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Protective Effects of Echinacoside, One of the Phenylethanoid Glycosides, on H_2O_2-Induced Cytotoxicity in PC12 Cells

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Key words
- echinacoside (ECH)
- apoptosis
- ROS
- MMP
- $[Ca^{2+}]i$
- Bax/Bcl-2

Abstract

We have investigated the protective effects of echinacoside (ECH), one of the phenylethanoid glycosides, on H_2O_2-induced cytotoxicity in the rat pheochromocytoma cell line (PC12 cells). Our data show that application of ECH to H_2O_2-injured PC12 cells (HIPCs) increased cell viability and decreased the apoptotic ratio. Flow cytometry (FCM) and laser scanning confocal microscopy (LSCM) analysis suggested that ECH exerted its inhibitory effects on the formation of reactive oxygen species (ROS) and the accumulation of intracellular free Ca^{2+} ($[Ca^{2+}]i$). In addition, ECH elevated the mitochondrial membrane potential (MMP) in HIPCs. Furthermore, Western blot analysis revealed that ECH prevented an H_2O_2-induced increase of the Bax/Bcl-2 ratio by downregulating Bax protein expression and upregulating Bcl-2 protein expression. In summary, ECH showed significant neuroprotective effects on HIPCs through the mitochondrial apoptotic pathway, and could be a potential candidate for intervention in neurodegenerative diseases such as Alzheimer's and Parkinson's disease.

Abbreviations

$[Ca^{2+}]i$:	intracellular free Ca^{2+}
DMEM:	Dulbecco's modified Eagle's medium
ECH:	echinacoside
FCM:	flow cytometry
FITC:	fluorescein isothiocyanate
HIPCs:	H_2O_2-injured PC12 cells
LSCM:	laser scanning confocal microscopy
MMP:	mitochondrial membrane potential
MPTP:	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MTT:	3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
PBS:	phosphate-buffered saline
PC12 cells:	rat pheochromocytoma cells
ROS:	reactive oxygen species
SDS:	sodium dodecyl sulfate
SDS-PAGE:	sodium dodecyl sulfate polyacrylamide gel electrophoresis
V_E:	vitamin E

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Introduction

Alzheimer's and Parkinson's diseases are neurodegenerative disorders characterized by apoptosis and neuron loss where the effects of oxidative stress are thought to play critical roles [1]. Oxidative stress-induced cell injury is mediated by ROS such as superoxide, hydroxyl radicals, and H_2O_2. H_2O_2 is a by-product of both normal and aberrant metabolic processes that utilize molecular oxygen, including amyloid aggregation [2], dopamine oxidation [3], and brain ischemia/reperfusion [4]. H_2O_2 can be converted into highly toxic hydroxyl radicals that attack proteins, deoxynucleic acid, and lipid membrane, thereby leading to mitochondrial dysfunction, calcium imbalance, and apoptosis of neuronal cells [5,6]. Because H_2O_2 has been used extensively to induce cell apoptosis, and PC12 cells have many advantages over primary cultured neuronal cells [7], the HIPCs model has been used widely to develop drugs for neurodegenerative diseases *in vitro* [8,9].

In China, *Cistanche tubulosa* glycosides isolated from the stems of *Cistanche tubulosa* (Schrenk) Wight have been approved as a treatment for vascular dementia. This is the first example of a government-approved drug containing phenylethanoid glycosides. Recent studies have shown that phenylethanoid glycosides are effective at scavenging free radicals and protecting against gluta-

