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·Original Article·

Neurotrophic effects of 7,8-dihydroxycoumarin in primary cultured rat cortical neurons

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Abstract: Objective Neuronal loss in the central nervous system is central to the occurrence of neurodegenerative diseases. Pharmaceutical companies have devoted much effort to developing new drugs against such diseases, since there are currently no effective drugs for neurodegenerative disease treatment. Promoting the capacity for nerve regeneration is an ideal treatment target. The present study aimed to investigate the neurotrophic effects of 7,8-dihydroxycoumarin (DHC) or daphnetin in primary cultured rat cortical neurons. **Methods** Cortical neurons were identified by microtubule-associated protein 2 (MAP2) immunostaining. Morphological observation was used to measure the average length of neurite outgrowth. MTT and lactate dehydrogenase assays were used to assess neuronal survival. The mRNA expression of MAP2 and brain-derived neurotrophic factor (BDNF) was measured by RT-PCR. **Results** MAP2 immunostaining showed that most of the cultured cells were neurons. Compared with the vehicle control group, DHC promoted neurite outgrowth and prolonged neuronal survival time at concentrations ranging from 2 to 8 μmol/L. Expression of both BDNF mRNA and MAP2 mRNA was increased in the groups treated with 2, 4 and 8 μmol/L DHC. **Conclusion** DHC significantly increases neurite outgrowth and promotes neuronal survival in primary cultured rat cortical neurons. The neurotrophic effects of DHC are probably associated with increased BDNF expression.

Keywords: 7,8-dihydroxycoumarin; daphnetin; rat cortical neurons; brain-derived neurotrophic factor; neurodegenerative disease

1 Introduction

Neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Huntington's disease, are characterized by neuronal loss and/or neuronal death[1]. Neurotrophic factors (NTFs), including nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) can be used for the treatment of neurodegenerative disease[2]. However, these large NTFs cannot cross the blood-brain barrier and are easily degraded[3]. To address this issue, researchers have been screening small molecular compounds that have neurotrophic properties or are able to enhance the action of NTFs[1-3].

Zushima is a common traditional Chinese medicine which is distributed in Sichuan, Shanxi and Gansu Provinces, China[4]. It contains many pharmacologically active com-

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pounds such as coumarins, lignans, flavonoids and diterpenoids[5]. Among these compounds, 7,8-dihydroxycoumarin (DHC), also called daphnetin, has been isolated from *Zushima* and used to treat coagulation disorders and rheumatoid arthritis[6,7]. A number of biological activities of DHC, such as anti-inflammatory, antioxidant[8], antimicrobial[9] and antimalarial[10] effects, have been demonstrated. However, the neurotrophic effects of DHC remain to be reported. The present study aimed to investigate the neurotrophic effects of DHC in primary cultured rat cortical neurons.

2 Materials and methods

2.1 Drugs DHC (>97% pure) was from the National Institute for the Control of Pharmaceutical and Biological Products, China (Fig. 1).

2.2 Cell culture Primary rat cortical neurons were prepared from neonatal Sprague-Dawley rats (<24 h old) from the Experimental Animal Center of Guangdong Province, China. Briefly, the neocortex was dissected from the cerebrum, transferred to D-Hank's solution and cut into small pieces (~1 mm^3) with eye scissors. Then, these tissue pieces were trypsinized in 3 mL solution (0.08%, *w*/*v*) for 10 min at 37°C, and filtered through a mesh screen (74 μm) to obtain a single-cell suspension, which was centrifuged at 1 000 g for 5 min at 25°C. Isolated neurons were diluted to the required concentrations as described below, seeded on poly-*L*-Lysine-coated 24- or 96-well culture plates (Gibco, Carlsbad, CA) with DMEM/F12 and 0.4% B27 (Gibco), and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

