Fructus Corni extract-induced neuritogenesis in PC12 cells is associated with the suppression of stromal interaction molecule 1 expression and inhibition of Ca^{2+} influx

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Abstract. Fructus Corni (*Cornus officinalis* Sieb. et Zucc.) is commonly prescribed as a traditional Chinese herbal medicine that possesses pharmacological actions against inflammation, diabetic nephropathy, tumors, oxidation and aging. However, its function and mode of action within the nervous system remain largely unclear. In this study, the effects of Fructus Corni extract (FCE) on neuronal differentiation were investigated. It was found that FCE significantly increased the percentage of PC12 cells bearing neurites ($P<0.001$). Following the generation of neurite outgrowth, FCE treatment decreased the mRNA expression of stromal interaction molecule 1 (STIM1; $P<0.05$) and suppressed the expression of STIM1 protein ($P<0.001$). In addition, extracellular calcium (Ca^{2+}) influx was inhibited resulting in a reduction in the intracellular Ca^{2+} level, suggesting that the inhibition of Ca^{2+} influx may be involved in the FCE-promoted neurite outgrowth of PC12 cells. These results demonstrate that FCE induces neurite outgrowth in PC12 cells and that this is associated with the suppression of STIM1 expression and the inhibition of Ca^{2+} influx, which may partially explain the FCE-induced neuritogenesis.

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Abbreviations: FCE, Fructus Corni extract; NGF, nerve growth factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; DMSO, dimethyl sulfoxide; SOCE, store-operated Ca^{2+} entry; ER, endoplasmic reticulum; STIM1, stromal interaction molecule 1; CRAC, Ca2$^+$ release-activated Ca2$^+$; VGCC, voltage-gated Ca^{2+} channel; IP3, inositol 1,4,5-trisphosphate; TRPC, transient receptor potential channel

Key words: Fructus Corni extract, neurite outgrowth, STIM1, Ca^{2+} influx, PC12 cells

Introduction

Calcium (Ca^{2+}) is a ubiquitous and essential second messenger in cells, which mediates a broad of cellular functions including secretion, excitation, contraction, metabolism, transcription, growth, proliferation, division and apoptosis (1). In general, Ca^{2+} signals are controlled by Ca^{2+} flux in (influx) and out (efflux) through plasma membrane channels in order to achieve Ca^{2+} homeostasis within the cell (2). Store-operated Ca^{2+} entry (SOCE) is a major mechanism of Ca^{2+} import from extracellular to intracellular space, which has been demonstrated to consistently increase intracellular Ca^{2+} levels (3).

Recently, two key factors involved in SOCE have been identified. These are the endoplasmic reticulum (ER) Ca^{2+} sensor protein stromal interaction molecule 1 (STIM1) and the plasma membrane Ca^{2+} channel protein Orai1 (4). STIM1 is a transmembrane protein located in the ER where it has a Ca^{2+} sensor function, and it has a Ca^{2+}-binding EF-hand motif located within the ER lumen (5). Orai1 is an essential component of Ca^{2+} release-activated Ca^{2+} (CRAC) channels in the plasma membrane (6). STIM1 is predominantly located in the ER lumina, the main intracellular Ca^{2+} store. When the store of Ca^{2+} is depleted and inositol 1,4,5-trisphosphate (IP3) receptors are activated, STIM1 translocates into ER/plasma membrane junctions where it couples to activate Orai1 and, possibly, transient receptor potential channels (TRPCs). At these junctions, STIM proteins bind to and activate the highly Ca^{2+}-selective Orai channels, thereby mediating finely controlled Ca^{2+} signals and homeostatically balancing cellular Ca^{2+} levels (7,8).

The traditional Chinese herbal medicine Fructus Corni (*Cornus officinalis* Sieb. et Zucc.), known as Shan-zhu-yu in Chinese, has a long history of use in the treatment of diabetes, cancer, inflammation and shock, and for hepatoprotection (9). Fructus Corni contains gallic acid, malic acid, tartaric acid, ursolic acid, morroniside, loganin and sweroside (10,11). Recent studies have shown Fructus Corni extract (FCE) to be effective against cerebral ischemia-reperfusion injury, and to have anti-oxidant and anti-aging functions (12-14). However, its role and effect in the nervous system remain unknown.

