	3.872±0.448 <sup>a</sup>	0.977±0.316 <sup>a</sup>
<i>Herba Epimedii</i>	2.294±1.106 <sup>ab</sup>	2.284±1.106 <sup>a</sup>	2.491±1.548 <sup>ab</sup>	1.384±1.141 <sup>b</sup>
<i>Semen Cuscutae</i>	2.006±0.485 <sup>b</sup>	2.006±0.485	2.015±0.561 <sup>b</sup>	1.049±0.481 <sup>a</sup>
<i>Herba Cistanches</i>	2.239±0.350 <sup>ab</sup>	2.239±0.357 <sup>a</sup>	2.191±0.405 <sup>b</sup>	1.231±0.311 <sup>ab</sup>
Selegiline	2.046±0.529 <sup>b</sup>	2.046±0.531	2.138±0.638 <sup>b</sup>	1.500±0.505 <sup>b</sup>

Results are expressed as mean ± SD. The experiment was conducted seven times. <sup>a</sup>*P* < 0.05, vs. blank serum group; <sup>b</sup>*P* < 0.05, vs. model group using one-way analysis of variance followed by the least significant difference test. Positive expression rate (%) = number of fluorescent cells (number of cells binding antibodies)/number of total cells × 100%<sup>[13]</sup>.

Table 4 Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) content changes (absorbance) in H<sub>2</sub>O<sub>2</sub>-induced MES23.5 cells following treatment with drug-containing serum for 24 hours

Group	NGF	BDNF	GDNF
BS	0.531±0.088	0.176±0.029	0.174±0.024
M	0.342±0.024 <sup>a</sup>	0.157±0.019 <sup>a</sup>	0.173±0.047
HE	0.451±0.123 <sup>bc</sup>	0.202±0.031 <sup>abc</sup>	0.179±0.024
SC	0.405±0.141 <sup>bc</sup>	0.205±0.048 <sup>abc</sup>	0.157±0.032 <sup>abc</sup>
HC	0.458±0.125 <sup>bc</sup>	0.208±0.026 <sup>abc</sup>	0.186±0.024 <sup>abc</sup>
S	0.368±0.090 <sup>a</sup>	0.169±0.021 <sup>a</sup>	0.179±0.030

Results are expressed as mean ± SD. The experiment was conducted six times. <sup>a</sup>*P* < 0.05, vs. blank serum group; <sup>b</sup>*P* < 0.05, vs. model group; <sup>c</sup>*P* < 0.05, vs. selegiline group using one-way analysis of variance followed by the least significant difference test. BS: Blank serum; M: model; HE: *Herba Epimedii*; SC: *Semen Cuscutae*; HC: *Herba Cistanches*; S: Selegiline.

## DISCUSSION

The pathological and neural biochemical changes in Parkinson's disease mainly include dopaminergic neuronal loss in the substantia nigra-corpora striatum, formation of eosinophilic Lewy bodies and reduction of dopamine transmitters in the corpora striatum<sup>[14]</sup>. Studies have shown that neuronal apoptosis is an important cause for the occurrence and progression of neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease<sup>[15-16]</sup>.

Modern pharmacological studies have addressed that the Chinese medicines *Herba Epimedii*, *Semen Cuscutae*, and *Herba Cistanches*, which can tonify the kidney, are neuroprotective. *Herba Epimedii*, an herbaceous plant of berberidaceae family, can protect nerve cells against amyloid beta peptide (25–35) injury and reduce apoptosis<sup>[17]</sup>. *Semen Cuscutae* is the dried mature seed of dodder. It can regulate immunity, delay aging, reduce

free radical production, promote superoxide dismutase generation, increase Ca<sup>2+</sup> content in serum, inhibit L-type calcium channels, control calcium influx, regulate osteoporosis and suppress nerve cell apoptosis<sup>[18-19]</sup>. *Herba Cistanches* is a chylacaulous of scale leaf in dry areas. It can prolong life span, inhibit oxidation, and improve central neurotransmitter content<sup>[20-21]</sup>. Selegiline, monoamine oxidase-B inhibitor, can also protect nerve cells by several pathways that include inhibition of monoamine oxidase-B activity, reduction of endogenous or exogenous dopamine degradation, maintenance of dopamine concentration in the synaptic terminal, reduction of monoamine oxidase-B, promotion of antioxidase activity, slow oxidation, decrease hydroxyl radical generation, and protect dopaminergic neurons<sup>[22]</sup>.

Fas, FasL, and the caspase and Bcl-2 protein family play important roles in regulating cell apoptosis<sup>[23-24]</sup>. Previous animal experiments from our group have shown that Chinese medicines that tonify the kidney can reduce caspase-3 and FasL content in the substantia nigra-corpora striatum of mice with Parkinson's disease<sup>[11]</sup>. This indicates important roles for Chinese medicines that tonify the kidney by downregulating apoptotic factors to protect neurons in mice with Parkinson's disease. This study used cultured cells *in vitro* and exposed them to drug-containing serum to directly observe the effects of Chinese medicines. Moreover, this method minimized the interference of direct exposure of cells to medicines. Results showed that *Herba Epimedii* and *Herba Cistanches* upregulated Bcl-2 expression, and *Herba Epimedii*, *Semen Cuscutae*, and *Herba Cistanches* reduced caspase-3 and FasL expression. This indicates that regulation of Bcl-2 and caspase-3 expression by *Herba Epimedii* and *Herba Cistanches* plays an important role in nerve cell apoptosis.

