Biological evaluations of DTS （田七杜仲精） and YHK （養生片仔癀）on neuronal differentiation in PC12 cell model

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Aug. 2, 2016

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1. Introduction

1.1 To explore the functions of DTS and YHK on nerve cells, the water extracts of DTS and YHK were prepared, and the effects of DTS and YHK were evaluated according to the neuronal etiology.

1.2 The neurobiological functions of DTS and YHK were evaluated on two aspects: (i) differentiation of neuronal cells and (ii) expression of synaptic proteins.

2. Material and methods

2.1. Preparation of TC samples

DTS and YHK samples were grinded into powder, weighed 50 mg into a 1.5-mL eppendorf, add 1 mL of water, sonication for 30 min. Then centrifuged at 13,200 rpm for 10 mins. The supernatant was collected and filtered by PTFE syringe filter (0.22 μm). The stock solution was 50 mg/mL and stored at -20 ^{o}C.

2.2. Cell culture

PC12 cells originated from rat adrenal medulla were purchased from American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 6% fetal bovine serum (FBS) and horse serum (HS), 100 units/mL of penicillin and 100 μg/mL of streptomycin, placed in a humidified CO2 (7.5%) incubator at 37 ^{o}C. Fresh medium was applied every other day. The medium was changed by fresh DMEM with 1% FBS and HS, 100 units/Ml of penicillin and 100 μg/mL of streptomycin 3 hours before extract treatment. In the treatment of herbal extract, the extracts were diluted by DMEM with 1% FBS and HS, 100 units/mL of penicillin and 100 μg/mL of streptomycin.

2.3. Cell viability assay

Cell viability was assessed by MTT [3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide] assay. Cells were plated in 96-well plate for 24 hours and treated with extracts of different concentrations for 48 hours. Then the cells were incubated with MTT for 3 hours at 37 ^{o}C. Then the medium was discarded and 100 µL of DMSO was added to each well. The plate was shaken in dark place for 15 min. After that, the absorbance under 570 nm was measured by a microplate reader (Thermo Scientific, Fremont, CA).

2.4. Extracts treatment

The effect of herbal extracts on neurite outgrowth was investigated in cultured PC12 cells, where 50 ng/mL of NGF was served as a positive control showing neurite-like processes. PC12 cells (5×10^4 cells/well) were seeded into 6-well plates. After 24 hours, the cells were changed with DMEM medium containing 1% FBS, 1% HS and 1% P/S. In the treatment, series concentrations of herbal extracts (0.1, 0.5 and 5 mg/mL) were treated every 24 hours twice. Then the cells were collected for further study.

2.5. Determination of neurite outgrowth

After the treatment, a light microscope (Diagnostic Instruments, Sterling Heights, MI), equipped with a phase-contrast condenser, 10X objective lens and a digital camera (Diagnostic Instruments), was used to capture the images with the manual setting. For analyzing the number and length of neurite, approximately 100 cells were counted from at least 10 randomly chosen visual fields for each culture. Using the photoshop software, the cells were then analyzed for the number and length of neurite. The cells were scored as differentiated if one or more neurite was longer than diameter of cell body, and they were also classified to different groups according to the length of neurite that it possessed, i.e. < 30 µm, 30-60 µm, 60-90 µm and > 90 µm. In this study, NGF (50 ng/mL) was used as a positive control.

2.6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

After the herbal extract treatment, the proteins for neuronal differentiation were extracted in low salt lysis buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, 5 mM benzamidine HCl, 10 μM aprotinin, 10 μM leupeptin). After centrifugation at 13,200 rpm (16,100 × g) for 20 min at 4 ^{o}C, the supernatant was collected to detect the total protein amount using protein assay. All the samples were normalized to the same level of protein, treated with 2 x SDS-PAGE sample buffer (0.125 M Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, 2% 2-mercaptoethanol and 0.02% bromophenol blue) and boiled for 15 min before being challenged to the SDS-PAGEs.