In the present study, the aim was to investigate the effects of FCE on neuronal differentiation. The effects of

FCE treatment on neurite outgrowth, intracellular Ca^{2+} and the expression of STIM1 provide useful information to the therapeutic potential of FCE in neurodegenerative diseases.

Materials and methods

Preparation of FCE. Fresh Fructus Corni (*C. officinalis* Sieb. et Zucc.) was purchased from Tong Ren Tang (Beijing, China). The Fructus Corni (100 g) was extracted by heating in 1,000 ml distilled water at 80°C for 2 h, and then concentrated using a rotary evaporator (Rotary evaporator R-480; Büchi Labortechnik AG, Flawil, Switzerland). The concentrated extracts were lyophilized using a freeze dryer (FDU-540; Elaya, Tokyo, Japan). Following the lyophilization, a powder was obtained (yield, 25.82 g) and was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) to provide a stock solution of concentration 100 mg/ml and stored at 4°C. The stock solution of FCE was then diluted with medium to the desired concentration prior to use.

Materials. Rat β-nerve growth factor (NGF) was purchased from R&D Systems (Minneapolis, MN, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) was obtained from Sigma-Aldrich. Rabbit polyclonal anti-STIM1 (S6072) and mouse monoclonal anti-β-actin (A5316) antibodies were purchased from Sigma-Aldrich.

Cell culture. PC12 (adrenal gland pheochromocytoma) cells (that were a gift from Professor Yanhong Liao of Huazhong University of Science and Technology, Wuhan, China) were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone™, GE Healthcare, Logan, UT, USA) supplemented with 10% heat-inactivated horse serum (Gibco, Grand Island, NY, USA), 5% heat-inactivated fetal bovine serum (Gibco), and antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin; Gibco) at 37°C in a 5% CO_2 incubator. Cells were supplied with fresh medium three times per week, and were split at a 1:3 ratio twice per week.

MTT assay. PC12 cells were seeded into 96-well plates (200 μl/well) at a density of $5x10^4$ cells/ml. They were treated with various concentrations (0, 10, 20, 40, 60, 80 and 100 μg/ml) of FCE for 48 h. At the end of the drug incubation period, MTT (Sigma-Aldrich) working solution (0.5 mg/ml) was added to each well, and the plates incubated for an additional 4 h at 37°C. Following centrifugation at 350 x g for 5 min, the medium was replaced with DMSO. The absorbance of reduced MTT at 570 nm was measured with a plate reader (Bio-Rad Laboratories, Hercules, CA, USA). The rate of inhibition of cell proliferation was calculated from the optical density (OD) values as follows: Inhibitory rate of PC12 cell proliferation (%) = (OD value control group - OD value test group) x 100/OD value control group. Based on the IC_{50} of FCE in PC12 cells, a single concentration of 60 μg/ml was determined according to the results and it was selected for evaluation in the following tests. The MTT assay was also conducted using 60 μg/ml FCE with various incubation times (0, 1, 6, 12, 18, 24, 48 and 72h) to evaluate whether the effect of FCE was time-dependent. Based on the results a 48-h culture time was used for the PC12 cells treated in different groups.

Lentivirus-delivered RNA interference. Lentiviral plasmids carrying STIM1 short hairpin RNA (shRNA) were purchased from Sigma-Aldrich. The lentivirus was produced by the Hope Center Viral Vectors Core of Washington University (St. Louis, MO, USA). For control infection, non-targeted shRNA was used.

Transduction of shRNA. STIM1 shRNA and non-targeted shRNA were transduced into PC12 cells using lentivirus (Hope Center Viral Vectors Core of Washington University) according to the instructions provided by the manufacturer. Briefly, PC12 cells were infected with lentivirus at day 8 *in vitro*, and selected with puromycin dihydrochloride (Santa Cruz Biotechnology, Inc.) for 1 week. At 3 days after infection, western blotting or immunofluorescence staining was performed.

