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Original Article

Effects of echinacoside on GDNF expression and mitochondrial oxidative stress levels in vascular dementia rats

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Abstract: Mitochondrial oxidation injury plays a critical role in pathogenesis of vascular dementia (VD) in rats. Glial cell line-derived neurotrophic factor (GDNF) is related with cognitive functions. Echinacoside (ECH) protects learning and memory functions of VD rats. This study thus investigated the effect of ECH on GDNF expression and mitochondrial oxidative stress level in VD rat brain at genetic and molecular levels. Healthy male SD rats (4 months old) were randomly assigned into sham, model and ECH groups (30 mg/kg). VD model was generated by the ligation of bilateral common carotid artery. ECH group received drug treatment for 4 consecutive weeks. Morris water-maze was used to test memory function of rats, while HE staining was used to observe morphology of brain tissues. Superoxide dismutase (SOD), reactive oxygen species (ROS) in mitochondrial were quantified from hippocampus. Western blot was used to test the expression of silent information regulator 3 (Sirt3), while hippocampal GDNF level was assayed by immunohistochemistry (IHC) staining. VD model rats had elongated escape latency and lower platform crossing times, plus significant injury of hippocampal CA1 regions. Model rats had elevated ROS contents, and suppressed SOD activity, Sirt3 or GDNF expression ($P<0.05$). ECH treated rats had shorter escape latency and more platform crossing times, significantly improved hippocampal injury, lower ROS contents, and elevated SOD activity, Sirt3 and GDNF expression ($P<0.05$). ECH can alleviate ischemia injury of VD rat neurons and improve learning and memory functions, via modulating oxidative response level of mitochondria, and upregulating hippocampal GDNF expression.

Keywords: Echinacoside, vascular dementia, glial cell line-derived neurotrophic factor, mitochondria, oxidative response

Introduction

Vascular dementia (VD) often occurs secondly to cerebrovascular atherosclerosis, or brain stroke, and is one cognitive dysfunction syndrome caused by various cerebrovascular diseases [1, 2]. Its pathogenesis mechanism is correlated with pathological processes including neuron degeneration, apoptosis or necrosis caused by ischemia brain damage [3, 4]. Currently no effective medicine or method has been described for VD [5, 6]. Previous study showed the close correlation between mitochondrial oxidative stress and apoptosis or necrosis of neurons after brain ischemia. After deprivation of blood and oxygen, reactive oxygen species (ROS) and NO are activated to induce mitochondrial apoptotic pathway. During occurrence and progression of VD, mitochondria have abnormal structures or functions, oxidative stress injury, and production of ROS, which works as the mediate bridge. Due to higher oxidative metabolic rate and lower anti-oxidant levels in brain tissues, they are predisposed to ROS-induced injury. Mitochondria are the source of body's free radicals, and may be the early target for ROS injury. Silent information regulator 3 (Sirt3) is closely correlated with energy metabolism of mitochondria, and can protect mitochondria via activating downstream target superoxide dismutase MnSOD, peroxisome proliferator-activated receptor-γ (PPAR-γ) coactivator-1α [7, 8]. Glial cell line-derived neurotrophic factor (GDNF) is closely

correlated with neuronal synaptic plasticity, glial proliferation and memory formation, and plays an important role in the participation in cognitive functions by nutrient neurons [9, 10]. Echinacoside (ECH) is one of major components of echinaceapurpurea, and is believed to be one anti-aging reagent in phenethyl alcohol glycoside family. Various Chinese medicines with anti-aging effects have phenethyl alcohol glycoside compounds including ECH, which has 30% contents in Cistanchetubulosa [11, 12]. ECH has multiple pharmaceutical activities including neuroprotection, immune modulation, anti-oxidation and improving learning/memory functions. It has significantly protective effects of anoxia injury endothelial cells and neural cells. Some studies showed that ECH could elevate learning and memory functions of VD rats, alleviate oxygen/nitrogen free radicals of cerebral cortex and hippocampus, inhibit oxidative stress injury, and alleviate hippocampal neuronal injury, thus preventing and treating VD related cognitive disorders [13, 14]. Currently the detailed mechanism of ECH on the inhibition of oxidative stress injury has not been fully illustrated. This study thus established a rat VD model, and investigated the effect of ECH on GDNF expression and mitochondrial oxidative stress level from genetic and molecular levels, in an attempt to analyze related mechanisms of improving learning and memory functions of VD rats.

