Protective effect of tubuloside B on TNFα-induced apoptosis in neuronal cells[1]

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KEY WORDS tubuloside B; apoptosis; tumor necrosis factor; SH-SY5Y cells; mitochondria; membrane potential; reactive oxygen species; caspase-3; calcium

ABSTRACT

AIM: To investigate the neuroprotective effect of tubuloside B, one of the phenylethanoids isolated from the stems of *Cistanche salsa*, on tumor necrosis factor-alpha (TNFα)-induced apoptosis in SH-SY5Y neuronal cells. **METHODS:** Cell viability was analyzed using MTT assay. Apoptotic cells were detected using Hoechst33342 staining, and confirmed by DNA fragmentation and flow cytometric analysis. The activity of caspase-3 was measured with special assay kit. The concentration of free intracellular calcium was determined with the probe Indo-1 by spectrometer. The level of intracellular reactive oxygen species and the potential of mitochondrial membrane were determined by laser scanning confocal microscopy (LSCM) combined with fluorescence probe H2DCFDA or JC-1 respectively. **RESULTS:** SH-SY5Y cells treated with TNFα 100 μg/L for 36 h showed typical morphological changes of apoptosis. DNA ladder could be observed by agarose gel electrophoresis. The highest percentage of apoptotic cells accumulated to 37.5 %. Following 36 h treatment with TNFα, accumulation of intracellular ROS and $[Ca^{2+}]_i$ and decrease in mitochondrial membrane potential were observed, and caspase-3 activity increased by about five-fold compared with controls. However, pretreatment with tubuloside B (1, 10, or 100 mg/L) for 2 h attenuated the TNFα-mediated apoptosis. The antiapoptotic action of tubuloside B was partially dependent on an anti-oxidative stress effects, maintain of mitochondria function, decrease of concentration of free intracellular calcium and inhibition of caspase-3 activity. **CONCLUSION:** Tubuloside B has the neuroprotective capacity to antagonize TNFα-induced apoptosis in SH-SY5Y cells and may be useful in treating some neurodegenerative diseases.

INTRODUCTION

Apoptosis plays an important homeostatic role in several cellular processes as well as in the development of the nervous systems[1]. Programmed cell death may also contribute to various pathological conditions, such as cerebral ischemia[2,3], neurodegenerative disorders, such as Alzheimer disease, Parkinson disease, Huntington disease, and amiotrophic lateral sclerosis[4-7]; as well as delayed encephalopathy after acute carbon monoxide poisoning[8]. Several lines of evidence have strongly suggested that oxidative stress, a cellular imbalance

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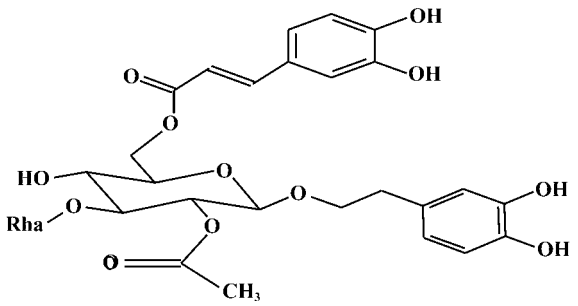

Chemical structure of tubuloside B

between the production and elimination of reactive oxygen species (ROS), leaded to neuronal apoptosis and necrosis[9-11]. Therefore, it is valuable to identify compounds that can antagonize the deleterious action of ROS and act as an antioxidant to protect neurons from apoptosis.

Tubuloside B is one of the phenylethanoids isolated from the stems of *Cistanche salsa*, a Chinese herbal medicine, which is an important crude drug used as both anti-senium and anti-fatigue agent[12]. Several phenylethanoids have been shown to possess free radical scavenging properties and protect oxidative stress induced toxic injuries[13-15]. Tumor necrosis factor-alpha (TNFα) is a toxic-interfering agent because of its important role in neurodegenerative diseases. Death by either necrosis or apoptosis has been seen in response to TNFα[16]. Many studies suggest that oxidative stress play a critical role in the mechanism of TNFα-mediated cell apoptosis. Increased levels of ROS have been documented in cells following treatment with TNFα[17,18] and TNFα-mediated death can be inhibited by free radical scavengers[19]. Thus, we studied whether tubuloside B can protect against TNFα-induced apoptosis and oxidative stress in cultured SH-SY5Y neuronal cells.

MATERIALS AND METHODS

Reagents Modified Eagle's medium (MEM) and fetal bovine serum (FBS) were purchased from GIBCO/BRL. Chromatin dye bisbenzimide (Hoechst 33342), Poly-L-lysine, 3-(4,5-dimethylthiazal-z-yl)-2,5-diphenylterazolium (MTT), TNFα and 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) were obtained from Sigma Chemical Company (St Louis, MO, USA). The annexin-V fluorescein isothcocyanate (FITC) apoptosis detection kit was purchased from Boehringer Mannheim (Indianapolis, IN, Germany). 5, 5′,6,6′-Tetrachloral-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) and Indo-1/AM were purchased from Molecular Probes (Leiden, The Netherlands). Caspase-3 assay and DNA extraction kits were obtained from Promega (USA). Tubuloside B from *Cistanche salsa* was kindly supplied by Dr Peng-fei TU (Peking University Modern Research Center for Traditional Chinese Medicine). The purity of the compounds was more than 98 % on high-performance liquid chromatography (HPLC). All other reagents or drugs were of analytical grade.