Evaluation of neurite outgrowth. PC12 cells ($1x10^5$ cells/ml) were seeded onto 24-well plates and cultured for 1 day, after which time 60 μg/ml FCE or 50 ng/ml NGF was added and the cells were cultured for an additional 2 days. The cells were then fixed with 4% paraformaldehyde (Sigma-Aldrich) in phosphate-buffered saline (PBS), and cell morphology was assessed under a phase-contrast microscope (IBE2003; Chongqing COIC Industrial Co., Ltd, Chongqing, China). STIM1 shRNA-treated cells were also cultured and assessed, without 60 μg/ml FCE or 50 ng/ml NGF addition. Neurite extension from the PC12 cells was regarded as an index of neuronal differentiation. Processes with a length equivalent to ≥1 diameters of the cell body were regarded as neurites. The differentiation of PC12 cells was evaluated by examining the proportion of neurite-bearing cells to total cells in randomly selected fields. The mean differentiation score was obtained for >100 PC12 cells in each well. In certain experiments, images of the cells were captured, and the total length of the neurite extension per positive cell and average length of neurites in positive cells were determined in randomly chosen fields using Motic Images Plus software (version 2.0S; Motic Instruments Inc., Richmond, Canada).

Immunofluorescence staining. Cells were blocked with 0.1% Tween-20 and 5% goat serum in PBS for 30 min and then stained for 2 h at room temperature with monoclonal mouse anti-Myc-Cy3 (1:200, cat. SAB4700448, Sigma-Aldrich) and polyclonal rabbit anti-GAP-43 (1:50, cat. BA0878, Boster, Pleasanton, CA, USA) antibodies. Primary antibody was then visualized using ECL™ donkey monoclonal anti-rabbit secondary antibody (1:1,000, cat. A-21206, Thermo Fisher Scientific Inc., Waltham, MA, USA). Fluorescent images were captured with a confocal microscope (Olympus Fluoview 500; Olympus, Tokyo, Japan).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and reverse transcribed using M-MLV-Reverse Transcriptase (Promega Corporation, Madison, WI, USA), according to the manufacturer's instructions. qPCR was performed using the SYBR-Green Master PCR mix (Applied Biosystems, Foster City, CA, USA) on the TP800 Real Time PCR System
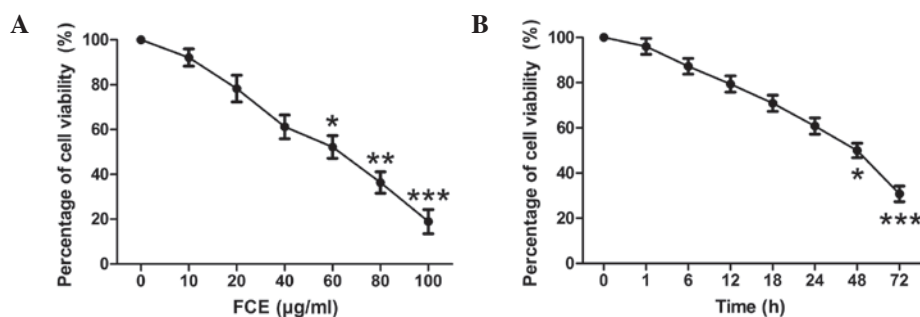
Figure 1. Effects of FCE on PC12 cell viability. PC12 cells were seeded in a 96-well plate at an initial density of 1×10^4 cells/well, and were (A) incubated with various concentrations of FCE for 48 h or (B) incubated with 60 µg/ml FCE for various periods of time. Cell viability was determined by MTT assay. The data are presented as a percentage of the vehicle treated control ± standard error of the mean of three independent experiments. A Student's t-test was used for the determination of significance. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared with the untreated control. FCE, Fructus Corni extract; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium.

(Takara Bio, Otsu, Japan). qPCR of cDNA was performed using the following forward (F) and reverse (R) primer sequences: STIM1: F, 5'-GGACGATGATGCCAATGG TGATGT-3'; R, 5'-TTCCACAGGTCCTCCACGCTGAT-3'; β-actin: F, 5'-TGGACATCCGCAAAGAC-3'; R, 5'-GAA AGGGTGTAACGCAACTA-3'. The amplification conditions were: 5 min at 94°C; 35 cycles of 45 sec at 94°C, 1 min at 56°C and 1 min at 72°C; followed by 10 min at 72°C. All quantification was normalized to an endogenous gene, β-actin. For relative quantification, $2^{-(Ct-Cc)}$ where Ct and Cc are the mean threshold cycle differences after normalizing to β-actin, was calculated and used as an indication of the relative expression levels (15).

