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# Echinacoside prevents the striatal extracellular levels of monoamine neurotransmitters from diminution in 6-hydroxydopamine lesion rats

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**Abstract**

We investigated the effects of echinacoside, a phenylethanoid glycoside isolated and purified from the stems of *Cistanche salsa*, a Chinese herbal medicine, on the striatal extracellular levels of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in 6-hydroxydopamine (6-OHDA) lesion rats. Seven days after 6-OHDA was injected into the right striatum of rats, the striatal extracellular levels of DA, DOPAC and HVA fell significantly ($P < 0.01$ vs. vehicle), as demonstrated by the method of cerebral microdialysis and high performance liquid chromatography with electrochemical detection. However, simultaneous treatment with echinacoside (7.0, 3.5 mg/kg) attenuated the diminution of them ($P < 0.01$ vs. model). The results implied that echinacoside could protect the striatal dopaminergic neurons from injury induced by 6-OHDA and may be useful in the prevention and treatment of Parkinson's disease (PD).


*Keywords:* Echinacoside; Dopamine; 3,4-Dihydroxyphenylethanoid acid; Homovanillic acid; 6-Hydroxydopamine; Parkinson's disease; Brain microdialysis; Rats

## 1. Introduction

Parkinson's disease (PD) is a well-known chronic neurodegenerative disease and has long been believed to be associated with the abnormal loss of dopamine (DA)-rich neurons in the central nervous system (CNS) (Kurth and Adler, 1998). The ensuing diminution of DA concentration leads to the imbalance of regulations to patient's motor function, which causes the special neurological motor symptoms such as bradykinesia, rest tremor and rigidity. Though the real pathogenesis of PD is still unknown to date, previously published studies showed that oxidative stress, a cellular dysfunction between the production and scavenging of free radicals, was the main reason related to the neuronal death (Lucio et al., 2003; Kelso et al., 2001; Freyer, 1998; You and Lin, 2002). 3, 4-Dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) are two important intermediates in the metabolic course of DA and can directly reflect the change of DA in CNS. Furthermore, experiments both in animal models and in patients suggested that there were decreases of DA and its metabolites, especially DOPAC and HVA, in the process of PD (Kish et al., 1992; Wang et al., 2005).

6-Hydroxydopmine (6-OHDA) is a selective dopaminergic neurotoxin (Ungerstedt, 1968), which produces reactive oxygen species (ROS) and thus damages the nigrostriatal dopaminergic neurons through oxidative stress (Cohen and Heikkila, 1974). When injected into the striatum, 6-OHDA may produce a continuous lesion right from several minutes to 1 week or month and can make the decrease of DA and its metabolites (Carmen et al., 2005; Zhang et al., 2003) in this region, which will finally lead to the emergence of some symptoms of PD.

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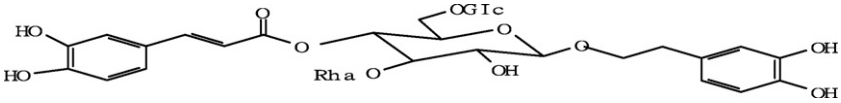


Fig. 1. The chemical structure of echinacoside.

Echinacoside (Fig. 1) is a phenylethanoid glycoside isolated and purified from the stems of *Cistanche salsa*, a parasitic plant native to northwest China, which is used as a traditional Chinese herbal medicine with antisenile and antifatigue effects (Xiong et al., 1999). It has been evident that echinacoside had neuroprotective effects and may be useful in the treatment of some neurodegenerative diseases (Deng et al., 2004a). Several other phenylethanoid glycosides were also proved to have similar actions and may be used to prevent neurons from oxidative stress-induced toxic injuries (Geng et al., 2004; Lee et al., 2006; Deng et al., 2004a; Sheng et al., 2002; Pu et al., 2003; Tian and Pu, 2005). However, the cellular and molecular mechanisms such as the changes of neurotransmitters and their metabolites that underlie the actions are not fully understood. Based on this reason, the present study aimed at the observation of the effects of echinacoside on the striatal extracellular levels of DA, DOPAC and HVA in 6-hydroxydopamine lesion rats.