Cell culture and treatment Neuronal SH-SY5Y cells obtained from ATCC were maintained in MEM supplemented with 10 % FBS, 100 KU/L benzylpenicillin and 100 mg/L streptomycin in a poly-L-lysine-coated culture flask in a humidified incubator with 5 % CO_2 at 37 ºC. The culture medium was renewed every 2 to 3 d. Cells were pretreated with various concentrations of tubuloside B (1, 10, or 100 mg/L) for 2 h and then treated with 100 μg/L TNFα for 36 h before analysis. Normal cells were considered as control.

Analysis of cell viability Cell viability was determined by use of an MTT assay[20]. Cells were seeded in 96-well plates at 1×10^4 cells per well and grown to 70 % confluence in culture medium. The medium was replaced by medium containing various concentrations of tubuloside B for 2 h and then with TNFα for 36 h. A total of 5 g/L MTT was added to each well after 36 h, and the culture continued to incubate for another 4 h at 37 ºC. After the medium was removed, cells and dye crystals were solubilized with 200 μL dimethylsulfoxide (Me_2SO), and absorbance was measured at 570 nm by use of a model ELX-800 microplate assay reader (One Lambda Inc).

Hoechst 33342 staining After treatment with tubuloside B and/or TNFα, cells were harvested and fixed with 4 % paraformaldyhyde for 30 min at 25 ºC, then washed with pre-chilled phosphate buffer saline (PBS) three times and exposed to 10 mg/L Hoechst 33342 at room temperature in the dark for 10 min. Samples were observed under a fluorescence microscopy (Olympus RX 400)[21].

Analysis of DNA fragmentation DNA was extracted by use of a DNA extraction kit (Promega) according to manufacturer's instructions. Briefly, a 10 μL DNA sample was loaded onto 1.5 % horizontal agarose gels containing ethidium bromide. Gels were run at 60 V for 1 h and DNA fragments were visualized using UV illumination[22].

Annexin-V binding experiment AnnexinV-FITC

apoptosis detection kit was used to bind annexin-V, which has a strong affinity for phosphatidylserine and can probe for apoptosis[23]. In brief, cells were harvested and suspended in binding buffer at a final cell concentration of 1×10^6 cells/ml. Approximately 1×10^5 cells were incubated in the dark with annexin-V and propidium iodide for 15 min. Then the suspension was analyzed with use of a FACS scan flow cytometer (Becton Dickinson, Heidelberg, Germany). Annexin-V FITC and propidum iodide-related fluorescence was recorded on FL1-H (525 nm) and FL2-H (575 nm) filters, respectively.

Caspase-3 activity assay Caspase-3 activity was detected by use of the Apo-ONE™ Homogeneous Caspase-3 assay kit (Promega). Briefly, cells were seeded into 96-well plates at 1×10^4 cells/well. After being exposed to various concentrations of tubuloside B and/or TNFα, cells were washed with ice-cold PBS. Then, 1 μL Z-DEVD-R110 and 99 μL caspase buffer were mixed to make the homogeneous caspase-3 reagent. A total of 100 μL homogeneous caspase-3 reagent was added to each well. The contents were gently mixed and incubated for 4 h at room temperature in the dark. The intensity of the fluorescence of the Z-DEVD-R110 substrate was measured at an excitation wavelength of 498 nm and an emission wavelength of 521 nm with use of a microplate spectrofluorometer (Wallac Victor$^2_{TM}$ 1420 Multilabel Counter, USA).

Determination of intracellular ROS levels Intracellular ROS levels were measured with use of the fluorescent dye H_2DCFDA staining method[24]. H_2DCFDA is a nonpolar compound that is converted into a nonfluorescent polar derivative (H_2DCF) by cellular esterases after incorporation into cells. H_2DCF is membrane impermeable and rapidly oxidized to the highly fluorescent 2,7-dichlorofluorescein (DCF) in the presence of intracellular ROS[25]. Cells cultured on glass coverslips in every group were incubated for 30 min at 37 ºC with H_2DCFDA 20 μmo/L dissolved in PBS. Cover slips were then washed three times with PBS and analyzed under a confocal laser microscopy (Leica, Germany). DCF was excited at 488 nm, and the emission filter was a 510 nm barrier filter. The fluorescent intensity and confocal laser microscopic images were held constant to allow for comparison of relative fluorescence intensities between control and experimental cells.