Neurotrophic factors are a type of polypeptide or protein released by organisms. Studies have shown that nerve growth factor, brain-derived neurotrophic factor and glial

cell line-derived neurotrophic factor can offer a nutritive microenvironment for the nervous system and play critical roles in maintaining normal development of the nervous system, neuronal survival and nervous system regulation<sup>[25-27]</sup>. Nerve growth factor is mainly responsible for the development, differentiation and survival of dopaminergic neurons, as well as repair of injured dopaminergic neurons<sup>[28-29]</sup>. Brain-derived neurotrophic factor can serve as an antioxidant and protect nerve cells by synergizing glial cell line-derived neurotrophic factor<sup>[30-31]</sup>. Glial cell line-derived neurotrophic factor has specific trophic actions on dopaminergic neurons and can nourish nerve cells by altering the volume of nerve cells, length of processes and influence specific protein production<sup>[32]</sup>. Our previous study showed that Chinese medicines that can tonify the kidney can upregulate the endogenous content of nerve growth factor, brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor in the substantia nigra-corpora striatum of mice with Parkinson's disease and effectively reduce nerve cell apoptosis<sup>[11]</sup>. Results from this study indicated that *Herba Cistanches* increased nerve growth factor and glial cell line-derived neurotrophic factor content, and that *Herba Epimedii*, *Semen Cuscutae*, and *Herba Cistanches* increased brain-derived neurotrophic factor content in MES23.5 cells.

In summary, Chinese medicines used for tonifying the kidney, such as *Herba Epimedii* and *Herba Cistanches*, can protect nerve cells by regulating the expression of apoptosis-related factors and neurotrophic factors. The action of *Semen Cuscutae* is slow and mild, so its effects on cell apoptosis are not obvious. However, we only monitored the apoptotic inhibitory effects of these medicines. Further studies are needed to investigate the neuroprotective mechanisms of these kidney tonifying Chinese medicines.

## MATERIALS AND METHODS

### Design

*In vitro* comparative observation of serum pharmacology.

### Time and setting

The experiments were conducted in the Laboratory of Cytobiology, Fujian University of Traditional Chinese Medicine, China from 2010 to 2011.

### Materials

#### Animals

A total of 60 male Wistar rats, 8 weeks old, weighing 200–220 g, were purchased from Slac Laboratory Animal Co., Ltd., Shanghai, China (license No. SCXK (Hu) 2007-0005). They were housed in the Laboratory Animal Center of Fujian University of Traditional Chinese Medicine in China at 20–22°C with a humidity of 40–60% and illumination from 7:00 a.m. to 7:00 p.m. Food was provided by the Laboratory Animal Center of Fujian University of Traditional Chinese Medicine. All experimental procedures were in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China<sup>[33]</sup>.

### Drugs

*Herba Epimedii*, the aerial part of the *Epimedium sagittatum Maxim* was from Sichuan Province, China; *Semen Cuscutae*, the dry mature seed of *Cuscuta chinensis Lam.*, was from Shandong Province, China; and *Herba Cistanches*, the chylous of scale leaf of *Cistanche deserticola Y.C.Ma*, was from Xinjiang Uygur Autonomous Region. All medicines were provided by Fujian Pharmaceutical Co., Ltd., Fujian Province, China. *Herba Epimedii*, *Semen Cuscutae*, and *Herba Cistanches*, 97.2 g each, were immersed in a decoction vessel containing 1 500 mL distilled water, rapidly heated, boiled, maintained at 100°C to allow the active components of the drugs to dissolve and condensed to 540 mL. The crude amount of drug in the drug solution was 0.180 g/mL. The solution was filtered, placed in a bottle, sealed, sterilized and stored at 4°C. Selegiline, 5 mg × 10 tablets per kit, was purchased from Nanjing Sike Pharmaceutical Co., Ltd. (Jiangsu Province, China). Selegiline, 21.6 mg, was dissolved in double distilled water to prepare a 540 mL water solution. The concentration of the drug solution was 0.04 mg/mL. The drug solution was placed in a bottle, sealed, sterilized and stored at 4°C.

### Methods

#### Preparation of drug-containing serum

The *Herba Epimedii*, *Semen Cuscutae*, *Herba Cistanches* and selegiline groups were intragastrically perfused with drug-containing serum containing 0.180 g/mL *Herba Epimedii*, *Semen Cuscutae*, or *Herba Cistanches*, or 0.04 mg/mL selegiline, twice a day, at a dose of 0.1 mL/kg, for 14 consecutive days. The blank serum group was administered an equal volume of distilled water. The animals were deprived of food and water for 12 hours following the final administration. The rats were anesthetized by intraperitoneal injection with 0.3 mL/100 g ketamine hydrochloride. Blood was harvested from the abdominal aorta, and centrifuged at 1 000 r/min for 15

minutes. The supernatant was harvested, deactivated in a water bath at 56°C for 30 minutes, filtered through a 0.22 µm microporous membrane and stored at -20 °C.