## 2. Materials and methods

### 2.1. Animals and reagents

Male Wistar rats, weighing 230–270 g, were used in this study and housed individually in cages with food and water consumed ad libitum. The animals were kept under the temperature of $24 \pm 1\,^{\circ}C$ and the relative humidity of $55 \pm 5\%$ with a 12-h light:12-h-dark cycle (lights on at 7:00 a.m.). All experiments were performed in accordance with the guidelines established by the European Community for the care and use of laboratory animals and were approved by the Animal Care Committee of the Shihezi University.

Echinacoside from *Cistanche salsa* was kindly provided by Dr. Peng Fei Tu (Peking University Modern Research Center for Traditional Chinese Medicine). The purity of the compounds was shown to be more than 98% on high performance liquid chromatography (HPLC). DA, DOPAC, HVA, 1-heptanesulfonic acid sodium salt (HSA), triethylamine (TEA, ≥99%) and ascorbic acid were purchased from Sigma (St. Louis, MO, USA). Acetonitrile (ACN, HPLC grade) was purchased from Fisher (New Jersey, USA). Phosphorate acid (PA) and EDTA tetrasodium salt were obtained from Guoyao Group Co., Ltd. (Shanghai, China). Ringer's fluid was prepared in our laboratory. All the solutions were prepared by deionized water of at least 18.2 MΩ cm specific resistance.

### 2.2. Experimental design

All animals were divided into five groups: vehicle, 6-OHDA, echinacoside high and low doses and medopar, among which the vehicle-treated rats were used as control, the 6-OHDA-treated as model and medopar-treated rats as positive drug group. On the operation day, rats accepted the injection of 4 μl 0.9% saline (for vehicle group) or 6-OHDA (12 μg/4 μl in 0.9% saline containing 0.1% ascorbic acid, for 6-OHDA, echinacoside and medopar groups) into the right striatum. After the operation each rat was given intraperitoneal injection of 0.9% saline (2 ml/kg, for vehicle and 6-OHDA groups), echinacoside (7.0 or 3.5 mg/kg for echinacoside high or low dose groups) or medopar (64 mg/kg), respectively and this administration was carried out one time a day at 8 o'clock a.m. during the following 7 consecutive days. At the end of the last administration, the microdialysis procedure was performed.

### 2.3. Surgery and microdialysis procedure

The rats were anesthetized with an intraperitoneal injection of 50 mg/kg of sodium pentobarbital and were fixed in a stereotaxic apparatus (SAS-4100, Bioanalytical Systems, Inc., West Lafayette, USA). After the skull was exposed, a burr hole was drilled for the accommodation of guide cannula (Microbiotech AB, Stockholm, Sweden). The cannula was implanted into the right striatum with the following coordinates: AP + 0.2 mm, ML −3.0 mm, DV −3.5 mm from bregma according to the brain atlas of Paxinos and Watson (1998), and was secured to the skull with screws and dental cement. Each rat was housed individually following the surgical operation.

Seven days later the dummy stylet in the guide cannula was pulled out and a microdialysis probe (MAB/6; o.d. 0.6 mm, membrane length 4 mm, cut-off 15,000 Da, Microbiotech AB, Stockholm, Sweden) was inserted into it while the rat was keeping in awake, freely moving status. The probe, which had been connected to a microinfusion pump (MD-1001 Baby Bee Syringe Drive and MD-1020 Bee Hive Controller, Bioanalytical Systems, Inc., West Lafayette, USA), was perfused at a constant flow rate of 1.5 μl/min with Ringer's solution composed of 125 mM NaCl, 3.3 mM KCl, 2.4 mM $Mg_2SO_4$, 1.25 mM $KH_2PO_4$, 1.85 mM $CaCl_2$ (pH 7.1). After an 80-min equilibrium period, dialysate samples were consecutively collected every 20 min into vials containing 5 μl saline of 0.1% ascorbic acid in order to prevent DA, DOPAC and HVA from oxidation. All samples were injected directly into the HPLC–ECD system and analyzed immediately or kept at $-70\,^{\circ}C$ until analysis.