2.7. Western blot analysis

After the electrophoresis, the proteins were transferred to a nitrocellulose membrane, using a Mini Trans-Blot® Cell. The transfer condition was: ~40 V and ~0.1 A for 16 hours in 1x transfer buffer (25 mM Tris, 192 mM glycine, 15% ethanol, 0.1% SDS). Transfer and equal loading of the samples was confirmed by staining with ponceau-S. The nitrocellulose membrane was blocked with 5% fat-free milk in Tris-buffer saline/0.1% Tween 20 (TBS-T) for 2 hours at room temperature, and then incubated in the primary antibodies diluted in 2.5% fat-free milk in TBS-T over night at 4 ^{o}C. After that, the nitrocellulose membrane was rinsed with TBS-T and incubated for 2 hours at the room temperature in peroxidase (HRP)-conjugated anti-mouse secondary antibody, or peroxidase (HRP)-conjugated anti-rabbit secondary antibody, diluted in the 2.5% fat-free milk in TBS-T. After intensive washing with TBS-T, the immune complexes were visualized using the enhanced chemiluminescence (ECL) method. The intensities of the bands in the control and different samples, run on the same gel and under strictly standardized ECL conditions, were compared on an image analyzer, using a calibration plot constructed from a parallel gel with serial dilutions of one of the sample.

The antibodies used in the study were shown as bellow:

Antibody	**Dilution**
Anti-neurofilament (NF) 160 antibody	1:5,000
Anti-neurofilament (NF) 68 antibody	1:2,500

Anti-GAPDH antibody	1:10,000,000
HRP-conjugated anti-rabbit secondary antibody	1:5,000
HRP-conjugated anti-mouse secondary antibody	1:5,000

3. Result Interpretation

3.1. Effect of DTS and YHK extracts on the growth of PC12 cells

PC12 cell is a well-studied cell model for neuronal differentiation, which can respond to various stimuli, e.g. NGF. A cell viability assay was first performed to determine a safe concentration range (0-5 mg/mL) of each herbal extract at which all extracts did not induce cell proliferation or death. Result showed that, the cell viability was not significantly affected by DTS and YHK extracts at concentrations from 0-5 mg/mL. Therefore, 0.1, 0.5 and 5 mg/mL (low, medium, high), three different concentrations were selected for following study (Fig. 1).

3.2. Biological evaluation of DTS and YHK extracts on neuronal differentiation

PC12 cells showed similar characters of sympathetic neurons, such as neurofilament expression and neurite outgrowth. Here, the number and length of neurite outgrowth and expression of neurofilaments (NF68 and NF160) of PC12 cells were tested to determine the morphological and biological changes after the treatment of DTS and YHK extracts.

3.2.1. Single application of DTS or YHK in PC12 cells

The neuronal differentiation of PC12 cells was determined morphologically in measuring the number and length of neurite, where 50 ng/mL of NGF was used as a positive control. After 50 ng/mL of NGF treatment for 48 hours, significant neurite outgrowth of PC12 cells was observed. Under the treatment of DTS or YHK extract, at 0.1, 0.5 and 5 mg/mL (a concentration chosen from previous study), the cultures did not show significant growth of neurite (Fig. 2A&B), further mechanism studies should be undertaken afterward.

3.2.2. Single application of YHK extract induce the expression of neurofilaments in PC12 cells at high concentration

The neuronal differentiation could be determined biochemically in analyzing the expression of neurofilaments that are the major structural components of differentiated neurons. During neurite outgrowth, the mammalian neurofilament subunits, NF68 (~68 kDa) and NF160 (~160 kDa) are believed to form hetero-dimers in making the structural domain of neurites.

Application of 50 ng/mL of NGF on cultured PC12 cells for 48 hours robustly induced the expression of both NF68 and NF160. The single application of DTS extracts at all concentrations, on cultured PC12 cells, caused no regulation on expression of NF68 and NF160. While single application of YHK extract at high concentration (5 mg/mL) up-regulated the protein expressions of NF68 and NF160 (~4 folds) (Fig. 3A&B).

3.2.3. DTS and YHK extracts could compensate NGF-induced neurite outgrowth

In this study, a suitable concentration of NGF was selected: this concentration should have no or very little effect on induction of neurite outgrowth or neurofilament expression. NGF concentration at 0.5 ng/mL was selected in following study.