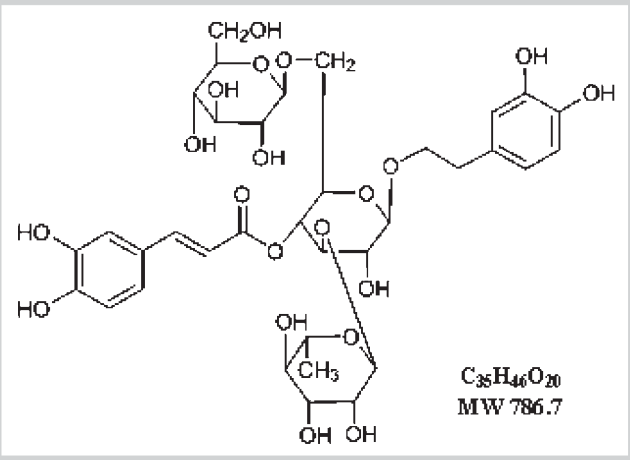

Fig. 1 Chemical structure of ECH.

mate-induced neurotoxicity [10,11]. *Cistanche tubulosa* glycosides also showed antioxidant properties and neuroprotective function with ECH (**Fig. 1**), {2-(3,4-dihydroxyphenyl)ethyl *O*-α-L-rhamnopyranosyl-(1→3)-[β-D-glucopyranosyl-(1→6)]-(4-*O*-*E*-caffeoyl)-β-D-glucopyranoside}, as a quality control compound (content is more than 25%) [12,13]. ECH is the first compound that was defined as a phenylethanoid glycoside. It was extracted from *Echinacea angustifolia* in 1950 and exists in several other plants. It has recently been demonstrated that ECH protects against hepatotoxicity and is a potent promoter of neuronal survival that is induced by TNF-α and MPTP [14–16]. However, these studies provide little information about the protective effects of ECH on oxidative stress-induced injury *in vitro*. In order to validate the neuroprotective effects of ECH on H_2O_2-induced cytotoxicity in PC12 cells, we determined rates of cell survival and apoptosis, accumulation of ROS, the Bax/Bcl-2 protein ratio, and changes in MMP and $[Ca^{2+}]i$.

Materials and Methods

Materials and cell culture

ECH (purity > 98%, HPLC) and vitamin E (V_E, purity > 98.8%, GC) were supplied by the National Institute for the Control of the Pharmaceutical and Biological Products (Nicpbp). PC12 cells were purchased from Shanghai Institute of Biochemistry and Cell Biology (Sibcb). Cells were maintained in medium supplemented with 5% heat-inactivated horse serum, 10% fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 U/mL streptomycin. Culture flasks were kept in humidified 5% CO_2/95% air at 37 °C. The medium was changed every three days.

Experimental protocols

PC12 cells were grown to 80–90% confluence and then replanted at an appropriate density (according to the particular experiment) on culture plates or dishes. To induce oxidative stress, fresh H_2O_2 was prepared from a 30% stock solution prior to each experiment. In all experiments, two doses (5, 10 µg/mL) of ECH or 10 µg/mL of V_E were pre-incubated with PC12 cells for 30 min, after which 400 µM H_2O_2 was added (except in $[Ca^{2+}]i$ evaluation). Assays were performed 4 h after H_2O_2 was added. V_E, a strong antioxidant that has anti-apoptosis properties [17], was used as a positive control.

Measurement of cell viability

PC12 cells were seeded in a 96-well plate at a density of 2×10^4 cells per well. After treatment with ECH and H_2O_2 as described above, cells were incubated with 0.5 mg/mL of MTT solution (Sigma) for 4 h at 37 °C. Formazan crystals that formed in intact cells were dissolved with 200 µL of dimethyl sulfoxide (DMSO), and the absorbance was measured at 570 nm using a microplate reader (Molecular Devices Co.; Spectra MAX 340). Cell viability measurements have been calculated as a percentage of the absorbance of the control sample.

Flow cytometric analysis of cell apoptosis

An annexin V kit (Caltag) and a flow cytometer (Becton-Dickinson) were used to detect apoptosis of PC12 cells. After treatment with ECH and H_2O_2, cells were assayed by analyzing the conjugation of annexin V to fluorescein isothiocyanate (FITC) in order to determine the translocation of phosphatidylserine from the inside to the outside of the plasma membrane. Cell staining was performed according to the manufacturer's instructions. For each sample, at least 10 000 cells were stained with annexin V and detected by FCM. Data were analyzed using CellQuest software (Becton-Dickinson).