### 2.4. Chemical assays

DA, DOPAC and HVA were determined by a Shimadzu (Kyoto, Japan) LC-10ADvp HPLC system with a Shimadzu L-ECD-6A amperometric detector. Separations were performed by a Hypersil GOLD $C_{18}$ column (ODS, 150 mm × 4.6 mm i.d., 5 μm, Thermo, UK). The column and detector were placed in the compartment of Shimadzu CTO-10A column oven under the temperature maintained at 40 °C. The analytes were detected

at an oxidation potential of 0.75 V vs. *in situ* Ag/AgCl reference electrode. The isocratic mobile phase (pH 2.1) consisted of 8.65 mM HSA, 0.35% TEA, 0.4% PA, 6.25% ACN and 0.26 mM EDTA tetrasodium salt and was delivered at a flow rate of 1.0 ml/min. The sample volume used to inject was 20 μl.

### 2.5. Probe position proof

After the completion of the microdialysis experiments, rats were sacrificed by rapid decapitation. The brains were removed immediately and immersed into 4% paraformaldehyde overnight. The probe implantation sites were verified by visual inspection carefully.

### 2.6. In vitro recovery experiments

Prior to *in vivo* microdialysis procedure, *in vitro* recovery experiments were performed in order to examine the recovery of DA, DOPAC and HVA through the dialysis membrane of the probe. The probe was immersed in the standard solution containing certain concentration of DA, DOPAC and HVA and then perfused at a flow rate of 1.5 μl/min with Ringer's solution under room temperature. The samples were collected at intervals of 20 min and measured with the same conditions described before by the HPLC–ECD system. The recovery of DA, DOPAC and HVA were 30.3%, 25.8% and 25.6%, respectively.

### 2.7. Statistical analysis

Pharmacological results were expressed as the mean ± S.E.M. of the concentrations. One way analysis of variance (ANOVA) followed by Dunnett' post hoc test was used to compare the difference of the means between groups and the statistical significance level was set at $P < 0.05$.

## 3. Results

### 3.1. Effect of echinacoside on the striatal extracellular level of DA

The effect of echinacoside on the striatal extracellular level of DA was shown in Fig. 2. Though the level of DA in each group changed little throughout the entire perfusion period, the differences between groups were significant ($P < 0.05$, $P < 0.01$, or $P < 0.001$ vs. model). Obviously, 6-OHDA damaged the dopaminergic neurons in the striatum and caused a great decrease of DA compared with the vehicle group. The levels of DA in the 6-OHDA-treated group were 38.5% below the vehicle group at the beginning and kept relatively stable during the whole microdialysis procedure ($P < 0.001$). Echinacoside high and low dose could both prevent the levels of DA from decrease caused by 6-OHDA ($P < 0.05$, $P < 0.01$, or $P < 0.001$ vs. model). It seemed that the effects were dose-dependent. Medopar increased the content of DA continuously ($P < 0.05$, $P < 0.01$, or $P < 0.001$ vs. model) and manifested the trend of time-dependent.

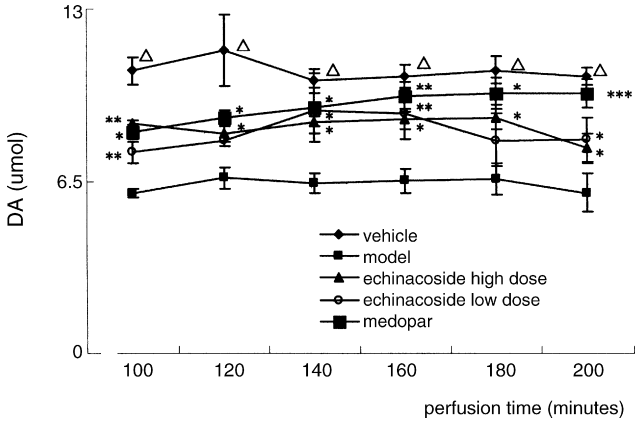


Fig. 2. Effects of echinacoside on the extracellular levels of DA in the striatum of awake, freely moving rats by 6-hydroxydopamine lesion ($n = 5$). Rats were treated with vehicle (2 ml/kg, for vehicle and 6-hydroxydopamine groups), echinacoside (7.0 or 3.5 mg/kg, i.p., for echinacoside high and low group, respectively) and medopar (64 mg/kg, for medopar group) during 7 days after surgical procedure. Data were expressed as mean ± S.E.M. of each group. The values of vehicle, echinacoside high and low dose and medopar group are compared with the corresponding values of the model group ($^{\Delta}P < 0.001$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. model).