2.3 Immunocytochemistry Immunocytochemical staining was used to identify cells in cultures[1,11]. Cortical cells were cultured as described above and seeded at 6×10^4 cells/well. The culture medium was changed from DMEM/F12 + 0.4% B27 to DMEM/F12 + 0.4% B27 + 2, 4 or 8 μmol/L DHC. In the positive control group, basic fibroblast growth factor (bFGF, 10 ng/mL) was used instead of DHC. After incubation for 3 days, cells (on 24-well plates) were washed three times with 0.1 mol/L phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 in PBS for 10 min. Endogenous peroxidase activity was blocked by fresh 1% bovine serum albumin in PBS for 20 min. The neurons were incubated with rabbit anti-microtubule-associated protein 2 (MAP2) primary antibody (1:200; Sigma, St. Louis, MO) overnight at 4°C, followed by incubation with Cy3-conjugated goat anti-rabbit IgG (1:500; Sigma). After three washes, Hoechst 33258 was added, and 20 min later, the slides were rinsed with PBS. Neurons were observed under an Olympus (IX71) inverted microscope, and the neurite length was quantified using Image-Pro plus software (Media Cybernetics Inc., Bethesda, MD). Only neurites greater than twice the length of the soma were counted. Twenty neurons per field of view were quantified in this assay.

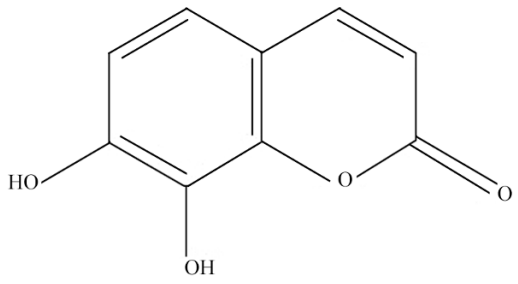

Fig. 1. Chemical structure of 7,8-dihydroxycoumarin.

2.4 Assessment of cortical neuron viability Cortical neurons were cultured as described above and seeded onto poly-*L*-lysine-coated 96-well plates at 3.3×10^4 cells/well. After 6 h, the medium was changed to DMEM/F12 + 0.4% B27 + 2, 4 or 8 μmol/L DHC. bFGF (10 ng/mL) was used as the positive control. After the cells were cultured for 3 days, MTT was added to each well at a final concentration of 0.5 mg/L (*w*/*w*) for 4-h incubation. Then the medium was replaced by DMSO. Finally the formazan produced was measured at 570 nm by a microplate reader (Bio-Rad, Japan). Neuron viability was calculated as follows: Neuron viability (%) = $OD_{(experimental\ group)}/OD_{(mean\ of\ control)} \times 100\%$.

Cell damage was also assessed by measuring the lactate dehydrogenase (LDH) activity or content in the medium. The cells were seeded onto poly-*L*-lysine-coated 24-well plates at 2.5×10^5 cells/well. After 6 h, the cortical cells were divided into 5 groups: vehicle control, 2,4, and 8

μmol/L DHC, and 10 ng/mL bFGF; all were cultured for 3 days. Then 450 μL culture solution was collected from each well to determine LDH using the LDH assay kit (Sichuan Maker Biotechnology Co., Ltd, Chengdu, China)

2.5 Reverse transcription quantitative PCR (RT-PCR) assay Both MAP2 mRNA and BDNF mRNA were detected by RT-PCR. Total RNA was extracted from the cultured cells using TRIzol reagent. Primers of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), MAP2 and BDNF genes were designed using Primer 5.0 software according to their sequences in GenBank, and synthesized by Invitrogen. The primers were as follows: rat GAPDH (206 bp) forward 5'-GGCAAGTTCAACGGCACA-3', reverse 5'- CCACAGCTTTCCAGAGGG-3'; rat MAP2 (429 bp) forward 5'-TGTACCTGGAGGTGGTAACG TAA-3', reverse 5'-ACCTGCTTGGCGACTGTGTG-3'; rat BDNF (259 bp) forward 5'-AGCCTCCTCTGCT CTTTCTG-3', reverse 5'-ATGGGATTACACTTG GTCTCG-3'. GAPDH was used as a reference gene. The total RNA was reverse-transcribed using the PrimeScript RT-PCR kit. PCR was performed as follows: GAPDH denaturation (94°C, 30 s), and 30 cycles of denaturation (94°C, 15 s), annealing (58°C, 15 s) and extension (72°C, 30 s); MAP2 denaturation (94°C, 30 s), and 35 cycles of denaturation (94°C, 15 s), annealing (58°C, 15 s) and extension (72°C, 30 s); BDNF denaturation (94°C, 30 s), and 38 cycles of denaturation (94°C, 15 s), annealing (58°C, 15 s) and extension (72°C, 30 s). The PCR products were analyzed in 1.2 % (*w*/*v*) agarose gels under UV light, and the band intensity of MAP2 and GAPDH was quantified using the Gel-Pro software (Media Cybernetics Inc.).