Western blotting. PC12 cells were collected, washed once with ice-cold PBS, and lysed with a lysis buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.2% deoxycholic acid and 1:100 protease inhibitor cocktail). Lysates were centrifuged for 10 min at 12,000 x g and supernatants were analyzed for protein concentration using a BCA Protein Assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Equal amounts of proteins were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with antibodies against STIM1 (1:1,000) and β-actin (1:1,000), respectively. Immunoreactive bands were visualized by enhanced chemiluminescence. Images of the bands were captured using a scanner (HP Scanjet 7400C; Hewlett-Packard, Palo Alto, CA, USA), and the intensities of the were quantified using Image J software (National Institutes of Health, Bethesda, MD, USA).

Intracellular Ca^{2+} measurements. Ratiometric imaging of intracellular Ca^{2+} using cells loaded with fura-2 was measured as previously described (16). All cells for these experiments were grown in round coverslips (30 mm) under normal tissue culture conditions, except where specified. Coverslips with cells were placed in a cation-safe solution composed of (in mM): 107 NaCl, 7.2 KCl, 1.2 $MgCl_2$, 11.5 glucose and 20 HEPES-NaOH pH 7.3, and loaded with fura-2-acetoxymethyl ester (2 µM final concentration) for 30 min at 37°C. Cells were washed, and de-esterification was allowed for a minimum of 15 min. Ca^{2+} measurements were made using a Leica DMI6000 B fluorescence microscope

(Leica Microsystems, Wetzlar, Germany) controlled by SlideBook software (Intelligent Imaging Innovations, Denver, CO, USA). Fluorescence emission at 505 nm was monitored while excitation wavelengths were alternated between 340 and 380 nm at a frequency of 0.5 Hz; intracellular Ca^{2+} measurements were determined as a 340/380 nm ratio obtained from groups (n=15-25) of single cells. External solutions were (in mM): 135 NaCl, 5.4 KCl, 10 HEPES, 0.02 NaH_2PO_4, 2 Mg^{2+} and 10 glucose pH 7.4. Measurements shown are representative of a minimum of three independent experiments.

Statistical analysis. All results are expressed as the means ± standard error of the mean of data obtained from triplicate experiments. Data in two groups were analyzed by Student's t-test. Multiple comparisons of the data were performed by analysis of variance followed by Tukey's test. All the analyses were performed using the SPSS software package, version 17.0 (SPSS, Inc., Chicago, IL, USA). Differences with $P<0.05$ were considered statistically significant.

Results

FCE treatment inhibits the proliferation of PC12 cells. To examine the effects of FCE treatment on cell proliferation, MTT assays were used to detect the viability of PC12 cells. When the PC12 cells were treated with various concentrations of FCE for 48 h, the proliferation of PC12 cells was inhibited in a concentration-dependent manner (Fig. 1A). According to the inhibitory rates of FCE at different concentrations on PC12 cells (data not shown), the IC_{50} was 58.248 µg/ml. A concentration of 60 µg/ml FCE was selected for use in this study. Furthermore, a time-dependent reduction in cell viability was observed (Fig. 1B). The PC12 cells were incubated for 48 h with different treatments prior to the following experiments.