### 3.2. Effect of echinacoside on the striatal extracellular level of DOPAC

The effect of echinacoside on the striatal extracellular level of DOPAC in 6-OHDA lesion rats was similar to that of DA (Fig. 3). The differences between groups were also significant ($P < 0.05$, $P < 0.01$, or $P < 0.001$ vs. model). Compared with the vehicle group, the level of DOPAC in the model group fell greatly and was down to 43.1% of that in the vehicle group at the beginning and kept relatively stable during the whole microdialysis procedure ($P < 0.001$). Echinacoside high and low dose could stop the levels of DOPAC from decrease caused by 6-OHDA ($P < 0.05$ or $P < 0.01$ vs. model). The effects were seemingly

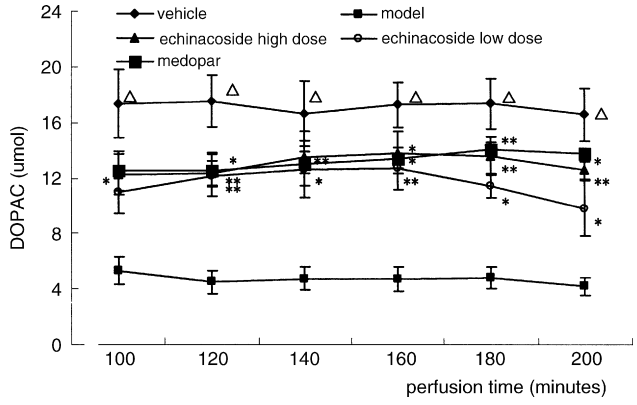


Fig. 3. Effects of echinacoside on the extracellular levels of DOPAC in the striatum of awake, freely moving rats by 6-hydroxydopamine lesion ($n = 5$). Rats were treated with vehicle (2 ml/kg, for vehicle and 6-hydroxydopamine groups), echinacoside (7.0 or 3.5 mg/kg, i.p., for echinacoside high and low group, respectively) and medopar (64 mg/kg, for medopar group) during 7 days after surgical procedure. Data were expressed as mean ± S.E.M. of each group. The values of vehicle, echinacoside high and low dose and medopar group are compared with the corresponding values of the model group ($^{\Delta}P < 0.001$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. model).

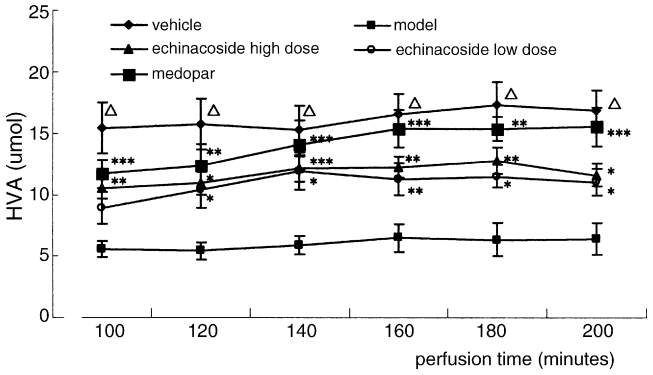


Fig. 4. Effects of echinacoside on the extracellular levels of HVA in the striatum of awake, freely moving rats by 6-hydroxydopamine lesion ($n=5$). Rats were treated with vehicle (2 ml/kg, for vehicle and 6-hydroxydopamine groups), echinacoside (7.0 or 3.5 mg/kg, i.p., for echinacoside high and low group, respectively) and medopar (64 mg/kg, for medopar group) during 7 days after surgical procedure. Data were expressed as mean ± S.E.M. of each group. The values of vehicle, echinacoside high and low dose and medopar group are compared with the corresponding values of the model group ($^{\Delta}P<0.001$, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. model).

dose-dependent. As to medopar, it increased the content of DOPAC ($P<0.01$ or $P<0.001$ vs. model) and manifested the characteristic of time-dependent.

### 3.3. Effect of echinacoside on the striatal extracellular level of HVA

As is shown in Fig. 4, echinacoside produced the same effect on the striatal extracellular level of HVA appropriately. The level of HVA in each group also changed little throughout the entire perfusion period, but the differences between groups were significant ($P<0.05$, $P<0.01$ or $P<0.001$ vs. model). 6-OHDA caused a great diminution of HVA compared with the vehicle group ($P<0.001$). The level of HVA in the model group was 55.9% below the vehicle group ($P<0.001$) at the beginning and kept relatively stable during the whole microdialysis procedure. Echinacoside high and low dose could both prevent the levels of HVA from decrease caused by 6-OHDA ($P<0.05$ or $P<0.01$ vs. model). It also seemed that the effects were dose-dependent. Medopar increased the content of HVA continuously ($P<0.01$ or $P<0.001$ vs. model) and manifested the trend of time-dependent.