Assessment of the intracellular ROS level and MMP

The intracellular ROS level and MMP were monitored by using the fluorescent probe DCFH-DA and rhodamine 123 (Molecular Probes), respectively. PC12 cells were treated as described above, then harvested, washed three times with phosphate-buffered saline (PBS), and incubated in the dark for 15 min or 30 min at 37 °C with DCFH-DA or rhodamine 123 (final concentration of 10 µM or 1 µM, respectively). Cells were then suspended in PBS and analyzed by FCM with 488 nm excitation and 525 nm or 575 nm emission wavelengths. A minimum of 10 000 events were detected for each data set.

Measurement of $[Ca^{2+}]i$ change

A LSCM (Zeiss 510) was used to evaluate the relative change of $[Ca^{2+}]i$ by detecting fluo-3 fluorescence after intracellular cleavage of fluo-3 AM (5 µM; Molecular Probes). Fluorescence was measured at an excitation of 488 nm and emission of > 515 nm. Following the addition of 200 µM H_2O_2 to the dish, laser scanning was used to obtain a series of images. Image frames were acquired every 20 seconds and image acquisition was completed within 25 min following H_2O_2 addition. Images were analyzed quantitatively using Zeiss LSM software to determine the change in fluorescence intensity within a cell (20–25 cells were analyzed in each group). The fluorescence at each time point (Ft) after H_2O_2 addition was divided by the fluorescence before H_2O_2 addition (F_0) to obtain the ratio Ft/F_0 [6]. ECH and V_E were incubated with PC12 cells for 30 min prior to H_2O_2 irritation.

Western blot analysis of Bax and Bcl-2 protein

After exposure to H_2O_2 and/or ECH for 4 h, 5×10^7 cells were rinsed twice with cold PBS and lysed in 40 µL lysis buffer containing a protease inhibitor cocktail [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1.0% Nonidet P-40, 2 mM EDTA, 0.1% SDS, 1 mM sodium orthovanadate (Na_3VO_4), 25 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 5 µg/mL leupeptin, 10 µg/mL aprotinin] to obtain whole cell proteins. Cell lysates were then

centrifuged at 12 000 rpm for 15 min at 4 °C. The supernatant was collected, and the protein concentration was determined with the Bradford method. Total cell lysates (50 µg protein) were mixed with 10 µL of 2× sample buffer (62.5 mM Tris, pH 6.8, 1% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.02% bromophenol blue), and the proteins were denatured by boiling for 10 min. Proteins were separated using a 10% SDS-PAGE gel and transferred electrophoretically onto nitrocellulose membranes (Bio Basic, Inc.). The transferred membranes were then blotted with antibodies for anti-Bcl-2 (1 : 1000; Santa Cruz), or anti-Bax (1 : 1000; Santa Cruz) and anti-β-actin (1 : 1000; Santa Cruz) at 4 °C overnight, followed by treatment with horseradish peroxidase-conjugated secondary antibodies. Chemiluminescence was detected using ECL detection kits (Chemicon) and the bands were visualized on X-ray film. The intensity of the bands was quantified by scanning densitometry using NIH Image 1.61 software.

Statistical analysis

Data are presented as mean ± SD. Differences between group mean values were calculated using analysis of variance, and comparisons between means were performed by the two-tailed Student's t-test. Statistical significance was defined as $p < 0.05$.