FCE treatment induces neurite outgrowth in PC12 cells. Following treatment with 60 µg/ml FCE, morphological changes indicating neurite outgrowth were observed in the PC12 cells (Fig. 2A). NGF, a prominent neurotrophic factor, was used to induce neurite outgrowth in PC12 cells as a positive control. In addition, the morphological changes induced by the knockdown of STIM1 expression with specifically targeted shRNA were observed to elucidate the effect of
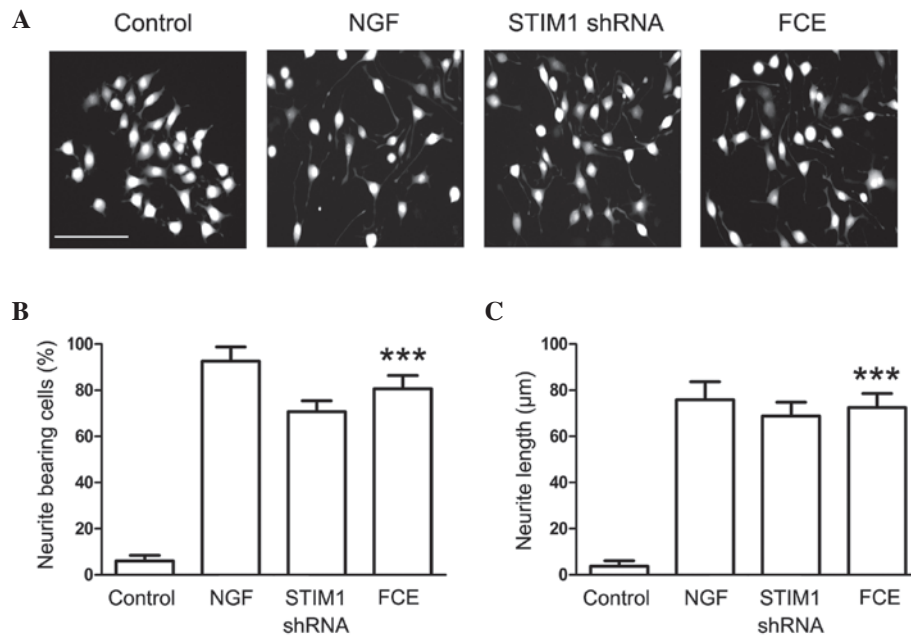
Figure 2. Effects of FCE on neurite outgrowth from PC12 cells. PC12 cells (1x10^5 cells/ml) were seeded onto 24-well plates. After 24 h of stabilization, NGF or FCE was added and the cells were cultured for an additional 48 h. STIM1 shRNA-treated PC12 cells were also observed. (A) Morphological observations of PC12 cells from the different groups (control, 50 ng/ml NGF-treated, STIM1 shRNA-treated and 60 μg/ml FCE-treated groups). Scale bar, 100 μm. (B) The percentage of neurite-bearing cells was calculated from 5 random fields per well, and the data are expressed as a mean percentage of 6 wells in a representative experiment. Data are presented as mean ± standard error of the mean (SEM). ***P<0.001 compared with the control group. (C) Neurite lengths in PC12 cells of the various groups were calculated. Data are presented as mean ± SEM. ***P<0.001 compared with the untreated control. FCE, Fructus Corni extract; NGF, nerve growth factor; STIM1, stromal interaction molecule 1; shRNA, short hairpin RNA.

STIM1 on neurite outgrowth from PC12 cells. The results demonstrated that FCE treatment stimulated neurite extension in PC12 cells in a similar manner to NGF. In addition, neurite outgrowth from the PC12 cells also was observed in the STIM1 shRNA-treated group, suggesting that STIM1 may be involved in the process of neural differentiation (Fig. 2A). Furthermore, FCE significantly increased the percentage of neurite-bearing cells and the length of the branches (Fig. 2B and C).

FCE treatment suppresses the expression of STIM1 mRNA and protein in PC12 cells. SOCCs, including STIM1, play a impor-tant role in cellular proliferation and differentiation. In this study, the knockdown of STIM1 expression with specifically targeted shRNA was carried out, and it effectively reduced the expression of STIM1 mRNA (Fig. 3) and protein (Fig. 4B). Treatment with FCE also reduced the mRNA expression of STIM1 in the PC12 cells (Fig. 3), concurrently with its inducing effect on neurite outgrowth. Immunofluorescence staining confirmed that FCE treatment suppressed the expression of STIM1 protein in PC12 cells (Fig. 4A). Consistent with these results, the expression of STIM1 protein was also found to be significantly decreased in the FCE treatment group compared with the control group by western blot assay (Fig. 4C).

