PROTECTIVE EFFECT OF EGCG ON ROTENONE-INDUCED NERVE CELL DAMAGE AND OXIDATIVE DAMAGE

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ABSTRACT

Objective: *: To analyze the protective effect of gallocatechin gallate (EGCG) on neuronal cell activity injury and oxidative damage induced by rotenone.*

Methods: *Human neuroblastoma cells SH-SY5Y cultured with rotenone were used to model Parkinson's disease cells in vitro, and then treated with EGCG 0,10,20,40μmol/L and a positive drug (pilagilan, 50μmol/L) respectively. The CCK-8 method was used to detect the survival rate of nerve cells in each group, while the Western blot method was used to detect protein (Bax, TH, Nrf2) expression in nerve cells and the content of oxidative damage factors in each group.*

Results: *The survival rate of nerve cells in the EGCG and positive control groups was significantly higher than that in the model control group (P<0.05). The expression of Bax protein in the EGCG 20,40μmol/L dose group and in the positive control group was significantly lower than that in the model control group (P<0.05). The expression of TH protein in the EGCG 10, 20μmol/L dose group and in the positive control group was significantly higher than that in the model group (P<0.05). The Nrf2 protein of nerve cells in the EGCG treatment group and in the positive control group was significantly higher than that in the model group (P<0.05).*

Conclusions: *EGCG seems to protect against neuronal cell damage and oxidative damage caused by rotenone through antioxidant stress injury, and this mechanism may be related to the signaling pathway by which EGCG promotes the activation of Nrf2/ARE protein expression.*

Keywords: *EGCG, rotenone, parkinson's disease, nerve cell, oxidative damage, protective effect.*

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Introduction

Parkinson's disease is a progressive neurodegenerative disease that occurs in middle and old age. The average age of onset is 60 years old, and the incidence rate in people over 65 years old in China is 1.7%. Parkinson's disease has become the second most common chronic neurological disease after Alzheimer's disease[(1)]. The main clinical manifestations are static tremor, motor retardation, muscle rigidity and slow movement. The main pathological features of Parkinson's disease are massive degeneration and loss of striatal dopaminergic neurons in the substantia nigra and the formation of Lewy bodies in the cytoplasm of midbrain substantia nigra neurons[(2)]. At present, the pathogenesis of Parkinson's disease is still unclear, but possible causes include oxidative stress, mitochondrial dysfunction, inflammatory response, excitotoxicity, and genetic factors, with oxidative stress considered to be one of the more significant pathogenic causes[(3)]. Rotenone is an active component

in the plant rotenone which has strong anesthetic and other toxic effects. In recent years, rotenone has been found to cause dopamine neural degeneration. For this reason, rotenone treatment cells at home and abroad are commonly used to prepare models of cells undergoing Parkinson's disease[4].

Epigallocatechin gallate (EGCG) is the most abundant and active component in green tea. It is part of an aromatic compound and contains a stereochemical structure of multiple phenolic hydroxyl groups. It has antioxidant, free radical scavenging, anti-inflammatory and anti-apoptotic effects[5]. Relevant data[6] show that EGCG can inhibit the proliferation and migration of tumor cells by triggering apoptosis, blocking the cell cycle and inhibiting the overexpression of proteasome and cyclooxygenase by regulating the epidermal growth factor receptor pathway and the MAPK pathway. Hence, the aim of this study was to analyze the protective effect of EGCG on neuronal cell activity damage and oxidative damage caused by rotenone.

Materials and methods

Experimental reagents and instruments

EGCG and rotenone were purchased from Sigma-Aldrich, USA; Pilagilan was purchased from Nanjing Sike Pharmaceutical Co., Ltd.; Fetal bovine serum was purchased from Hangzhou Sijiqing Company; Dimethyl sulfoxide was purchased from Solebo Technology Co., Ltd.; a CCK-8 kit, BCA protein concentration determination kit and SDS-PAGE protein loading buffer were purchased from Biyuntian Biotechnology Co., Ltd.; a PVDF membrane was purchased from Millipore; Trizol total RNA extraction reagent was purchased from Solebao Technology Co., Ltd.; Bax, TH, Nrf2 polyclonal antibodies were purchased from Cell Signaling Technology, Inc., USA. A Super Clean Worktable was purchased from Shanghai Sujing Industrial co., Ltd.; a carbon dioxide incubator was purchased from Binder GmbH, Germany; an inverted microscope was purchased from Chongqing Chongguang Industrial Co., Ltd.; a gel imaging system was purchased from Shanghai Shanfu Scientific Instrument Co., Ltd.; a vertical electrophoresis tank and electrophoresis apparatus were purchased from Bio-Rad, USA; an ultraviolet analyzer was purchased from Beijing Liuyi Instrument Factory; a micro-centrifuge was purchased from Hunan Xiangyi Experimental Instrument Development Co., Ltd.; and an electronic analysis balance was purchased from Nikon, Japan.