Materials and methods

Animals and grouping

Healthy male SD rats (4 months old, body weight 240~260 g) were purchased from Laboratory Animal Center of Nanchang University (Certificate No, SYXK-2013-0025), and were kept in an SPF grade facility with food and water *ad libitum*. Animals were randomly divided into sham, model and ECH groups (N=20 each). According to related literatures of ECH in anti-VD [13, 14] and pilot study, 30 mg/kg/d ECH has significant effects on anti-VD and improving central nervous system transmitter. After generating VD model, ECH group received 30 mg/kg/d drugs via intraperitoneal injection for 4 weeks at 1 ml/100 g volume. Equal volume of saline was given to sham and model groups.

Drugs and reagents

ECH (95% purity, Senbujia Bio, China), Chloral hydrate and paraformaldehyde were purchased from KemiouChem (China). Rabbit anti-GDNF antibody was purchased from Boster Bio (China). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody was purchased from CST (US). Commercial kit for measuring SOD and ROS were purchased from Jiancheng Bio (China). Sirt3 antibody was purchased from Santa Cruz (US). DAB staining kit was purchased from Jinqiao Bio (China). Morris water maze apparatus (Model DMS-2) was provided by Pharmacological Institute, Chinese Medicine Academy. Brain mitochondria isolation kit was purchased from Beijing Biosea Biotech Company (China).

Animal model preparation

Morris water maze was firstly performed to screen out 80 rats with normal learning and memory functions. 60 of them were randomly chosen to generate VD model using bilateral ligation of common carotid artery as previously described [15]. In brief, bilateral common carotid artery (2-VO) was permeant ligated in two surgeries with 72 h time interval. Before each surgery, rats were fasted for 8 h. After anesthesia by 10% chloral hydrate, rats were laid in supine position, with a middle incision in the neck. Bilateral common carotid artery was separated carefully to leaving nerves intact. Double ligations were performed using surgical silks, followed by sutures. The remaining 20 rats were included in sham group, which received only vascular separation but not ligation. During surgery, rectal temperature of rats was kept at 36.5~37.5°C. Penicillin sodium was applied on focal region to prevent infection, plus intramuscular injection (200000 U) daily for 3 days. Six weeks after surgery, Morris water maze was used to select 60 rats with successful VD model. The judgement criteria were: (averaged escape latency-escape latency in control group)/escape latency of target group ≥20%. These animals were randomly divided into model and ECH groups (N=20), the latter of which received ECH (30 mg/kg) via intraperitoneal injection for four consecutive weeks (1 ml/100 g volume). Sham and model groups received equal volumes of saline.

Behavioral test

Water-maze test includes both navigation session and exploration session. The water temperature was maintained at 20±2°C while water depth was 30 cm. During the navigation

test, rats were trained in 5 consecutive days to record the escape latency from entering the pool to climbing onto the platform. In navigation task, swimming path and times of crossing the original platform within 2 min were recorded.

HE staining for brain tissue pathology

Rats were fixed in 4% paraformaldehyde, and were decapitated. Cerebellum, olfactory bulb and lower brain stem were removed. Brain section from 4 mm posterior of chiasm toward cerebellum were immersed in paraformaldehyde, embedded in paraffin sections (5 μm thickness), and were stained by hematoxylin-eosin method. A light field microscope was used to observe tissue morphology.

SOD and ROS assay of hippocampal mitochondria

After sacrifice, rats were extracted for brain tissues, in which hippocampus was separated, and homogenized with mitochondrial separation reagent A (1 ml/100 mg) following manual instruction. After 4°C centrifugation at 1000 g for 5 min, supernatants were saved and re-centrifuged at 3500 g for 10 min at 4°C. Mitochondria were collected from precipitations. Parts of samples were re-suspended in storage buffer to test oxidative stress indexes. Parts of samples were tested in Western blot. SOD and ROS were tested in mitochondrial resuspensions following manual instruction of the kit. Absorbance value was measured using UV spectrometry.