## 4. Discussion

*Cistanche salsa* is a medicinal plant native to northwest China and used as a crude drug for the treatment of insomnia, kidney deficiency, neurasthenia and senile constipation. The phenylethanoid glycosides are the major components of this herb (Lei et al., 2001). As one of them, echinacoside was found to have neuroprotective effects recently (Deng et al., 2004b). However, the cellular and molecular mechanisms that underlie the actions are not fully understood, especially in its influence on neurotransmitters and their metabolites. The present study utilized the method of cerebral microdialysis to collect and detect the striatal extracellular DA, DOPAC and HVA directly. The results showed that echinacoside could obviously prevent the extracellular levels of DA, DOPAC and HVA from decrease in the striatum of 6-OHDA lesion rats. The mechanisms behind these effects may be owed to its individual neuroprotective actions or its interaction with other factors so that it may protect the dopaminergic neurons from the oxidative injuries caused by 6-OHDA.

Oxidative stress plays an important role in the neuronal apoptosis, a programmed cell death in the development of central nervous system. The important role of oxidative stress in apoptosis is strongly supported by the ability of various cellular antioxidants to block apoptosis induced by diverse agents other than oxidants (Lucio et al., 2003). Evidence showed free radicals may produce oxidative damage to lipid, deoxyribonucleic acid (DNA), and proteins in the substantia nigra of PD patients (Carmen et al., 2005) and the loss of neurons in human brain tissue and animal models of PD was mainly the dopaminergic neuronal apoptosis (Lawrence and Roger, 2000). In addition to PD, oxidative stress has also been suggested to be involved in other neurodegenerative diseases such as Alzheimer's disease and amyotrophic lateral sclerosis, which implied that the inhibition of oxidative stress might be a vital step to break the cascade of events that cause cell death (Julie, 2004). 6-OHDA is a selective catecholamine neurotoxin and could easily undergo autoxidation to yield hydrogen peroxide and superoxide radicals which take part in a secondary metal-catalyzed Haber–Weiss reaction producing hydroxyl free radicals (Opacka-Juffry et al., 1998). As a result, the mitochondrial respiratory enzymes are potently inhibited and the neurons can no longer exert their normal physiological function and will consequently die (Ronald et al., 2002). In line with this principle, 6-OHDA was usually used to make the animal model of PD. Previous study suggested that the concentration of DA, DOPAC and HVA in the injected striatum decreased to 12.4%, 15.5% and 22.5% of the intact side at 7 days after the 6-OHDA injection, respectively (Carmen et al., 2005). Our study demonstrated that their levels in the 6-OHDA (20 μg) group were down to 38.5%, 56.9% and 55.9% of the vehicle, respectively. This may be because we used a smaller dose of 6-OHDA (12 μg) to inject.

Echinacoside was reported to have antiapoptotic action, which was partially dependent on its antioxidative stress effects, maintenance of mitochondria function and inhibition of caspase-3 activity (Deng et al., 2004a). This may constitute to the basis of its neuroprotective effects. Theoretically, it can be inferred from the above study that echinacoside may inhibit the 6-OHDA-induced catecholamine neurotoxicity and maintain the extracellular concentration of DA and its metabolites at normality or close to normality. The present study demonstrated that consecutive administration of echinacoside for 7 days could prevent the striatal extracellular levels of DA, DOPAC and HVA from diminution induced by 6-OHDA. The results implicated that echinacoside may protect dopaminergic neurons in the striatum from apoptosis caused by 6-OHDA. Besides, we noticed that the extracellular concentrations of DA, DOPAC and HVA in the striatum of rats of the medopar group were time-dependent. Because medopar is a central DA supplementary agent, this phenomenon can be explained as the quantity of medopar inside brain was time-dependently elevated after its intraperitoneal

injection. In conclusion, the results of this research showed that echinacoside might be possibly a promising drug in the prevention and treatment of PD.

## Acknowledgement

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