Cell culture and grouping

SH-SY5Y human neuroblastoma cells were cultured in vitro after being purchased from the stem cell library of the Chinese Academy of Sciences. The cells were placed in a DMEM/F12 culture medium and cultured in a cell incubator at 37°C with 5% CO_2. When the cell adherence rate reached 78-80%, the cells were transferred. The cell model of Parkinson's disease was prepared using rotenone culture cells, which were randomly divided into EGCG 0, 10, 20, 40 μmol/L groups and a positive control group (slaregilan 50 μmol/L).

Observation indicators

• The CCK-8 method was used to detect the effect of EGCG on the survival rate of rotenone-induced nerve cells. The logarithmic-growing nerve cells were taken to prepare cell suspension and inoculated in 96-well plates. The density of 5×103/well was cultured for 24h. 10 μL CCK-8 solution was added to each well and further cultured for 1h. Cell activity = (experimental group OD – blank group OD)/(control group OD- blank group OD) × 100%.

• The western blot method was used to detect the expression of Bax and TH proteins in nerve cells. The logarithmically growing nerve cells in each group were collected and the total protein was extracted by adding RIPA lysate and stored at -80°C. The BCA method was used to detect protein content and calculate protein concentration of samples. The protein sample was melted at room temperature, mixed with 5×SDS sample buffer 100 mmol/L, and heated in a 100°C water bath for 8 min to make the proteins fully denatured. The treated samples were added to the upper sample hole by microsampler, and electrophoresis was started when bubbles appeared. After 2h, the membrane was electrotransferred to the PVDF membrane, which was sealed at room temperature for 2h in the TBS blocking solution, which contained 5% skim milk powder. The PVDF membrane was removed, washed at TBST for 3 times, then slowly shaken at room temperature in the blocking solution. Diluted rabbit anti-Bax, TH, and Nrf2 polyclonal antibodies were added and the solution was incubated at 4°C overnight. After washing the film, the diluted secondary antibody solution was added and slowly shaken at room temperature for 1h. HRP substrate chemiluminescence solution was prepared, covering the protein surface of PVDF membrane, developed by a chemiluminescence gel imager, and the gray level of the protein strip was analyzed by Image J software.

• Detection of oxidative damage factor: The lytic cells were centrifuged to take the supernatant to determine the protein concentration of the sample. Glutathione peroxidase (GPx) detection buffer was added to the 96-well plate, when the sample was mixed with the GPx detection working solution, 4μ/L peroxide reagent solution was added to start the reaction, and the absorbance value of the sample was detected at 340 nm UV spectrophotometer after 20 min. The steps of Superoxide dismutase (SOD) detection are the same as above, then SOD detection buffer, NBT/enzyme working solution, and reaction starting solution were added to determine the absorbance value at 560 nm of enzyme labeling instrument. A malondialdehyde (MDA) test was carried out according to the instructions, and the absorptivity of 532 nm was measured by centrifugation after sample treatment.

The reactive oxygen species (ROS) detection was carried out according to the following steps: after dilution of the DCFH-DA probe, the collected cells were added and mixed, then the PBS were added to resuspend cells; finally, the fluorescence intensity was detected by an enzyme labeling instrument at the wavelengths 488 nm and 525nm, respectively.

Statistical menthods

All of the data in this study were analyzed using the SPSS21.0 software package, and all of the measurement data were represented by ($\bar{x}\pm s$).

The two groups of data were compared with a t-test, and the comparison of data among multiple groups was analyzed by ANOVA. $P<0.05$ was considered to be statistically significant.

Results

Effects of EGCG on survival rate of rotenone-induced nerve cells

The survival rate of nerve cells in the EGCG treatment group and the positive control group was significantly higher than that in the model group, and the difference was statistically significant ($P<0.05$), as shown in Table 1.