2.6 Statistical analysis All data are expressed as mean ± SEM from at least 3 independent experiments, and analyzed using one-way ANOVA, followed by the Bonferroni *post hoc* test. $P < 0.05$ was considered to be statistically significant.

3 Results

3.1 Identification of neurons Cortical cells from newborn rats were cultured for 3 days, and then underwent immunocytochemical staining using anti-MAP2 antibody and Hoechst 33258 to identify the neurons. Under the inverted microscope, the cells showed clear cell bodies and processes; most stained for MAP2 and so were neurons (Fig. 2).

3.2 Neurite outgrowth assay As shown by MAP2 immunofluorescence staining, neurons treated with different doses of DHC had longer processes than those of vehicle control (Fig. 3). Only neurites greater than twice the length of the soma were counted. Compared with 10 ng/mL bFGF, 4 and 8 μmol/L DHC had a stronger effect in promoting neurite outgrowth. These observations suggest that DHC enhanced neurite outgrowth in a dose-dependent manner (Figs. 3, 4).

3.3 Cortical neuron viability As revealed by MTT assay, the viability of cortical neurons significantly increased after treatment with 2, 4, and 8 μmol/L DHC compared to vehicle control (Fig. 5). LDH release assay further confirmed the neurotrophic effect of DHC on neuronal survival. Consistently, LDH release decreased significantly after

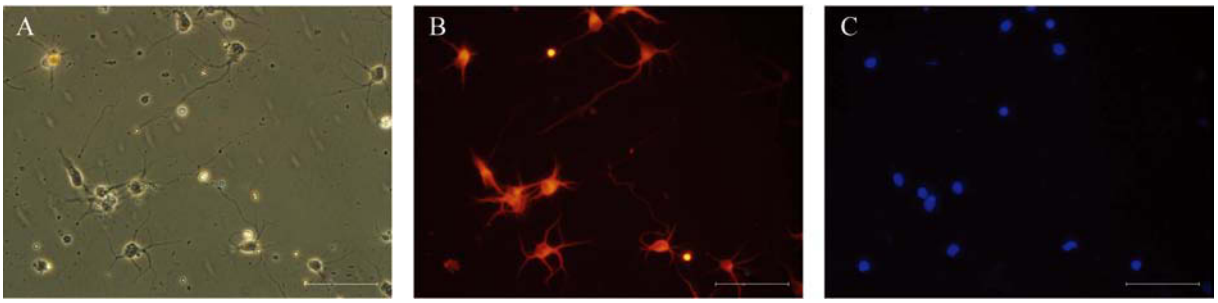

Fig. 2. Immunocytochemical staining of cultured cells. A: Cultured neurons under inverted phase-contrast microscope; B: The same neurons stained by MAP2 immunofluorescence; C: The same neurons stained by Hoechst 33258. Based on these stains, the cultured cells were mainly neurons. Scale bars: 80 μm.