Mitochondrial membrane potential assay The mitochondrial membrane potential of cells was measured with use of the probe JC-1[26]. JC-1 is able to enter mitochondria selectively, which appears green at low concentrations or at low membrane potential as a monomer. However, at high concentrations, mitochondria shows as red fluorescent aggregates. JC-1 is sensitive to mitochondrial membrane potential and the changes in the ratio between green and red fluorescence can provide information regarding the mitochondrial membrane potential. After the treated cells were loaded with JC-1 1 μmol/L for 10 min at 37 ºC, the fluorescent dye was excited at 490 nm, and the fluorescence intensities of both monomer and aggregated molecules were recorded at 590 nm under a confocal scanning laser microscopy.

$[Ca^{2+}]_i$ measurements The free intracellular calcium concentration ($[Ca^{2+}]_i$) of SHSYY cells was determined with the probe Indo-1 by following the method described by Rego *et al*[27]. After TNFα treatment (in the presence or absence of tubuloside B), SHSYY cells were loaded with 3 μmol Indo-1/AM in MEM for 45 min, at 37 ºC and further incubated for 15 min in MEM to allow the hydrolysis of the acetoxymethylester precursor of the probe. After rinsing with sodium saline solution (with 1.5 mmol/L $CaCl_2$), cell fluorescence was measured at 37 ºC in a luminescence spectrometer, with excitation at 335 nm and emission at 410 nm. The $[Ca^{2+}]_i$ was calculated according to the equation:

$[Ca^{2+}]_i$=250 nmol/L×[(F-F_{min})/(F_{max}-F)] (nmol/L), where 250 nmol/L corresponds to the dissociation constant of the complex Indo-1–Ca^{2+}, F_{max} is the maximal fluorescence obtained upon addition of 3 ¦Ìmol/L ionomycin, and F_{min} is the minimal fluorescence determined following the equation: F_{min}=AF+1/12(F_{max}-AF), where AF is the autofluorescence obtained upon addition of 3 mmol/L $MnCl_2$.

Statistical analysis Results were expressed as mean±SD of triplicate values for each experiment. Statistical comparisons involved use of the Student's *t*-test. $P<0.05$ was considered to be statistically significant.

RESULTS

Effect of tubuloside B on cell nuclear morphology The Hoechst 33342 staining that is sensitive to DNA was used to assess changes in nuclear morphology following tubuloside B and TNFα treatment. The nuclei in normal cells were normal and exhibited diffused staining of the chromatin (Fig 1A). However,

after exposure to TNFα 100 μg/L for 36 h, SH-SY5Y cells underwent typical morphologic changes of apoptosis such as condensed chromatin and shrunken nucleus (Fig 1B). A marked decrease was observed in the cells pretreated with tubuloside B (Fig 1C-1E).

Neuroprotective effect of tubuloside B on cell viability After incubation with TNFα, approximately 45.6 % of the cells underwent apoptosis death. Pretreatment with tubuloside B (1, 10, or 100 mg/L) decreased the cell death rate in a dose-dependant manner (cell death rate was 30 %, 19.5 %, and 6.2 %, respectively; Fig 2), but tubuloside B alone did not cause any apparent cytotoxicity (data not shown). .

Effect of tubuloside B on DNA fragmentation After exposure of SH-SY5Y cells with TNFα for 36 h, typically pronounced DNA laddering was observed.

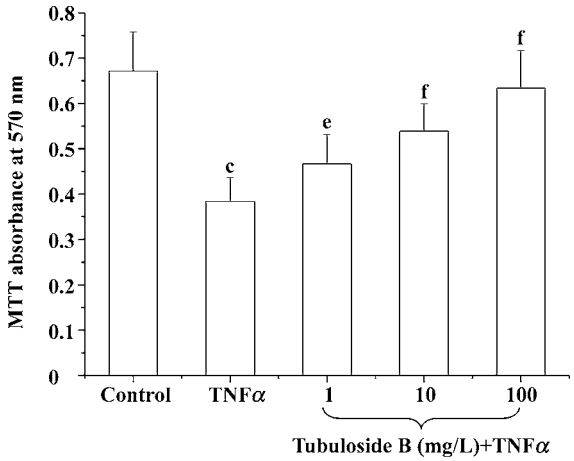

Fig 2. Effect of tubuloside B on TNFα-induced decrease in SH-SY5Y cell viability. *n*=8. Mean±SD. $^{c}P<0.01$ *vs* control cells. $^{e}P<0.05$, $^{f}P<0.01$ *vs* TNFα.