Western blotting for protein levels of Sirt3 in mitochondria

Rat brain tissues were lysed in lysis buffer and mitochondria was isolated using commercial kit according to the manufacturer's instructions followed by mitochondria protein quantification by BCA protein assay kit. Protein samples were separated by SDS-PAGE (8 μl loading sample, 75 V electrophoresis for 25 min until bromophenol blue reached the bottom of separation gel), and were transferred to PVDF membrane (110 V for 45 min). After blocking for 1 h, primary antibody against Sirt3 (1:100) or β-actin (1:1000) was added for 4°C overnight incubation. After TBST washing for three times, secondary antibody (1:8000) was added for 1 h incubation. Chromogenic substrate was then added for development, followed by exposure in a dark room. Quantity One software was used to analyze protein bands, which were expressed as relative level against internal reference: absorbance value ratio of target protein bands against internal reference bands.

Immunohistochemistry for GDNF protein expression

Rats were sacrificed and extracted for brain. After removing cerebellum and olfactory bulb, cerebral cortex and hippocampus were collected on ice and embedded in paraffin (8 μm thickness). After rinsing in PBS (pH 7.4) for three times (3 min each), antigen retrieval was then carried out. One drop of 3% H_2O_2 was added for 10 min incubation at room temperature to quench the activity of endogenous peroxidase. Rabbit anti-mouse GDNF polyclonal antibody (1:1000 dilutions) was added for 2 h incubation at room temperature, followed by polymer enhancing and 20 min room temperature incubation. Enzyme labelling anti-mouse/rabbit polymer was added for 30 min room temperature incubation. Freshly prepared DAB buffer was added for 5 min observation under microscope, followed by hematoxylin counterstaining and 0.1% HCl differentiation. The slide was rinsed in tap water, and was dehydrated in gradient ethanol, followed by xylene treatment and resin mounting. Immunohistochemistry staining (Ultra Vision Detection System) was used to detect GDNF protein level. Image-pro plus software was used to analyze the image.

Statistical methods

SPSS20.0 software was used to analyze all data, in which measurement data were firstly tested for normal distribution. Those fitted normal distribution were presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was employed to compare means across multiple groups, followed by LSD test in paired comparison. A statistical significance was defined when $P<0.05$.

Results

ECH improved the learning and memory functions of VD rats

Compared to sham group, VD model rats had elongated escape latency and lower platform

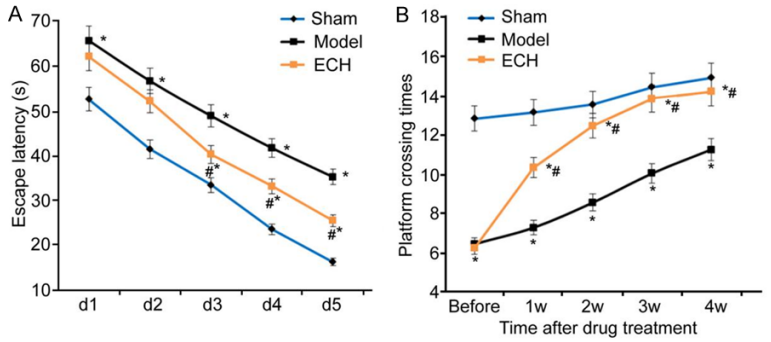

Figure 1. Escape latency and platform crossing times of VD rats. *P<0.05 compared to sham group; #P<0.05 compared to model group.