Inhibition of extracellular Ca^{2+} influx is involved in FCE-induced neurite growth. To examine whether sustained Ca^{2+} influx is critical for neurite growth in PC12 cells with FCE treatment, cytosolic Ca^{2+} was measured by the fura 2 assay (Fig. 5). FCE treatment inhibited Ca^{2+} influx in a time-dependent manner in PC12 cells, which differed from the NGF and STIM1 shRNA groups. These results suggest that
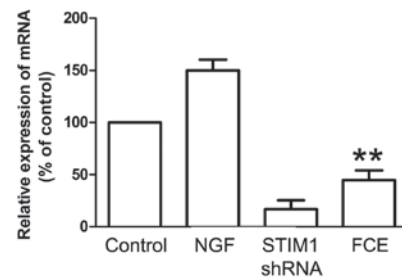

Figure 3. FCE treatment reduces the expression of STIM1 mRNA in PC12 cells. The relative mRNA level of STIM1 expression in PC12 cells of dif-ferent groups (control, 50 ng/ml NGF-treated, STIM1 shRNA-treated and 60 μg/ml FCE-treated groups) was measured by reverse transcription-quan-titative polymerase chain reaction. Data are presented as mean ± standard error of the mean from three experiments. **P<0.05, comparison with the untreated control. FCE, Fructus Corni extract; NGF, nerve growth factor; STIM1, stromal interaction molecule 1; shRNA, short hairpin RNA.

FCE-induced neurite growth may involve a signaling pathway and mechanism different from that of NGF.

Discussion

In the present study, it was shown that FCE promotes neurite outgrowth and differentiation of PC12 cells. Notably, the FCE-induced neuritogenesis was demonstrated to be associ-ated with the downregulation of STIM1 expression at the mRNA and protein levels, as well as the inhibition of extracel-lular Ca^{2+} influx.

The second messenger Ca^{2+} plays a crucial role in regu-lating a number of different cellular processes by modulating
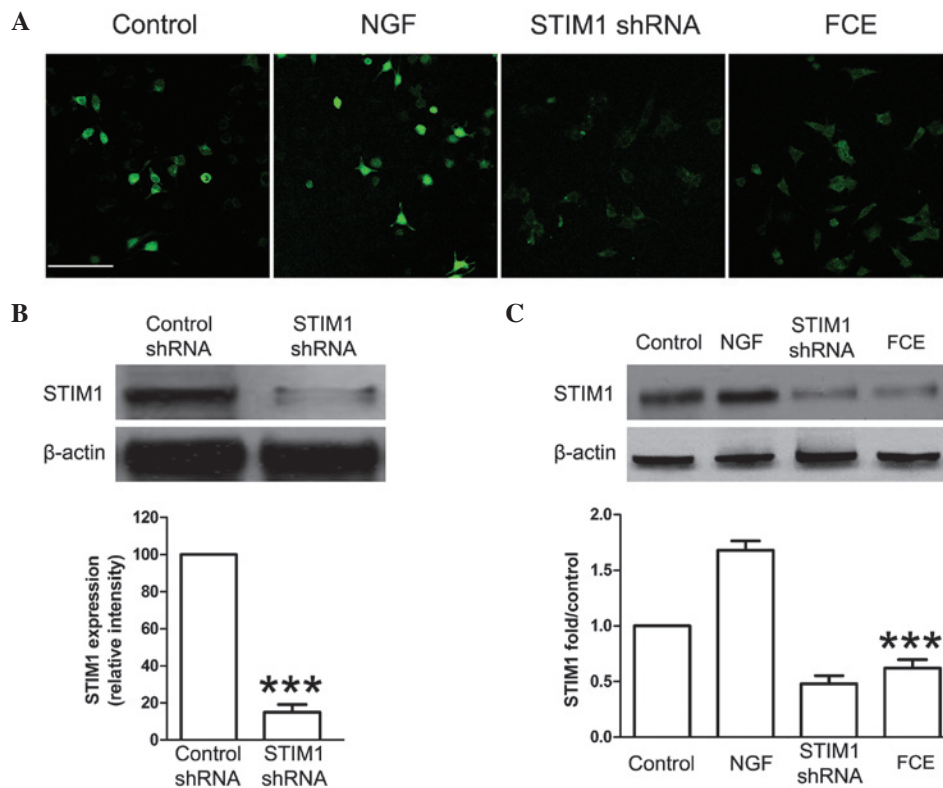
Figure 4. FCE treatment suppresses the expression of STIM1 protein in PC12 cells. (A) Expression of STIM1 protein in the PC12 cells of different groups (control, 50 ng/ml NGF-treated, STIM1 shRNA-treated and 60 μg/ml FCE-treated groups) was observed through immunofluorescence staining. Scale bar, 100 μm. (B) PC12 cells were treated with STIM1 shRNA or control shRNA, and the expression of STIM1 protein was detected by western blotting with specific anti-STIM1 antibody. Data are expressed as mean ± standard error of the mean (SEM). ***$P<0.001$ compared with the control group. (C) The expression of STIM1 protein in PC12 cells of different groups was detected by western blotting, and assessed by semi-quantitative analysis. All experiments were conducted in triplicate. Data are expressed as mean ± SEM. ***$P<0.001$ compared with the untreated control. FCE, Fructus Corni extract; NGF, nerve growth factor; STIM1, stromal interaction molecule 1; shRNA, short hairpin RNA.
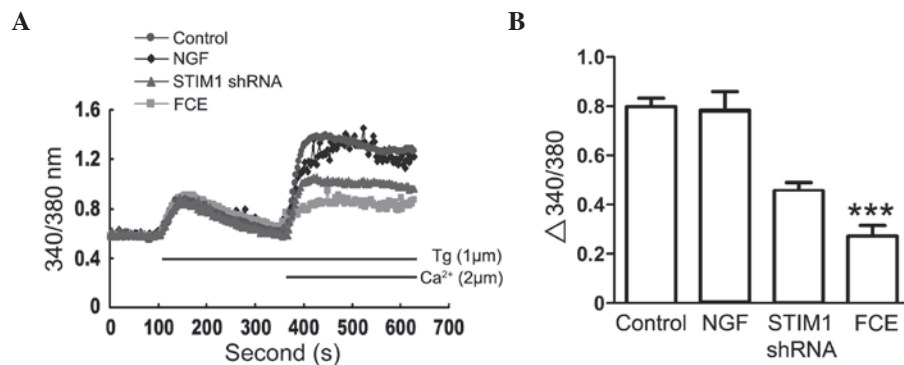