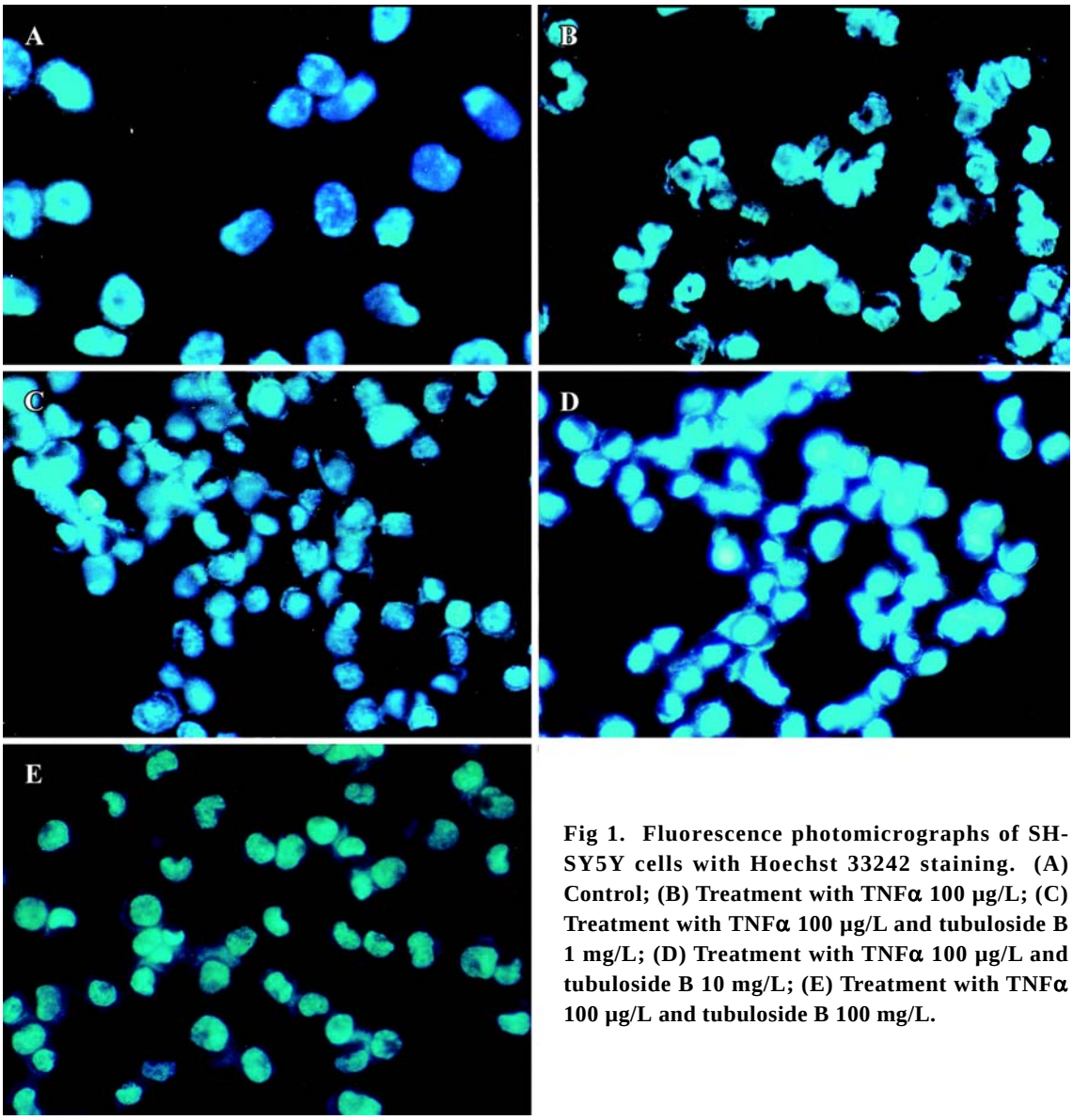

Fig 1. Fluorescence photomicrographs of SH-SY5Y cells with Hoechst 33242 staining. (A) Control; (B) Treatment with TNFα 100 μg/L; (C) Treatment with TNFα 100 μg/L and tubuloside B 1 mg/L; (D) Treatment with TNFα 100 μg/L and tubuloside B 10 mg/L; (E) Treatment with TNFα 100 μg/L and tubuloside B 100 mg/L.

However, pretreatment cells with tubuloside B inhibited TNFα-mediated DNA laddering, especially at the concentration of 100 mg/L, which completely inhibit DNA fragmentation (Fig 3).

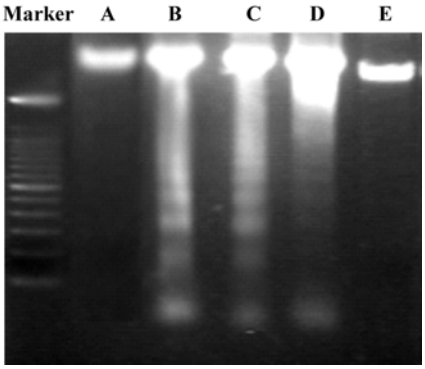

Fig 3. Effect of tubuloside B on TNFα-induced DNA fragmentation in SH-SY5Y cells. A) Control; B) Treatment with TNFα 100 μg/L; C) Treatment with TNFα 100 μg/L and tubuloside B 1 mg/L; D) Treatment with TNFα 100 μg/L and tubuloside B 10 mg/L; E) Treatment with TNFα 100 μg/L and tubuloside B 100 mg/L.