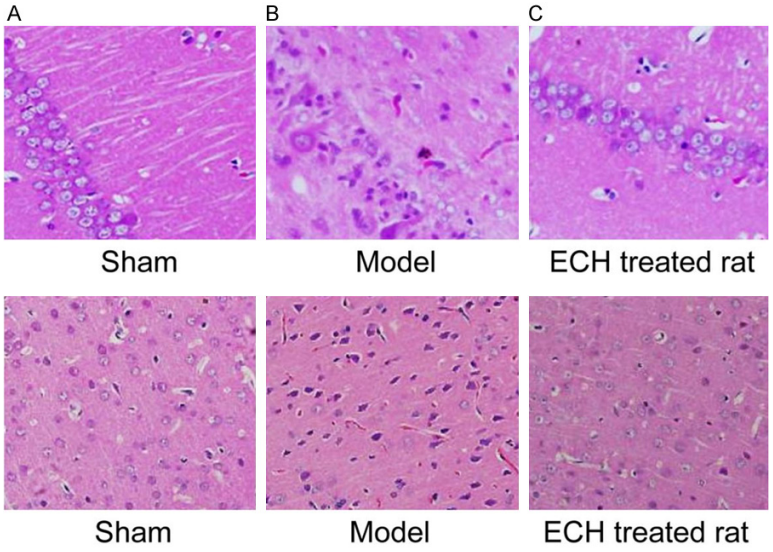

Figure 2. Pathology morphology of VD rat hippocampal CA1 (upper panels) and cortex (lower panels). HE staining, ×400. A. Sham; B. Model; C. ECH treated rat.

crossing times (P<0.05). ECH treatment shortened escape latency and increased platform crossing times (P<0.05 compared to model group, **Figure 1**).

Ameliorated brain injury of VD rat brains by ECH

HE staining results showed normal cortical structure and morphology of sham rats, which had regular arrangement of hippocampal tissues with intact morphology, plenty of cell number, normal structure and clear nucleus. VD model rats had diffused injury in both cortical and hippocampal regions, the latter of which had irregular distribution of cells, with incomplete morphology, less cell number, abnormal structure, condensed nucleus, plus glial hypertrophy. ECH treatment had alleviated injury compared to model group, as shown by improvement of neuron nucleus staining and morphology (**Figure 2**).

Reduced SOD and ROS level of hippocampal mitochondria after ECH treatment

Compared to sham group, VD model rats had elevated ROS contents and lower SOD level (P<0.05). ECH treatment depressed ROS level in mitochondria and elevated SOD activity (P<0.05, **Figure 3**).

Increased mitochondrial Sirt3 expression level by ECH

Compared to sham group, model rats had decreased Sirt3 protein levels (P<0.05). ECH treated elevated brain expression of Sirt3 proteins (P<0.05, **Figure 4**).

Elevated hippocampal GDNF expression level after ECH treatment

Under IHC staining of hippocampal tissues, positive staining signals were shown as brown-yellow color. VD model rats had lower number of GDNF-positive cells in hippocampus, while ECH treatment depressed positive cell number (P<0.05, **Figure 5**).

Discussion

Currently the patho-physiological mechanism of VD has not been fully illustrated. GDNF is closely related with learning and memory process, and is one important mediator of synaptic plasticity. The synaptic stimuli lead to axonal release of GDNF via N-type calcium channel and intracellular calcium ion peak [16]. Voltage gated ion channel can modulate GDNF release, whose biological effects depend on the specific binding onto tyrosine receptor kinase B (TrkB). Some studies showed that the co-activation of GDNF and receptor TrkB potentiated synaptic

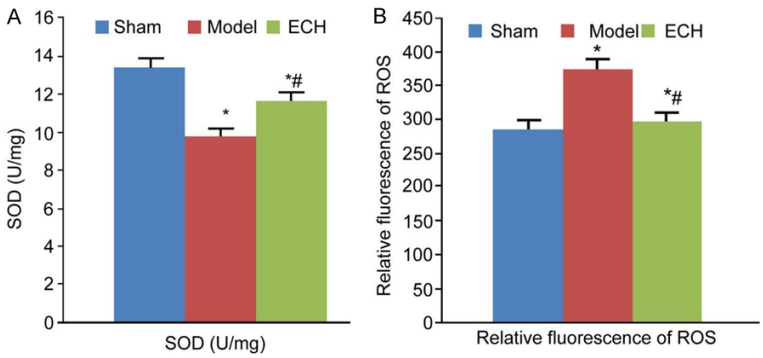

Figure 3. SOD and ROS activity of hippocampal mitochondria. *P<0.05 compared to sham group; #P<0.05 compared to model group.