Effects of EGCG on the expression of Bax and TH proteins in rotenone-induced nerve cells

Western blot results showed that the Bax protein expression of nerve cells in the EGCG 20 and 40 mol/L dose group and the positive control group was significantly lower than that in the model group ($P<0.05$), and the TH protein expression of nerve cells in the EGCG 10 and 20 mol/L dose group and the positive control group was significantly higher than that in the model group, with statistically significant differences ($P<0.05$) as shown in Table 2 and Figure 1.

Group	Sample (n)	Survival rate
EGCG 10μmol/L	5	0.70±0.07*
EGCG 20μmol/L	5	0.75±0.10*
EGCG 40μmol/L	5	0.67±0.06*
Positive control group	5	0.86±0.08*
Model control group	5	0.45±0.05

Table 1: Effect of EGCG on survival rate of rotenone-induced nerve cells.
*Note: compared with the model group *P<0.05.*

Group	Sample (n)	Bax	TH
EGCG 10μmol/L	5	2.38±0.35	0.80±0.12*
EGCG 20μmol/L	5	1.50±0.12*	0.79±0.15*
EGCG 40μmol/L	5	1.65±0.32*	0.72±0.13
Positive control group	5	1.46±0.15*	0.92±0.14*
Model control group	5	2.44±1.04	0.68±0.10

Table 2: Effect of EGCG on the expression of Bax and TH proteins in rotenone-induced nerve cells.
*Note: compared with the model group *P<0.05.*

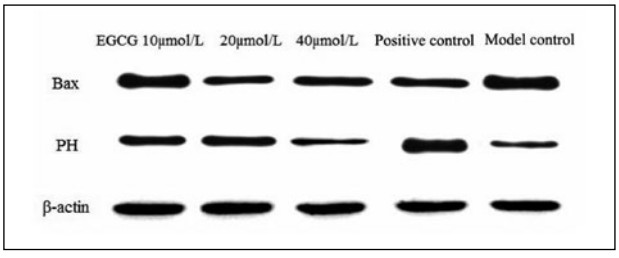

Figure 1: Effect of EGCG on the expression of Bax and TH proteins in rotenone-induced nerve cells.

Effects of EGCG on oxidative damage of nerve cells induced by rotenone

The contents of GPx, SOD, MDA and ROS of nerve cells in the EGCG treatment group and the positive control group were significantly higher than those in the model group, with statistically significant differences ($P<0.05$), as shown in Table 3.

Effects of EGCG on the expression of Nrf2 protein in rotenone-induced nerve cells

Nrf2 protein in nerve cells in the EGCG treatment group and in the positive control group was significantly higher than that in the model group, and the difference was statistically significant ($P<0.05$), as shown in Table 4 and Figure 2.

Group	Sample (n)	GPx (mU/mg)	SOD (units)	MDA (nmol/mg)	ROS (%)
EGCG 10μmol/L	5	700.21±125.37*	1.87±0.45*	5.06±0.52*	1.50±0.14*
EGCG 20μmol/L	5	742.36±114.75*	2.05±0.72*	4.00±0.41*	1.16±0.09*
EGCG 40μmol/L	5	802.36±136.42*	2.16±1.03*	4.51±0.48*	1.08±0.07*
Positive control group	5	705.63±120.39*	2.35±0.89*	3.10±0.92*	1.25±0.08*
Model control group	5	572.69±105.36	1.47±0.75	6.23±1.05	1.78±0.16

Table 3: effects of EGCG on oxidative damage of nerve cells induced by rotenone.
Note: compared with the model group $^{*}P<0.05$.

Group	Sample (n)	Nrf2
EGCG 10μmol/L	5	1.50±0.16
EGCG 20μmol/L	5	2.20±1.78
EGCG 40μmol/L	5	2.28±1.52
Positive control group	5	2.30±1.46
Model control group	5	1.12±0.07

Table 4: effects of EGCG on the expression of Nrf2 protein in rotenone-induced nerve cells.

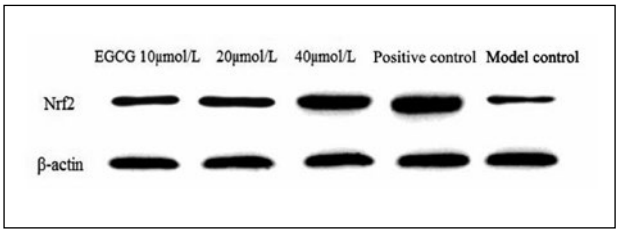

Figure 2: Effect of EGCG on the expression of Nrf2 protein in rotenone-induced nerve cells.