Figure 5. FCE-induced neurite growth in PC12 cells is associated with the inhibition of Ca^{2+} influx. (A) Cytosolic Ca^{2+} was measured in the PC12 cells of different groups (control, 50 ng/ml NGF-treated, STIM1 shRNA-treated and 60 μg/ml FCE-treated groups), and representative traces of intracellular Ca^{2+} changes (fluorescence emission at 505 nm with alternating excitation wavelengths of 340 and 380 nm) in PC12 cells were observed. (B) Involvement of inhibiting extracellular Ca^{2+} influx in FCE-induced neurite growth of PC12 cells. Fluorescence emission at 505 nm was monitored while excitation wavelengths were alternated between 340 and 380 nm at a frequency of 0.5 Hz The intracellular Ca^{2+} measurements were determined as a 340/380 nm ratio obtained from different groups. All data shown are from individual experiments representative of at least three independent replicates. Data are expressed as mean ± standard error of the mean. **$P<0.001$ compared with the untreated control. FCE, Fructus Corni extract; NGF, nerve growth factor; STIM1, stromal interaction molecule 1; shRNA, short hairpin RNA.

Ca^{2+}-regulated proteins and the corresponding signaling pathways. There are two main sources of intracellular free Ca^{2+}: Ca^{2+} released from intracellular Ca^{2+} storage organelles, most notably the ER, and Ca^{2+} influx from sources external to the cell (17). It has been recognized that Ca^{2+} influx into neuronal subcellular compartments (for example, dendrites, somata,

spines and axons) is mediated by two principal means of Ca^{2+} entry. These routes are voltage-gated Ca^{2+} channels (VGCCs) and ionotropic neurotransmitter receptors; both routes elicit crucial rises in cytosolic Ca^{2+} levels in response to different stimuli (18). VGCCs are widely expressed in excitable cells and they trigger Ca^{2+} influx over specific ranges of membrane