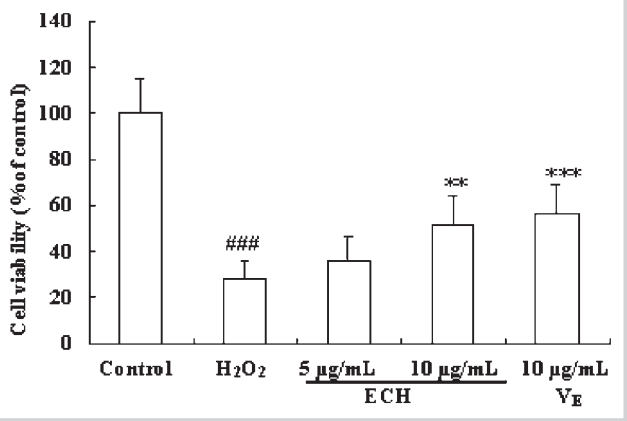

Fig. 2 Effect of ECH on the loss of viability in PC12 cells induced by H_2O_2. After PC12 cells were pretreated with ECH (5, 10 µg/mL) for 30 min and co-incubated with 400 µM H_2O_2 for 4 h, 0.5 mg/mL MTT was added and allowed to react for another 4 h. Data are expressed as percentages of cell viability relative to the control, with mean ± SD (n = 8). ### $P < 0.001$ compared to control; ** $p < 0.01$, *** $p < 0.001$ compared to H_2O_2-treated cells.

Results

When PC12 cells were exposed to 400 µM H_2O_2 for 4 h, cell viability decreased to 27.9 ± 8.0% relative to cells without treatment. This effect was significantly reduced ($p < 0.01$) by ECH (10 µg/mL), with the survival percentage increasing to 51.0 ± 8.0% (**Fig. 2**).

In the control samples, 3.8 ± 0.2% of cells stained positive for annexin V-FITC. H_2O_2 exposure showed an increase in apoptosis: 19.3 ± 3.4% of cells were annexin V positive. Pre- and co-incubation with ECH (5, 10 µg/mL) significantly reduced the number of cells labeled with annexin V. The apoptotic percentages were 14.7 ± 1.8% ($p < 0.05$) and 11.7 ± 3.0% ($p < 0.01$), respectively (**Fig. 3**).

As shown in **Fig. 4**, when PC12 cells were exposed to H_2O_2, the intracellular ROS level increased from 100.0 ± 37.7% to 215.2 ± 62.5%, indicating that H_2O_2 enhances the ROS concentration in PC12 cells. However, treatment with ECH (doses of 5 or 10 µg/mL) was able to effectively reduce ROS generation, with the DCF fluorescence decreasing to 190.1 ± 38.6% and 130.3 ± 30.2% ($p < 0.05$), respectively.

The decrease of MMP and the subsequent cytochrome c release have been proposed as early phenomena exhibited in the apoptotic process. To address this, we measured the effect of ECH on the disruption of MMP that was caused by H_2O_2 in PC12 cells. When PC12 cells were subjected to 400 µM H_2O_2 for 4 h, a decrease in rhodamine 123 fluorescence intensity (75.0 ± 7.0%, $p < 0.01$) was detected. Treatment with ECH (10 µg/mL) resulted in a marked increase in rhodamine 123 fluorescence intensity compared with incubation with H_2O_2 alone (91.1 ± 6.7%, $p < 0.01$) (**Fig. 5**).

During the 25-min exposure to 200 µM H_2O_2 irritation, $[Ca^{2+}]i$ increased rapidly during the first 23 min and then attained a stable state. Treatment with ECH (5, 10 µg/mL) 30 min before addition of H_2O_2 depressed the time course curve (**Fig. 6A**). Moreover, ECH application caused a marked decrease in the peak value of $[Ca^{2+}]i$ (reduced by 19.1%, $p < 0.05$ and 39.9%, $p < 0.01$, respectively) (**Fig. 6B**). These results provide convincing evidence that ECH slows the influx of Ca^{2+} and reduces the $[Ca^{2+}]i$ level induced by H_2O_2.