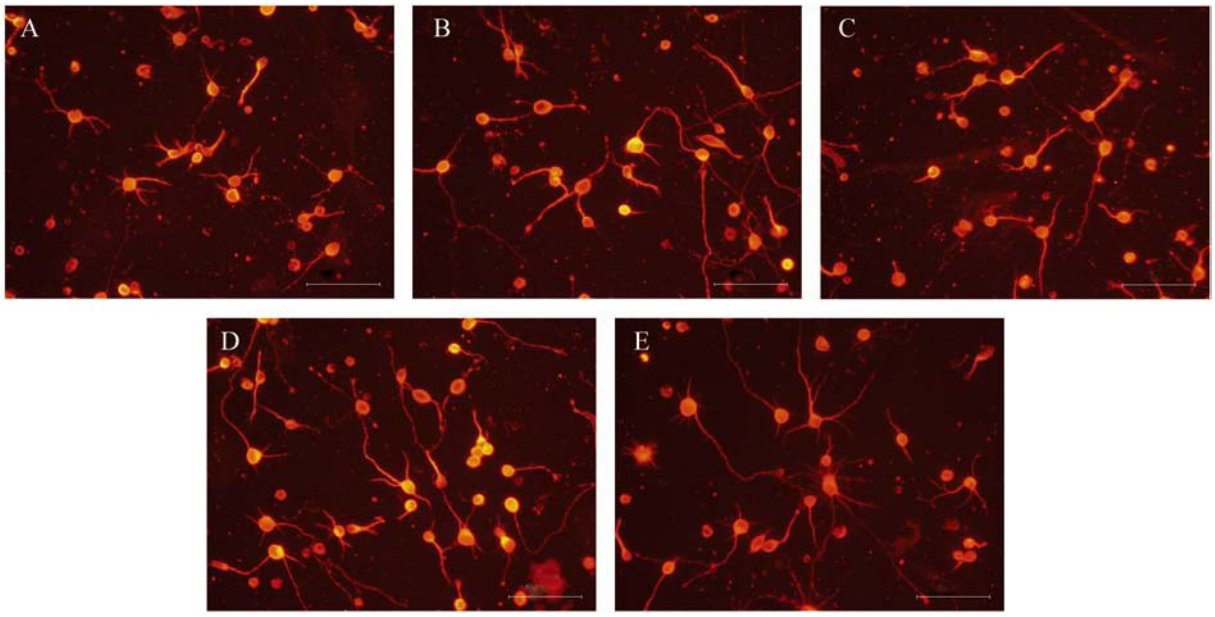

Fig. 3. MAP 2 immunofluorescence staining of primary cultured rat cortical neurons treated with 0.1% DMSO (A), 10 ng/mL bFGF (B), 2 μmol/L DHC (C), 4 μmol/L DHC (D), and 8 μmol/L DHC (E). Scale bars: 80 μm.

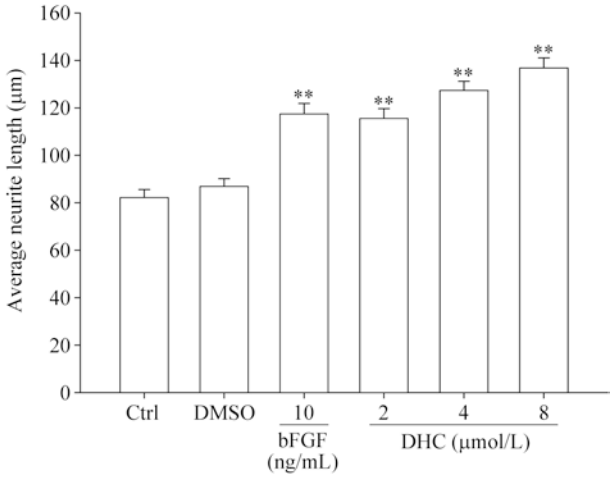

Fig. 4. Morphometric analysis of the average neurite length in culture. $^{**}P < 0.01$ compared with control (Ctrl). $n = 4$.