In neurite outgrowth assay, the percentage of differentiated cells by co-treatment of DTS or YHK extract (0.1, 0.5 and 5 mg/mL) and NGF (0.5 ng/mL) was in a dose-dependent manner. After co-treatment of DTS and low dose of NGF, PC12 cells showed longer neurite, e.g. 30-60, 60-90 and >90 μm. Similar observation was shown under the co-treatment of YHK and NGF (Fig. 4A&B).

3.2.4. DTS and YHK extracts could compensate NGF-induced neurofilament expression

To further study the compensate effect of DTS and YHK extracts in neuronal differentiation, DTS or YHK extract (0.1, 0.5 and 5 mg/mL) with NGF (0.5 ng/mL) were co-treated to cultured PC12 cells. The expression of NF68 was

slightly enhanced under the co-treatment of DTS (at all concentrations) plus NGF, or, 0.1 and 0.5 mg/mL of YHK plus NGF (~2-3 folds). While a significant increase of NF68 was observed under the co-treatment of 5 mg/mL of YHK plus NGF (~8 folds). The expression of NF160 was enhanced better than NF68 in all groups. Under the co-treatment of DTS plus NGF, 0.1 mg/mL of DTS showed the highest effect on NF160 expression (~6 folds). While Under the co-treatment of YHK plus NGF, 5 mg/mL of YHK showed the highest effect on NF160 expression (~9 folds) (Fig. 5A&B).

4. Results

Single application of DTS or YHK extracts did not directly induce neurite outgrowth under the treatment for 48 hours, but slightly increase the expression of neurofilaments in PC12 cells. Furthermore, the co-treatment of DTS or YHK extract with NGF (at non-effective concentration) could induce neurite outgrowth and enhance neurofilament expression.

Appendix I

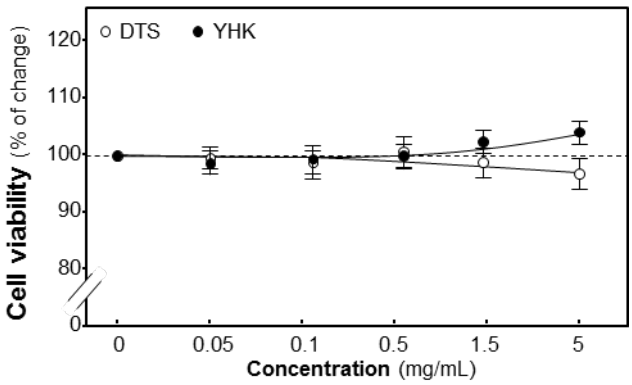

Fig. 1 Effect of DTS and YHK extracts on the growth of PC12 cells

Cultured PC12 cells were treated with DTS and YHK extracts for 48 hours. A cell viability and proliferation test (using the colorimetric MTT assay) was performed. Values are in Means ± SEM, each with five samples, *n* = 4.