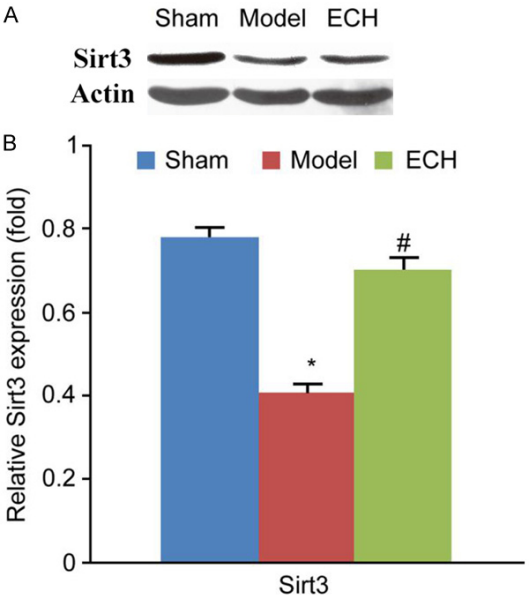

Figure 4. Sirt3 protein expression. A. Western blot bands; B. Relative expression level of Sirt3 protein. *P<0.05 compared to sham group; #P<0.05 compared to model group.

plasticity and facilitated axonal and dendritic growth, thus increasing terminal density of synapse [17, 18]. After binding of GDNF, receptor TrkB undergoes phospholipase hydration to expose binding domain. With PI3K activation, signals are further transmitted downstream to bind with extracellular ERK for the information integration into neurons. Under the direction of mitochondrial MAPK, signals were integrated and stored, with the binding of calcium ion channels for exerting biological effects, thus modulating GDNF differentiation and transmission. By phosphorylation of tyrosine kinase TrkB, GDNF participates in learning and memory process via intermediate signals [19, 20]. In this study, ECH treated rats had shortened escape latency and more platform crossing times, plus alleviated neuronal injury compared to model group, with lower cell denature and necrosis, and elevated GDNF expression, which suggest alleviation of brain tissue injury and neurological functions after ECH treatment, and improved learning and memory functions. The neuroprotective role of ECH on VD rats may work via GDNF-induced PI3K/Akt signal pathway, to facilitate regeneration and repair of injured neurons and to prevent late onset neuronal apoptosis, thus protecting neurocognitive functions and synaptic transmission. Endogenous neuroprotective mechanism plays an important role in ischemia brain injury. Such endogenous protective role, however, had slow initiation without exogenous intervention. ECH as exogenous drug thus protect neuronal integrity, facilitate synaptic plasticity and improve learning and memory function of rats.

Modern pharmaceutical study has illustrated the anti-oxidative, neuroprotective, anti-tissue injury, and learning and memory improvement roles of ECH. Phenolic hydroxyl and di-phenolic hydroxyl group in ECH molecule can protect against lipid peroxidation, clear free radicals, and ROS, as one natural anti-oxidants [21]. ECH can alleviate MPTP-induced neurotoxicity of dopaminergic neurons, or inhibit tumor necrosis factor (TNF)-induced SHSY5Y neuron apoptosis. Such neuroprotective roles are correlated with lower intracellular ROS level, maintaining mitochondrial function and high energetic status of membrane potential [22]. Via inhibiting oxidative stress and lowering free radical production, ECH can prevent neuron apoptosis. ECH can also decrease the overexpression of biliverdinreductase B in mice with dopaminergic neuron damage, and inhibit oxidative stress to protect these dopaminergic neurons [23]. ECH significantly decreases glutamate induced neuron toxicity [24], and inhibits oxygen/nitrogen free radicals, elevates SOD activity, lowers malondialdehyde level, thus