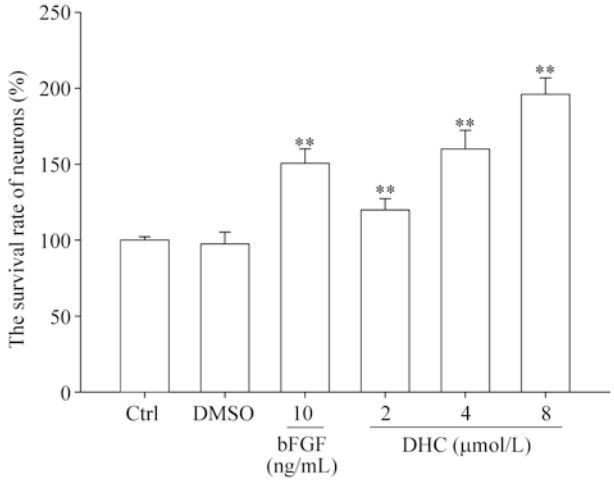

Fig. 5. MTT assay of primary cultured cortical neurons treated with vehicle, BDNF (10 ng/mL) or 2, 4 or 8 μmol/L DHC. $^{**}P < 0.01$ compared with control (Ctrl). $n = 6$.

3-day treatment with DHC (2, 4, and 8 μmol/L) compared to the vehicle control (Fig. 6).

3.4 RT-PCR for MAP2 and BDNF mRNA To further evaluate the neurite outgrowth effect of DHC on MAP2 expression, we treated cortical neurons with 2 to 8 μmol/L DHC. The results of RT-PCR analysis showed that the primary cultured cortical neurons treated with DHC showed increased MAP2 mRNA expression (Fig. 7). We also found that BDNF mRNA expression was significantly enhanced in cortical neurons by DHC in a dose-dependent manner (Fig. 8).

4 Discussion

The system of cortical neuronal culture uses a serum-

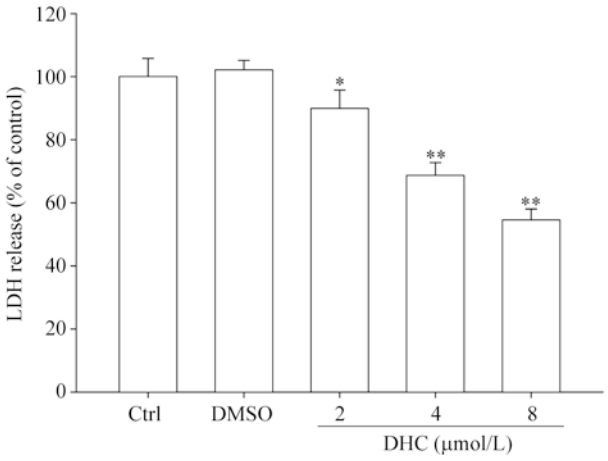

Fig. 6. LDH assay of the medium from primary cultured cortical neurons treated with vehicle, or 2, 4 or 8 μmol/L DHC. *P <0.05 **P <0.01 compared with control (Ctrl). n = 4.

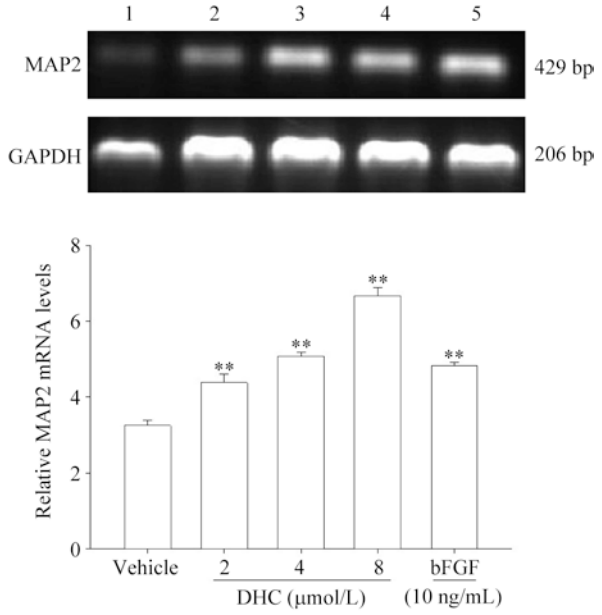

Fig. 7. Effects of DHC on MAP2 mRNA expression, as assessed by RT-PCR. Lane 1, vehicle control; lane 2, 2 μmol/L DHC; lane 3, 4 μmol/L DHC; lane 4, 8 μmol/L DHC; lane 5, 10 ng/mL bFGF. **P <0.01 compared with control. n = 4.