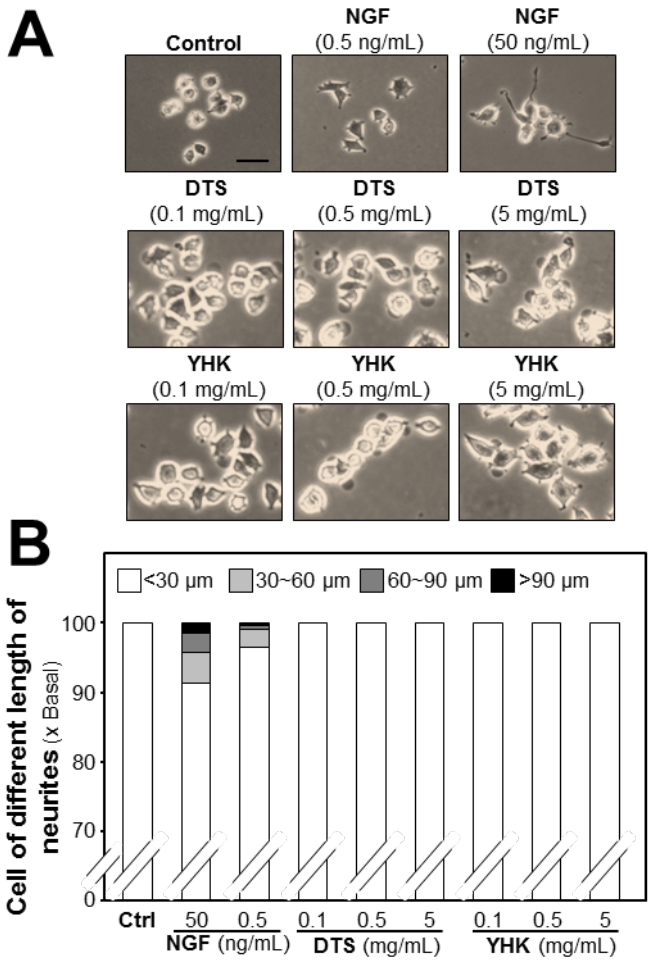

Fig. 2 Single application of DTS or YHK extract does not induce neurite outgrowth in PC12 cells

(A): DTS or YHK extract (0.1, 0.5 and 5 mg/mL) was applied onto cultured PC12 cells for 48 hours. Cells were fixed with ice-cold 4% paraformaldehyde. Bar = 50 µm. NGF served as a positive control. Representative images from four independent experiments are shown.

(B): Cultured PC12 cells were treated as in (A). The percentage of differentiated cells and length of neurite were counted as described in the method section. Values areexpressed as percentage of total cells in 100 counted cells, Mean ± SEM, *n* = 4.

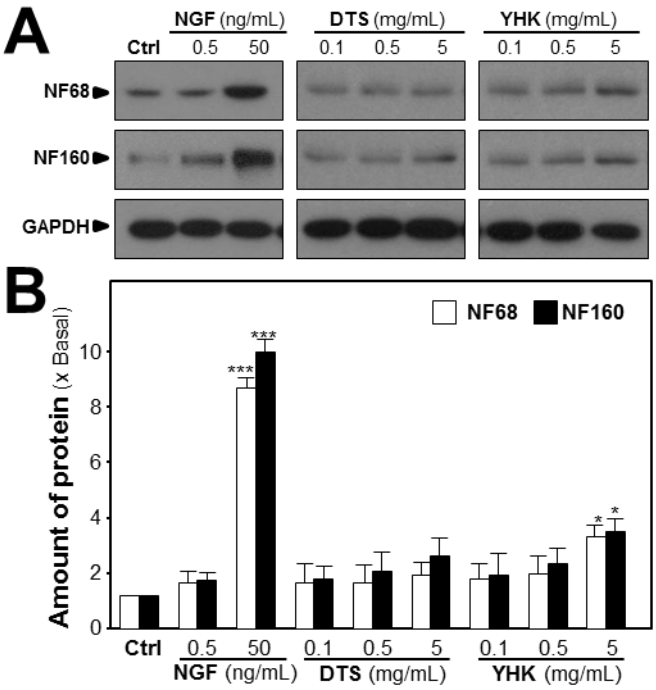

Fig. 3 Single application of YHK extract induces the expression of neurofilaments in PC12 cells at high concentration

(A): DTS or YHK extract (0.1, 0.5 and 5 mg/mL) was applied onto cultured PC12 cells for 48 hours, respectively. The expressions of neurofilaments (NF68 and NF160) were determined by specific antibodies. GAPDH served as a loading control. Representative images from four independent experiments are shown.
(B): Quantification plot was shown in histograms. Values are expressed as x Basal, where control value is set as 1, Mean ± SEM, *n* = 4. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared to the control.