We also measured the effect of ECH on H_2O_2-induced upregulation of the Bax/Bcl-2 ratio by Western blot. As shown in **Fig. 7B**, Bax protein expression in PC12 cells increased ($p < 0.01$) when cells were treated with 400 µM H_2O_2. At the same time, the Bcl-2 level decreased ($p < 0.01$). ECH (10 µg/mL) dramatically reduced the upregulated Bax/Bcl ratio that was induced by H_2O_2. The Bax/Bcl-2 ratio was 188.1 ± 29.8% in cells that received H_2O_2 treatment ($p < 0.01$); the addition of ECH prevented the H_2O_2-induced increase, and the ratio decreased to 113.0 ± 21.4% (**Fig. 7C**).

Discussion

Our results suggest that ECH may protect PC12 cells against H_2O_2-induced apoptosis and increase cell viability. Experiments revealed that several mechanisms, working individually or in concert, may be involved in ECH protection. First, our data further supports previous findings that H_2O_2 may directly induce the formation of ROS and mitochondrial dysfunction [18,19]. ROS cause cell injury through cell membrane lipid destruction, cleavage of DNA, and an elevation of $[Ca^{2+}]i$ level [20,21]. These factors are critical mechanisms for inducing cell apoptosis. ROS, including H_2O_2, may be partly responsible for opening mitochondrial permeability transition pores and causing the collapse of MMP, which is essential for cell survival [22]. Our data showed that ECH was highly effective at inhibiting ROS formation and MMP loss in HIPCs.

A possible mechanism by which ECH combats H_2O_2-induced PC12 cell injury is suggested by its polyphenolic structure. Polyphenolic compounds are known to be potent antioxidants and free radical scavengers. Additionally, $[Ca^{2+}]i$ may also contribute to the neuroprotective effect of ECH on HIPCs. Elevated $[Ca^{2+}]i$ impairs mitochondrial function and activates phospholipase, protease, and endonucleases, eventually leading to cell death [2,6]. H_2O_2 exposure causes membrane depolarization and the opening of ion channels, and increases Ca^{2+} influx through Ca^{2+} chan-