#### **Preparation of 50×Sato's solution**

A mixture of 60 mL DMEM/F12 (Gibco, Carlsbad, CA, USA), 15 mg bovine insulin (Sigma, St. Louis, MO, USA), 15 mg transferrin (Sigma), 145.8 mg sodium pyruvate (Biosharp, Korea), 12 mg putrescine (Solarbio, Beijing, China), 25 µL sodium selenate (1 mg/mL, 100 mg/100 mL H<sub>2</sub>O; Sigma), and 100 µL progesterone (0.315 mg/mL, 15.75 mg/50 mL; Biosharp) was subjected to ultrasound in an ice bath for 1.5 hours, filtered through 0.22 µm filter, aliquoted and stored at -20°C.

#### **Cell culture**

Cells were seeded in DMEM/F12 supplemented with 5% (v/v) fetal bovine serum (Gibco), 1% (v/v) glutamine (Sigma), 2% (v/v) 50×Sato's solution, and 2% (v/v) penicillin/streptomycin. Cells were then incubated in 5% (v/v) CO<sub>2</sub> and maintained at saturated humidity at 37°C. Cells were trypsinized with 0.25% (w/v) trypsin and passaged. The cells in the logarithmic phase were collected for subsequent experiments.

#### **Effect of H<sub>2</sub>O<sub>2</sub> on the growth of MES23.5 cells**

Cells were seeded in 96-well culture plates (5 × 10<sup>5</sup> cells/well), incubated with 50, 100, 150, 200, 300, and 400 µmol/L H<sub>2</sub>O<sub>2</sub> culture solution (Shanghai Shiyi Chemicals Reagent Co., Ltd., Shanghai, China) after 24 hours for 1, 3, 6, 9, 12, 24 hours, followed by MTT (Sigma) for 4 hours. The solution in the wells was discarded, and 150 µL dimethyl sulfoxide (Sigma) was added. The culture plate was shaken for 10 minutes, and the absorbance of samples at 570 nm was determined using an automatic microplate reader (EXL800; Biotek, Winooski, VT, USA). Freshly prepared DMEM/F12 culture solution was used as a negative control. Two parallel wells were set at each time point. The experiments were conducted in triplicate.

#### **Influence of drug-containing serum at different concentrations on the growth of H<sub>2</sub>O<sub>2</sub>-treated MES23.5 cells**

Cells were seeded and exposed to drug-containing serum containing 5, 10, 15, 20, 25, and 30% (v/v) *Herba Epimedii*, *Semen Cuscutae*, *Herba Cistanches* and seligiline or blank serum for 24 hours. Cells were further cultured with culture solution containing 100 µmol/L H<sub>2</sub>O<sub>2</sub> for 3 hours, followed by MTT for 4 hours. Three parallel wells were set in each group. Freshly prepared DMEM/F12 culture solution was used as a negative control. Cells cultured in culture solution containing

100 µmol/L H<sub>2</sub>O<sub>2</sub> and blank serum served as the model group, and cells cultured in blank serum alone served as the blank serum group. The experiments were conducted in triplicate. The mean absorbance value of each group was calculated as the survival rate of cells.

#### **Flow cytometry for FasL, Fas, caspase-3 and Bcl-2 expression in MES23.5 cells**

Cells were seeded and treated as before. Cells cultured in 100 µmol/L H<sub>2</sub>O<sub>2</sub> alone were regarded as the model group. The phycoerythrin-labeled FasL antibody and FasL isotype control (5 µL each), were added into two tubes (BD, San Jose, CA, USA), mixed evenly with cells, stored at room temperature in the dark for 15 minutes, centrifuged at 1 000 r/min for 5 minutes after addition of 2 mL PBS. The supernatant was discarded, and cells were resuspended using 500 µL PBS. The wavelength of the laser device for flow cytometry (FacsCalibur; BD) was 488 nm. The expression rate of FasL-positive cells was analyzed and calculated by Cell Quest software (BD). The expression rate of Fas-, caspase-3-, and Bcl-2-positive cells was detected using the same method.

#### **ELISA for content of nerve growth factor, brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor in cells**

Cells were seeded and treated as described previously. The supernatant was harvested, and nerve growth factor, brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor content was measured according to the manufacturer's instructions (BD). The absorbance was measured at 450 nm using an automatic microplate reader (EXL800; Biotek, Winooski, VT, USA).

#### **Statistical analysis**

Results were expressed as mean ± SD. Data were analyzed using SPSS 18.0 software (SPSS, Chicago, IL, USA). One-way analysis of variance was conducted followed by multiple comparison least significant difference test. A value of *P* < 0.05 was considered statistically significant.

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