Effect of tubuloside B on phospholipid phosphatidylserine exposure The antiapoptotic action of tubuloside B was also confirmed by measuring the presence of phosphatidylserine on the outer cell membrane. In the control, about 96.3 % of the cells are healthy. Fig 4 showed the results of annexin-V binding experiments to measure the presence of phosphatidylserine on the outer cell membrane. The percentage of apoptosis was 37.5 % after TNFα treatment (Fig 4B), but the proportion decreased to 28.2 %, 19.29 %, and 7.9 % with 1, 10 or 100 mg/L tubuloside B pretreatment, respectively (Fig 4C-4E).

Inhibitory effect of tubuloside B on ROS formation In the present study, typical confocal laser micrographs were obtained from SH-SY5Y cells for 36 h in the absence or presence of TNFα and tubuloside B. The level of ROS generated in TNFα-induced cells increased markedly compared with controls (Fig 5B). However, the fluorecent intensity in tubuloside B-treated cells decreased by 16.2 %, 56.4 % and 89.7 % with 1, 10, or 100 mg/L tubuloside B treatment compared with TNFα-treated cells, respectively (Fig 5C-5E).

Effect of tubuloside B on mitochondrial membrane potential Untreated control cells exhibited numerous, brightly staining mitochondria that emitted red-orange fluorescence and red/green-ratio was

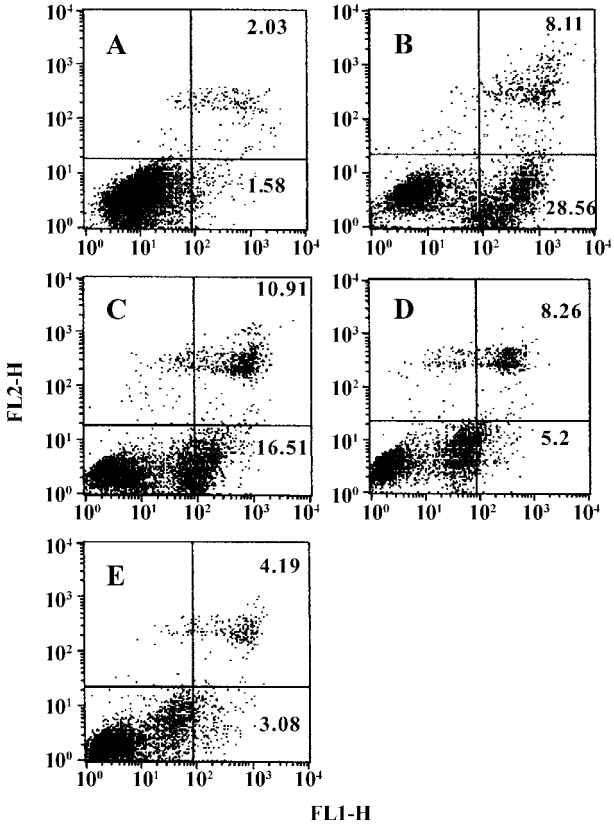

Fig 4. Flow cytometric histograms of control SH-SY5Y cells and cells exposed to TNFα alone or with tubuloside B. (A) Control cells; (B) Treatment with 100 μg/L TNFα; (C) Treatment with 100 μg/L TNFα and 1 mg/L tubuloside B; (D) Treatment with 100 μg/L TNFα and 10 mg/L tubuloside B; (E) Treatment with 100 μg/L TNFα and 100 mg/L tubuloside. Healthy cells are in the bottom left quadrant, apoptotic cells in the bottom right quadrant, and necrotic and late apoptotic cells in the upper right. Numbers in each quadrant are percentage of cells they contain.

5.97±0.21, which was indicative of normal high membrane potential (Fig 5A). TNFα-treatment induced a transition in mitochondria permeability and a significant loss of membrane potential (red/green ratio was 0.35±0.02) (Fig 5B). Tubuloside B treatment inhibited the collapse of mitochondrial membrane potential induced by TNFα in SH-SY5Y cells with increasing dosage. Tubuloside B gradually resumed the mitochondrial membrane potential with increasing concentrations as indicated via the reappearance of red mitochondrial staining (Fig 6C-6E).