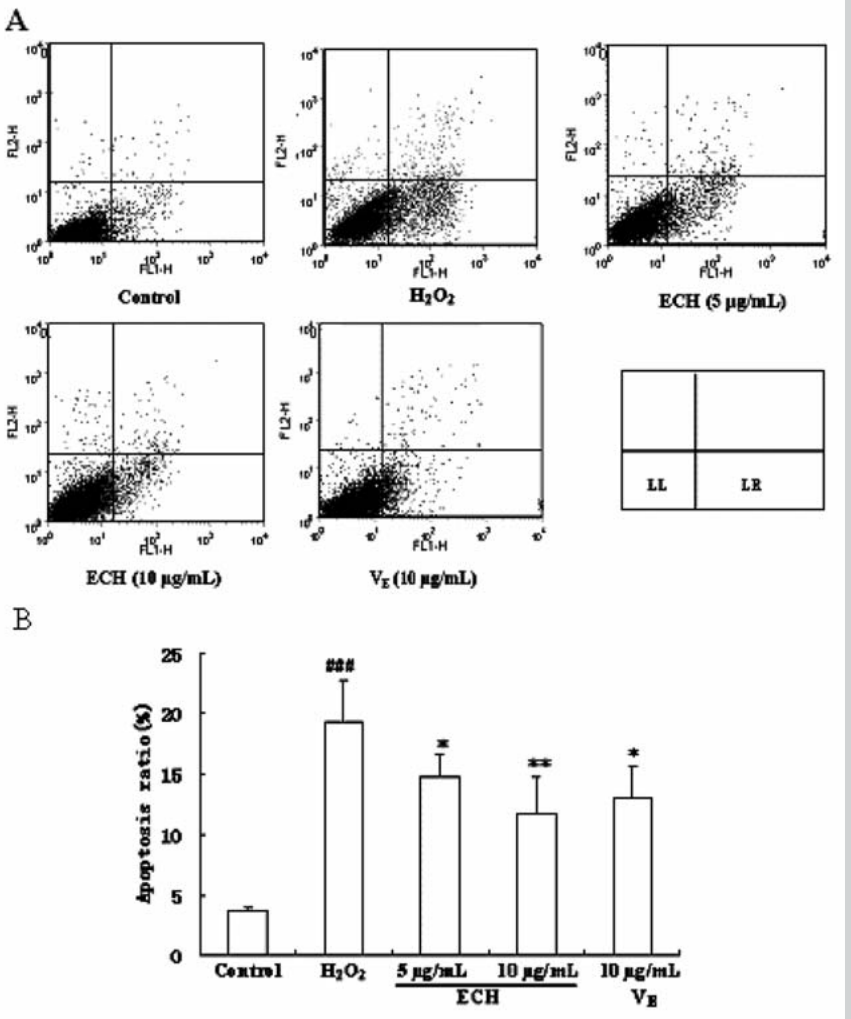

Fig. 3 Flow cytometry measurements of the effect of ECH on H_2O_2-induced apoptosis in PC12 cells. After pretreatment with ECH (5, 10 μg/mL) for 30 min, cells were co-incubated with or without 400 μM H_2O_2 for 4 h. Cells were then harvested and labeled with annexin V-FITC to detect apoptosis. (**A**) Shows the typical apoptotic histograms of all dose groups in which LL are the normal cells with annexin V-negitive, LR are the apoptotic cells with annexin V-positive. The apoptotic percentages were analyzed using CellQuest software (Becton-Dickinson) in (**B**). Data are expressed as percentages relative to the control, with mean ± SD (n = 5). ### $P < 0.001$ compared to control; * $p < 0.05$, ** $p < 0.01$ compared to H_2O_2-treated cells.

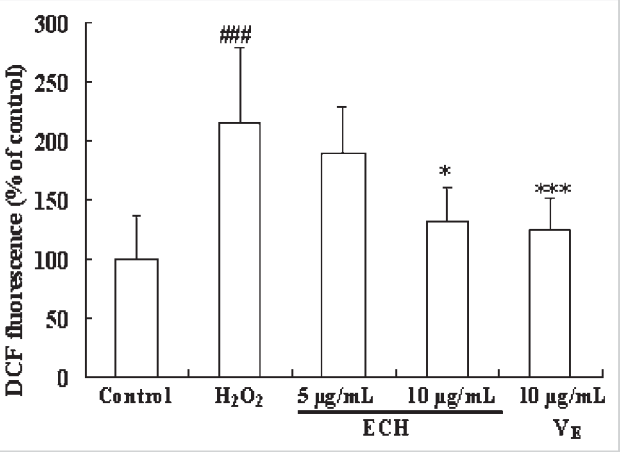

Fig. 4 Effect of ECH on ROS formation due to H_2O_2. The fluorescence intensity of DCF was measured after PC12 cells were exposed to ECH and/or 400 μM H_2O_2, as indicated in the protocol. Data are expressed as percentages relative to control, with mean ± SD (n = 5). ### $P < 0.001$ compared to control; * $p < 0.05$, *** $p < 0.001$ compared to H_2O_2-treated cells.

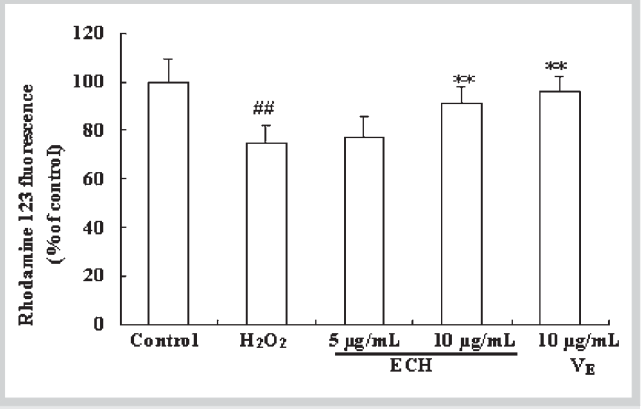

Fig. 5 Effect of ECH on the disruption of MMP in H_2O_2-induced PC12 cells. ECH (5, 10 μg/mL) was added to the culture medium 30 min prior to H_2O_2 addition. After a 4-h exposure to 400 μM H_2O_2, cells were stained with rhodamine 123, and the fluorescence intensity was measured. Data are expressed as percentages relative to control, with mean ± SD (n = 5). ## $P < 0.001$ compared to control; * $p < 0.05$, ** $p < 0.01$ compared to H_2O_2-treated cells.