free medium[12]. This is a common and useful model for studying neurotrophic effects, neuroprotective properties and other neurobiological events[13]. DHC, administered at different concentrations, promoted neurite outgrowth

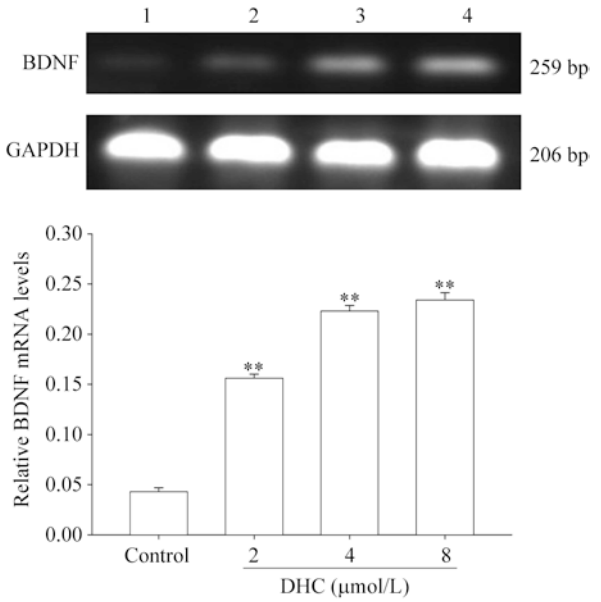

Fig. 8. Effects of DHC on BDNF mRNA expression, as assessed by RT-PCR. Lane 1, vehicle control; lane 2, 2 μmol/L DHC; lane 3, 4 μmol/L DHC; lane 4, 8 μmol/L DHC. **P <0.01 compared with control. n = 4.

and neuronal survival. By MTT assay and LDH assay, we found that DHC enhanced neuronal viability and promoted neurite outgrowth at 2, 4 and 8 μmol/L.

It is known that neurons abundantly express MAP2, which can be used as specific marker to identify immature and mature neurons[14]. Our results showed that most of the cultured cells were neurons (Fig. 2). MAP2 guides microtubules to extend towards specific cellular spaces and cross-link with other microtubules, and also modulates the interactions of microtubules with other proteins in neurons[15]. Therefore, MAP2 plays an important role in maintaining neuronal shape and stabilization, and regulating neurite growth[16].

BDNF is widely found in the cortex, hippocampus and basal forebrain, and is associated with several neurological disorders[17,18], contributing to the survival of neurons, promoting neuronal growth and differentiation, and inducing the formation of new synapses[19]. Thus, BDNF is also associated with learning and memory[20]. Research on the mechanism of action of BDNF showed that it acts on various neurotransmitter receptors, including TrkA, TrkB,

LNGFR and the alpha-7 nicotinic receptor[17]. The Trk receptor and p75NTR are receptors for neurotrophin, and have a special structural base suitable for neurotrophin signaling. Many studies have noted that an increase of BDNF secretion in the cortex has potentially neuroprotective and neurotrophic effects[17-19,21]. In this study, we found that the mRNA expression of both BDNF and MAP2 in the DHC-treated groups was enhanced compared with that in the vehicle groups.

In conclusion, the present study evaluated the neurotrophic effects of DHC isolated from *Zushima*. DHC increased BDNF expression in a dose-dependent manner, which might be the mechanism underlying its neurotrophic effects. Therefore, DHC can be regarded as a potential drug for the treatment of neurodegenerative diseases.

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