Effect of tubuloside B on caspase-3 activity Caspase-3 activity was increased by about five-fold compared with controls after TNFα exposure (Fig 7). In contrast, SH-SY5Y cells which were simultaneously

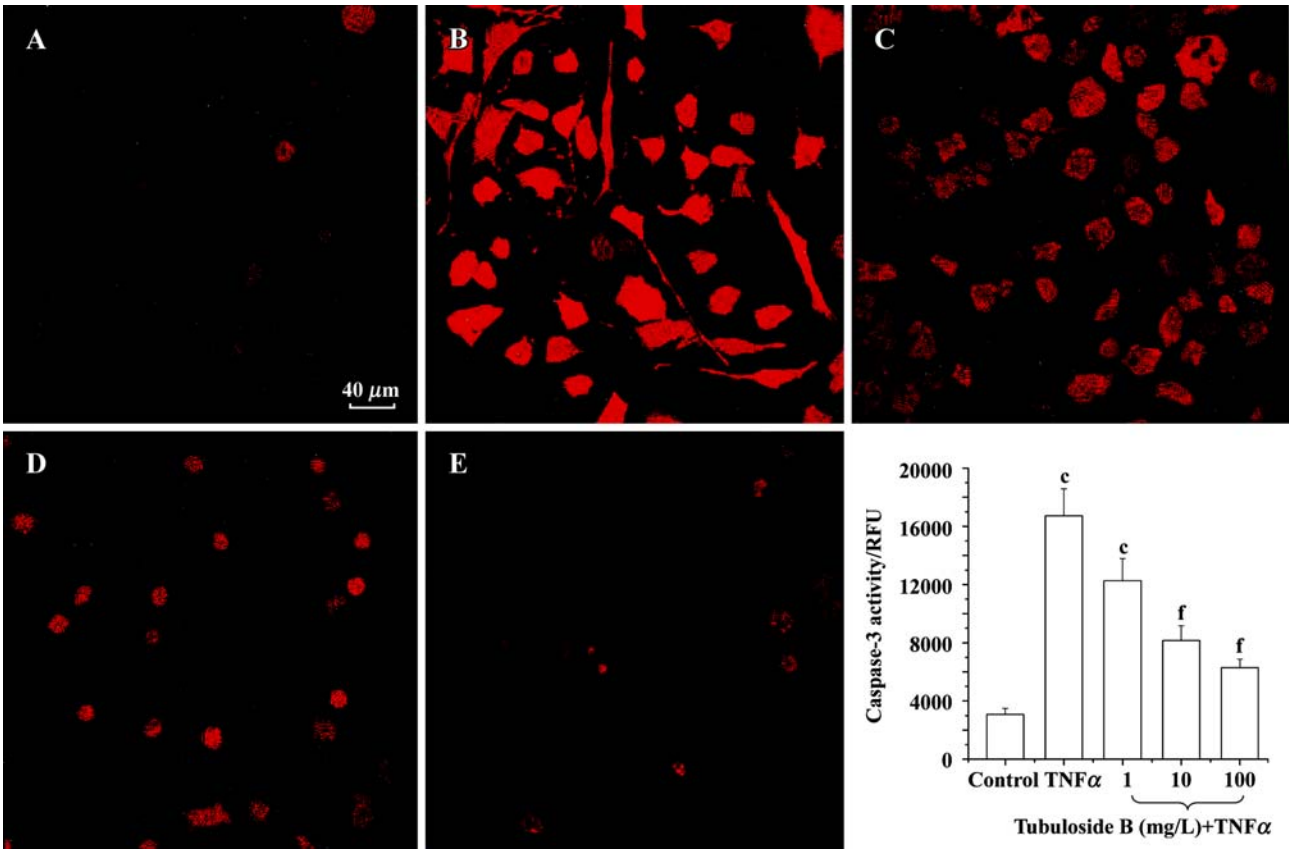

Fig 5. Effect of tubuloside B on level of intracellular ROS in TNFα-induced SH-SY5Y cells. (A) Control cells; (B) Treatment with 100 μg/L TNFα; (C) Treatment with 100 μg/L TNFα and 1 mg/L tubuloside B; (D) Treatment with 100 μg/L TNFα and 10 mg/L tubuloside B; (E) Treatment with 100 μg/L TNFα and 100 mg/L tubuloside B. *n*=8. Mean±SD. $^{c}P<0.01$ *vs* control cells; $^{e}P<0.05$, $^{f}P<0.01$ *vs* TNFα.