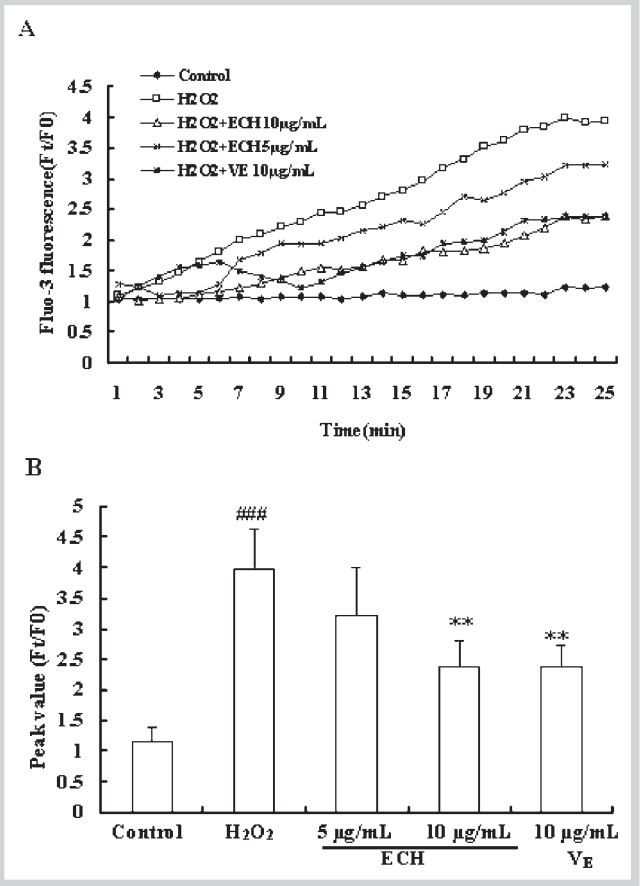

Fig. 6 Effect of ECH on $[Ca^{2+}]i$ levels over time (n = 20–25). After PC12 cells were treated with or without ECH (5, 10 μg/mL) for 30 min and incubated with fluo-3 for 15 min in the dark, 200 μM H_2O_2 was added and time-series data were collected using laser scanning confocal microscopy (**A**). (**B**) Shows the peak values of $[Ca^{2+}]i$. Data are expressed as ratios of Ft/F0, with mean ± SD. ### $P < 0.001$ compared to control; * $p < 0.05$, ** $p < 0.01$ compared to H_2O_2–treated cells.