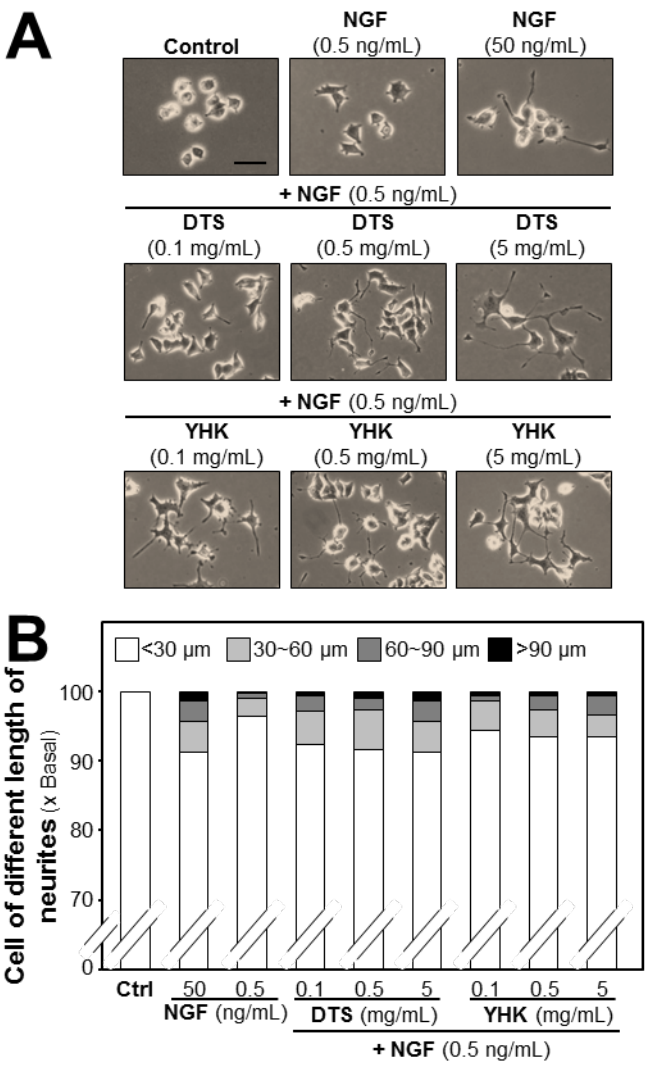

Fig. 4 DTS and YHK extracts could compensate NGF-induced neurite outgrowth

(A): DTS or YHK extract (0.1, 0.5 and 5 mg/mL) was co-treated with NGF (0.5 ng/mL) onto cultured PC12 cells for 48 hours. NGF (50 ng/mL) was applied as a positive control. Cells were fixed with ice-cold 4% paraformaldehyde and the extension of neuritis was revealed. Bar = 50 µm.

(B): Cultured PC12 cells were treated as in (A). The percentage of differentiated cells and length of neuritis were counted as described in the method section. Values are expressed as percentage of total cells in 100 counted cells, Mean ± SEM, *n* = 4.

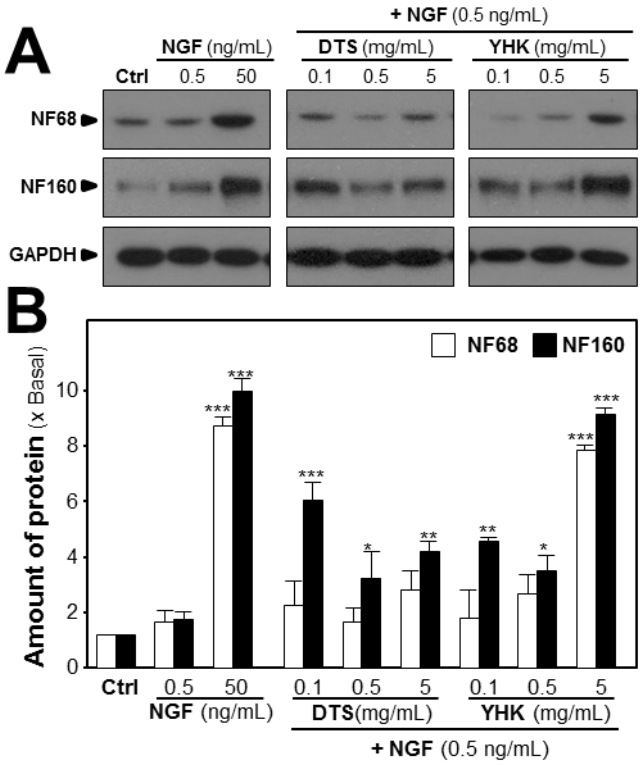

Fig. 5 DTS and YHK extracts could compensate NGF-induced neurofilament expression

(A): DTS or YHK extract (0.1, 0.5 and 5 mg/mL) was co-treated with NGF (0.5 ng/mL) onto cultured PC12 cells for 48 hours. The expressions of neurofilaments (NF68 and NF160) were determined by specific antibodies. GAPDH served as a loading control. Representative images from four independent experiments are shown.
(B): Quantification plot was shown in histograms. Values are expressed as x Basal, where control value is set as 1, Mean ± SEM, *n* = 4. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared to the control.

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