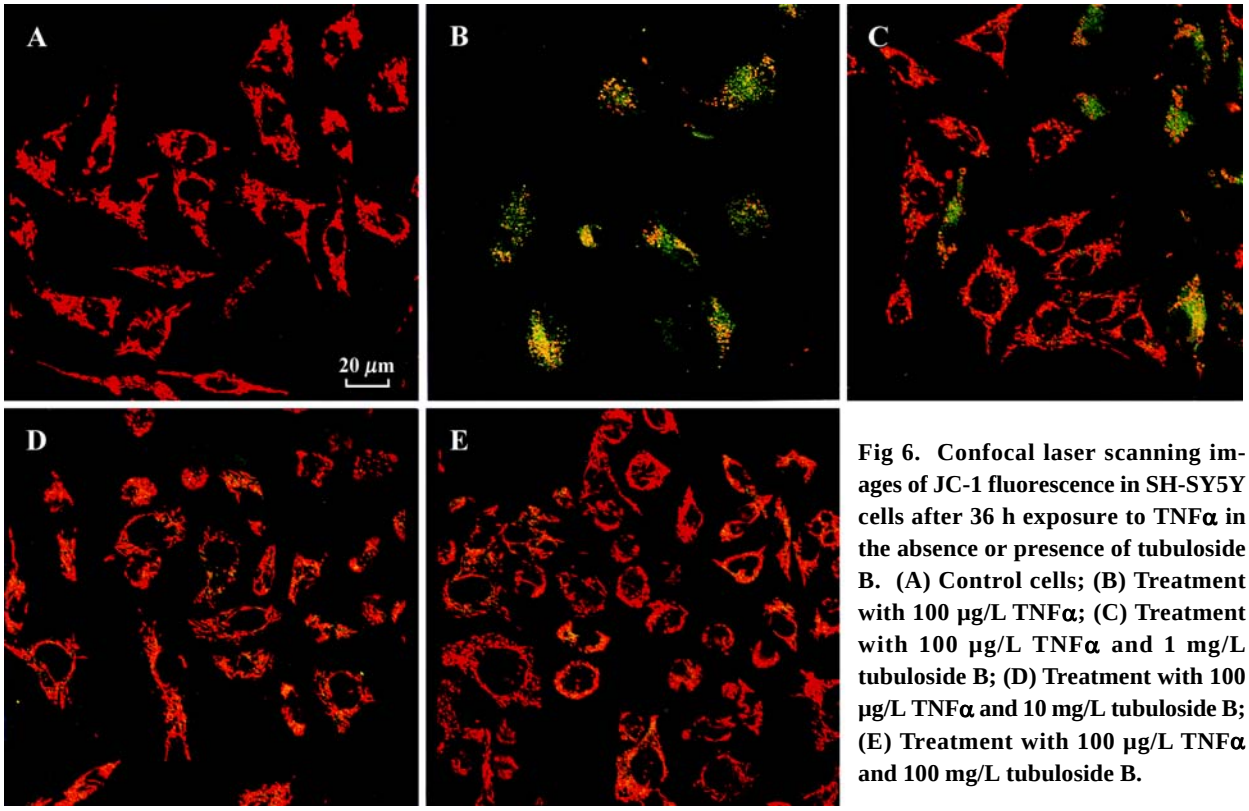

Fig 6. Confocal laser scanning images of JC-1 fluorescence in SH-SY5Y cells after 36 h exposure to TNFα in the absence or presence of tubuloside B. (A) Control cells; (B) Treatment with 100 μg/L TNFα; (C) Treatment with 100 μg/L TNFα and 1 mg/L tubuloside B; (D) Treatment with 100 μg/L TNFα and 10 mg/L tubuloside B; (E) Treatment with 100 μg/L TNFα and 100 mg/L tubuloside B.

pretreated with tubuloside B 1, 10, or 100 mg/L showed a significant decrease in caspase-3 activity compared with TNFα-Ctreated cells at the same time point (Fig 7).

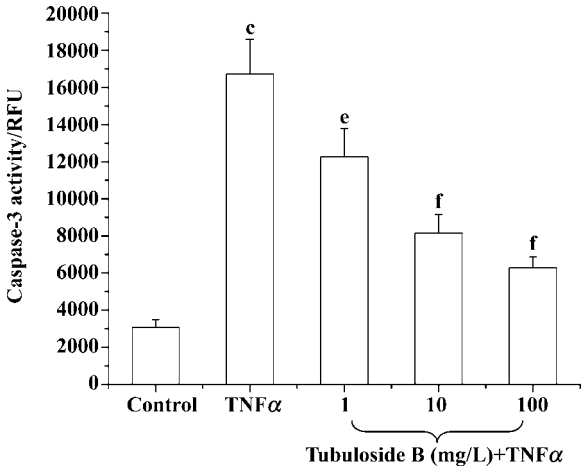

Fig 7. Effect of tubuloside B on TNFα-induced increase of caspase-3 activity. *n*= 8. Mean±SD. $^{c}P<0.01$ *vs* control cells. $^{e}P<0.05$, $^{f}P<0.01$ *vs* TNFα.

Effect of tubuloside B on the level of $[Ca^{2+}]_i$

The level of $[Ca^{2+}]_i$ in TNFα-induced cells increased markedly compared with controls (control: 105.5±11.2 nmol/L; 100 μg/L TNFα: 238.4±18.9 nmol/L), which was partially reduced by pretreated with tubuloside B (10 or 100 mg/L) (Fig 8).