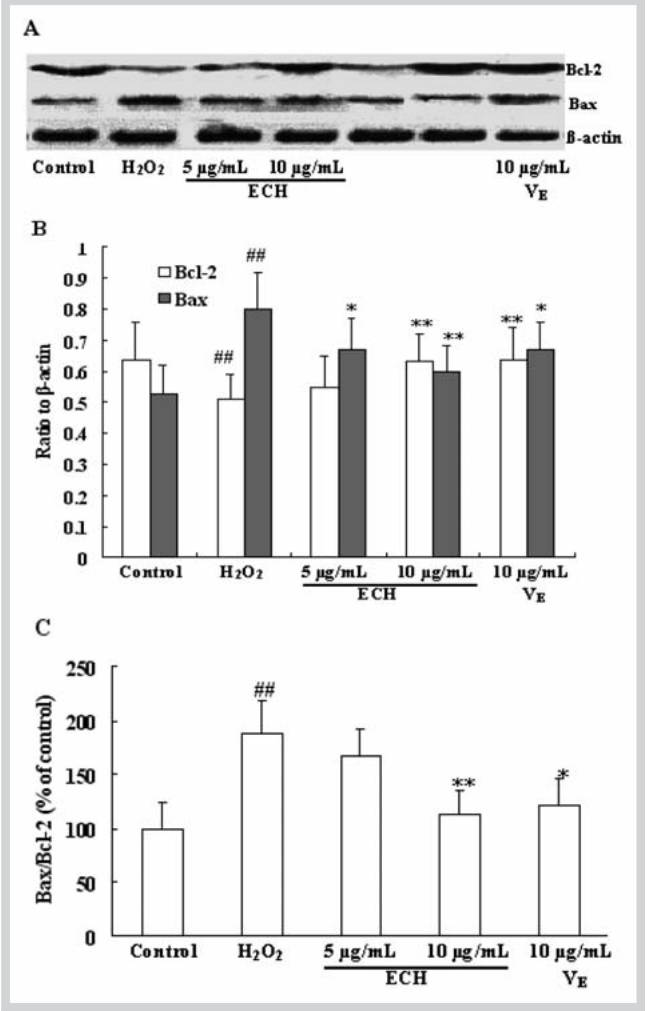

Fig. 7 Effect of ECH on the expression of Bax and Bcl-2 in PC12 cells. After treatment with ECH and H_2O_2, cell lysates were analyzed on a Western blot. (**A**) Top panel shows typical Western blot traces. The levels of Bax and Bcl-2 were quantified by densitometric analysis (**B**), and the Bax/Bcl-2 ratio was determined (**C**). Data are expressed as mean ± SD. ## $P < 0.01$ compared to control; * $p < 0.05$, ** $p < 0.01$ compared to H_2O_2-treated cells.

nels [23]. In reverse, elevated $[Ca^{2+}]i$ accelerate ROS formation due to the enhanced rate of oxygen consumption [24], resulting in a vicious cycle. Our results are highly consistent with previous reports of increased $[Ca^{2+}]i$ in HIPCs [15,25]. Unlike V_E, ECH is soluble in water, making absorption through the gastrointestinal tract possible. ECH may interact directly with the Ca^{2+} channel and block its opening which V_E cannot. Alternatively, ECH may enter cells through the injured membrane, affecting the signaling pathway between ROS and the opening of Ca^{2+} channel.

Bcl-2 family members such as Bax and Bcl-2 have been implicated in apoptosis induced by ROS through the mitochondrial apoptotic pathway [26]. Bcl-2 is located in the mitochondria membrane and prevents membrane depolarization and the production of ROS, therefore preventing apoptosis [27]. In contrast, Bax has been demonstrated to increase the formation of ROS, but decrease the MMP [28]. Cell survival in the early phases of the apoptotic cascade depends heavily on the balance between Bax and Bcl-2. The relative Bax/Bcl-2 ratio may predict the apoptotic fate of the cell better than the absolute concentration of either one alone [29]. As in previous studies [30], we showed that H_2O_2 significantly increased the ratio of the pro-apoptotic Bax to the anti-apoptotic Bcl-2. However, treatment with ECH reduced the expression of Bax, but increased the expression of Bcl-2. Thus, cells were protected from damage due to decreased sensitivity to apoptotic signals such as ROS. These results suggest that down-regulation of the Bax/Bcl-2 ratio may be involved in the protective action of ECH on H_2O_2-induced PC12 cell injury.

In this study, our results show that ECH inhibited H_2O_2-induced ROS production, attenuated the MMP loss, prevented the influx of Ca^{2+}, and down-regulated the Bax/Bcl-2 ratio. Taken together, these findings support the hypothesis that cytoprotective effects mediated by ECH are due partially to inhibition of oxidative stress-induced cell injury through the mitochondrial apoptotic pathway.

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