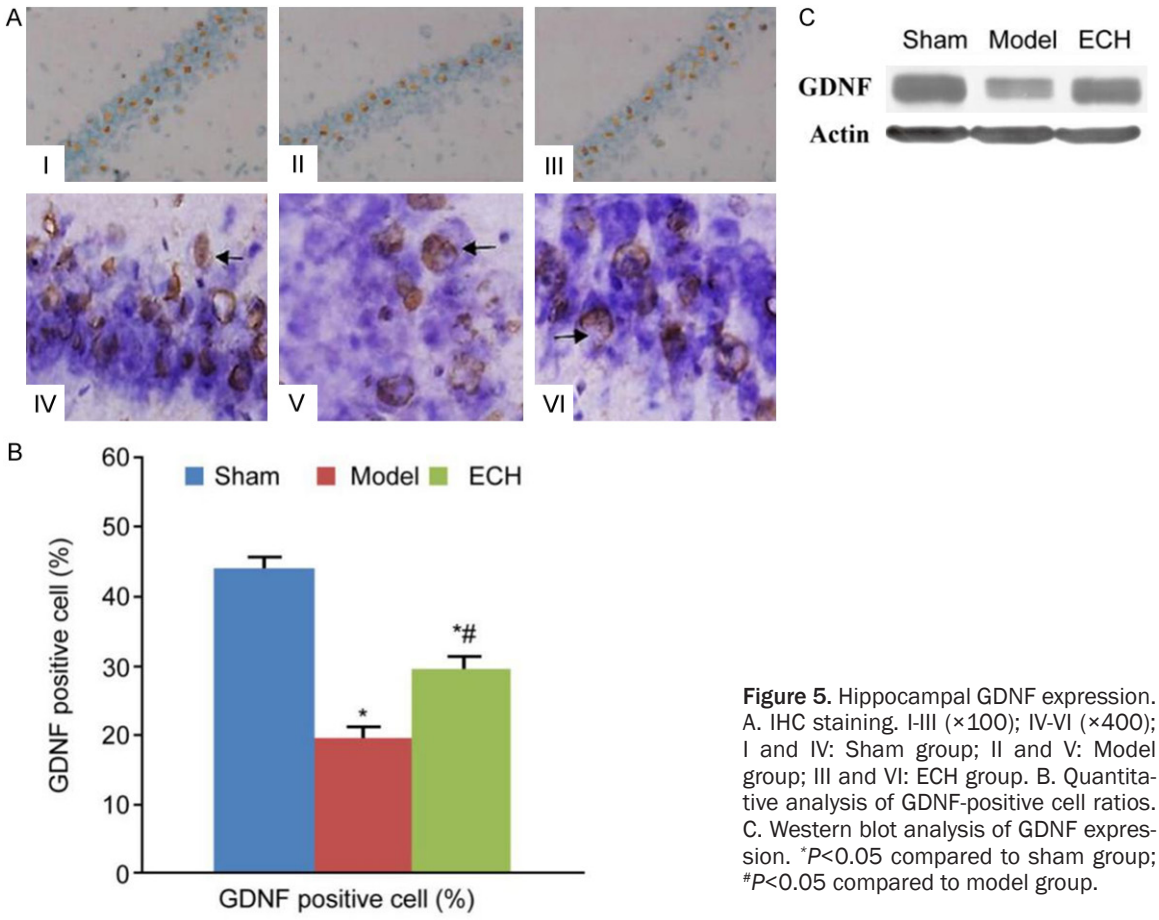

Figure 5. Hippocampal GDNF expression. A. IHC staining. I-III (×100); IV-VI (×400); I and IV: Sham group; II and V: Model group; III and VI: ECH group. B. Quantitative analysis of GDNF-positive cell ratios. C. Western blot analysis of GDNF expression. $^{*}P<0.05$ compared to sham group; $^{\#}P<0.05$ compared to model group.

exerting anti-aging effects. It can also potentiate cholinergic function of CNS, modulate immune functions, and improve learning and memory function of VD rats [25]. Sirt3 is related with cell metabolism, oxidative stress and apoptosis. Study has confirmed that Sirt3 could alleviate oxidative stress of neurons [8]. This study further investigated the effect of ECH on oxidative stress level at genetic and molecular levels. Results showed elevated ROS and depressed SOD activity, Sirt3 expression in VD model rats. ECH treated significantly depressed ROS contents, and elevated SOD and Sirt3 expressions. During VD progression, elevated Sirt3 expression enhanced the inhibition of oxidative stress caused by ROS, suggesting that ECH could exert anti-oxidative stress functions via modulating Sirt3 protein expression, mediating SOD level, potentiating clearance of ROS, and regulating oxidative stress level of mitochondria, although its detailed functional pathway requires further illustration.

Conclusion

ECH can alleviate ischemia injury of VD rat neurons and improve learning and memory functions via modulating mitochondrial oxidative stress level, and up-regulating hippocampal GDNF expression.

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Disclosure of conflict of interest

None.

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