potentials (19). Neurons along with other cell types display an alternative Ca^{2+} entry mode that is coupled to intracellular Ca^{2+} stores. This alternative type of entry, known as capacitative Ca^{2+} entry, is triggered upon the depletion of Ca^{2+} stores to facilitate SOCE (20). SOCE is a major mechanism by which Ca^{2+} is imported from extracellular to intracellular space. In general, the stimulation of cell surface receptors leads to the activation of IP3, which evokes a rapid and transient release of Ca^{2+} from the ER store through IP3 receptor channels. The resulting reduction of Ca^{2+} concentration in the ER is detected by the EF-hand motif of STIMs. This causes the STIMs to translocate to the plasma membrane, where they interact with Orai (also known as CRACM1) Ca^{2+} channel subunits, resulting in an influx of Ca^{2+} from the extracellular space in order to restore the Ca^{2+} concentration in the ER (21).

The essential roles of STIM1 and Orai1 in SOCE have been confirmed in a number of studies. STIM1 is a single-pass transmembrane protein in the ER membrane that functions as a Ca^{2+} sensor in the ER, while Orai1 is the pore-forming subunit of SOC channels in cell membranes (22). STIM1 predominantly exists in the sarcoplasmic reticulum (SR) or ER and has its N-terminal sensing Ca^{2+} domain in the SR/ER lumen and its C-terminal Orai1 coupling site in the cytosol (23). STIM1 acts as both an ER Ca^{2+} sensor and activator of SOCE. When Ca^{2+} in the ER is depleted and STIM1 migrates to the plasma membrane, STIM1 forms aggregates at plasma membrane sites of Ca^{2+} entry, where it interacts with and activates Orai1 channels to induce SOCE (24). Several studies have investigated the potential involvement of SOCE-mediated Ca^{2+} metabolism with a focus on STIM1. For example, STIM proteins and Orai channels have been indicated to be associated with neuronal development and memory, and it has been shown that abnormal SOCE may participate in the Ca^{2+} overload of neurons in the early stages following diffuse axonal injury via an increase in STIM1 expression (25). In addition, it has been observed that STIM1- and Orai1-regulated SOCE is associated with changes in the proliferation of endothelial cells (26). In vascular smooth muscle cells, the knockdown of either STIM1 or Orai1 has been demonstrated to inhibit the proliferation and migration of the cells (27). Furthermore, STIM1-knockdown inhibited serum-induced epidermoid carcinoma cell migration (28). The data from the present study demonstrate that STIM1 plays an important role in FCE-induced neuritogenesis, suggesting that inhibiting STIM1-mediated SOCE-associated Ca^{2+} metabolism dysfunction might be a strategy for the treatment of neuronal injury.

The upstream pathway leading to SOCE in PC12 cells has not been elucidated. Ca^{2+} levels in the ER are mainly controlled by the phospholipase C (PLC)/IP3/IP3 receptor pathway with activation by tyrosine kinase-type or Gq-related G protein-coupled receptors (29). In colorectal cancer cells, SOCE has been shown to be controlled by tyrosine kinase-type receptors rather than by Gq-coupled receptors (30). In pulmonary arterial smooth muscle cells, platelet-derived growth factor was found to activate SOCE via Akt signaling (31). In addition, the phosphorylation of STIM1 at extracellular signal-regulated kinase 1/2 (ERK1/2) target sites has been demonstrated to regulate the association of STIM1 with EB1 (end-binding protein 1), a regulator of growing microtubule ends (32). Another study revealed that ERK1/2 phosphory-

lates STIM1 *in vitro* at Ser575, Ser608 and Ser621, and that STIM1 regulates SOCE in HEK293 cells via phosphorylation at ERK1/2 target sites (33). An investigation of the functional association among tyrosine kinase-type receptors, SOCE and ERK signaling in PC12 cells would be of interest.

In conclusion, the findings of the present study provide the first evidence of induction of neurite outgrowth in PC12 cells by the traditional Chinese herbal medicine FCE. Additionally, the results demonstrate that FCE-induced neuritogenesis is associated with the suppression of STIM1 expression and the inhibition of Ca^{2+} influx. These results provide a useful information regarding the therapeutic potential of FCE for the treatment of neurodegenerative disease.

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