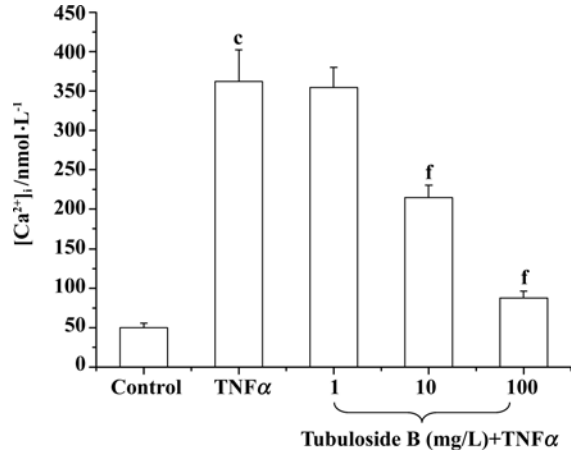

Fig 8. Determination of $[Ca^{2+}]_i$ in TNFα-treated SH-SY5Y cells in the absence or presence of tubuloside B. The fluorescence of the probe Indo-1/AM was monitored and the $[Ca^{2+}]_i$ was calculated following determination of F_{max} and AF. *n*= 8. Mean±SD. $^{c}P<0.01$ *vs* control cells. $^{f}P<0.01$ *vs* TNFα.

DISCUSSION

Cistanches salsa (CA Mey) G Beck, one species of *Cistanches* which belong to *Orobanchaceae* family, is a parasitic plant native in the northwest of China. The stem of this plant is an important traditional Chinese medicine and used for kidney deficiency, female infertility, morbid leucorrhea, neurasthenia, and senile constipation due to colonic inertia. The major active constituents of this herb are phenylethanoid glycoside[28]. In recent years, results of several studies highlighted the function of tubuloside B in promoting various pharmacological and biological activities[13-15]. However, the cellular and molecular mechanisms that underlie the actions are not fully understood. The present study demonstrated that tubuloside B had significant neuroprotective effects on TNFα-induced apoptosis in SH-SY5Y neuronal cells by maintaining mitochondrial function, decreasing the generation of ROS, reducing the level of intracellular calcium and inhibiting caspase-3 activity through an antioxidation mechanism. These mechanisms may be through the individual neuroprotective effect of tubuloside B or through its interaction with other factors and then may lead to a decreased ratio of apoptosis in cells.

Previous studies have shown that oxidants or prooxidants are important regulators of apoptosis and can induce apoptosis[29,30]. Oxidative stress is a common element of apoptosis induced by various stimuli such as TNFα and environmental toxin exposure, which usually do not exert a direct oxidant action. The central role for oxidative stress in apoptosis is strongly supported by the ability of various cellular antioxidants to block apoptosis induced by diverse agents[31]. Compounds with antioxidant properties could have a protective effect in different situations of cellular dysfunction through the scavenging of free radicals[32]. Moreover, several phenylpropanoid glycosides was reported to possess free radical scavenging properties and protect oxidative stress-induced toxic injuries. In line with this idea, we found that tubuloside B decreased the level of ROS induced by TNFα, it might protect neurons against apoptosis by directly scavenging intracellular reactive oxygen species.

Much evidence suggested that major alterations in mitochondrial function were critically involved in the apoptotic process[33]. Disorders of calcium homeostasis and alterations of mitochondrial membrane potential was found to promote the opening of the mitochondrial permeability transition pore (MPTP), or induce cyto-

chrome c release through MPTP-independent mechanisms, being directly response for the activation of the apoptotic cascade and may precede nuclear signs of apoptosis[34]. These biochemical changes may result from alternations in the function of mitochondria[35]. The facts that tubuloside B inhibited the reduction of mitochondrial membrane potential and reduced the rise in intracellular clcium induced by TNFα suggest that tubuloside B may have the capacity to counteract the toxicity of TNFα by inhibiting the opening of MPTP and suppressing the dysfunction of mitochondria.

Outer stimuli can initiate apoptosis through the above mechanisms and may converge on the caspase pathway to execute the final phase of the apoptotic process[36]. The caspase family of proteases consists of at least 14 mammalian members that are constitutively expressed in almost all cell types as inactive proenzymes (zymogens) that become processed and activated in response to a variety of pro-apoptotic stimuli[37]. Caspase-3 is a downstream member of the caspase cascade and acts as a central effector in the execution phase. When caspase-3 precursor protein CPP32 is activated by upstream signals such as the release of mitochondrial cytochrome c, the active caspase-3 cleaves specific aspartate residues in proteins with various structural, housekeeping and regulatory functions[38-40]. These proteolytic events can lead to cell apoptosis and contribute to DNA fragmentation and nuclear morphologic changes. Thus, substances that can inhibit the activity of caspase-3 might protect cells from apoptosis[2,31]. Since tubuloside B markedly inhibited the caspase-3 activity in the TNFα-treated cells, it has the neuroprotective capacity.

In conclusion, tubuloside B had a multifunctionally protective effect on damaged neurons. Because of its powerful anti-apoptosis and antioxidative stress activities, it might be for clinical use in neurodegenerative and neurologic disabilities involving neuron apoptosis.

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