Discussion

The pathogenesis and potential causes of Parkinson's disease are relatively complex. Oxidative stress and apoptosis are key factors. In recent years, a large number of studies have found that oxidative stress can cause the death of dopamine neurons in the substantia nigra[7]. Oxidative stress is the stress damage state caused by the imbalance between oxidation and antioxidation in cells. It can regulate gene expression, ontogeny, and cell differentiation by increasing the production and elimination of ROS and acting as a signal transduction medium in normal physiological activities of the body. Under normal circumstances, harmful free radicals are constantly produced in the process of cell metabolism, but the body's defense system limits the level of free radicals and the damage they do to cells. When the ROS level in the body exceeds the level of antioxidant defense, the brain is the organ with the most active oxidative metabolism in the body and therefore is most vulnerable to the invasion of free radicals[8-9]. Due to the high concentration of unsaturated fatty acids in brain tissues and the relatively weak self-protection mechanism, neuromelanin in dopamine neurons has a high affinity for iron trivalent, which has been converted into activated iron divalent. It has been speculated that the occurrence of Parkinson's disease together with imbalance of free radical release and clearance can induce cell death in dopamine neurons. Therefore, the application of antioxidants has become a hot spot in the clinical treatment of Parkinson's disease[10].

Tea polyphenols are the main chemical components in tea, and catechins account for 70%~80% of tea polyphenols, which have recently attracted attention from clinical researchers. EGCG is a polyhydroxyphenolic compound, which is a unique component of catechins and has a strong ability to scavenge free radicals, as well as having anti-aging, anti-radiation and anti-tumor effects[11]. EGCG plays a very important role in antioxidant activity. It is more soluble in water and is oxidized into quinones to provide hydrogen ions. The antioxidant activity of EGCG is 20 times higher than that of vitamin E or vitamin C[12]. In addition, EGCG can inhibit the proliferation and metastasis of tumor cells. Some scholars have found that EGCG can significantly inhibit the conversion of the breast cancer cell cycle, thus inhibiting the proliferation of breast cancer cells[13]. Free radicals can cause abnormal metabolism of nucleic acids and protein. In one study, rats were given EGCG to drink, together with an intraperitoneal injection of carcinogen 2-nitropane for 2 weeks.

The results showed that EGCG could inhibit the formation of 8-hydroxydeoxyguanosine in the nucleus of liver cells, as well as inhibit serum lactate dehydrogenase and lipid peroxidation of liver tissue and hepatocyte degeneration, suggesting that the anti-tumor effect of EGCG is related to antioxidant and scavenging free radicals[14]. The preparation of a Parkinson's disease model is based on this pharmacological research. Rotenone is a selective inhibitor of the mitochondrial respiratory chain complex I, and its lipid soluble cavity is easily entered from the cytoplasm through the cell membrane. Rotenone can selectively block the function of coenzyme Q and reduce the level of ATP. In addition, rotenone can induce the production of ROS in mitochondria and induce oxidative stress and apoptosis of nerve cells[15]. This is why rotenone

was used to prepare the cell model of Parkinson's disease. The results of this study on two aspects of cell activity injury and oxidative damage, evaluating the protective effect of EGCG on nerve cells affected by Parkinson's disease, show that within the EGCG treatment group and the positive control group each nerve cell's survival rate was significantly higher than within the model group ($P<0.05$). Moreover, EGCG could inhibit the expression of the protein Bax, which is a key factor in apoptosis, and increase the expression of PH protein in nerve cells. But this study also found that after a certain dosage of EGCG, any further increase of dosage may lead to a gradual decrease of the EGCG's protective effect on nerve cells or even an inhibitory effect. By analyzing the effect of EGCG on rotenone-induced oxidative damage of nerve cells, it was found that the contents of GPx, SOD, MDA and ROS of nerve cells in each treatment group and the positive control group of EGCG were significantly higher than that in the model group ($P<0.05$), suggesting that EGCG could reduce the oxidative damage of Parkinson's disease model by upregulating the activity of antioxidant enzymes. Additionally, it was found that Nrf2 protein in nerve cells of the EGCG treatment group and the positive control group was significantly higher than that of the model group ($P<0.05$), suggesting that the Nrf2/ARE signaling pathway may be one of the significant pathways for this protective role of e.

In summary, EGCG can protect rotenone-induced nerve cell activity from oxidative stress damage, and its mechanism may be related to the activation of the Nrf2/ARE signaling pathway through EGCG promoting Nrf2 protein expression.

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