

RESEARCH ARTICLE

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Danggui Buxue Tang, an ancient Chinese herbal decoction, protects β -amyloid-induced cell death in cultured cortical neurons

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Abstract

Background: Danggui Buxue Tang (DBT) is a historical Chinese herbal decoction, and which has more than 800 years of applications. This herbal decoction solely contains two materials: Astragali Radix (AR) and Angelicae Sinensis Radix (ASR) at a weight ratio of 5:1. Clinically, DBT aims to improve anemia syndrome. In complementary and alternative medicine theory, the cause of neurodegenerative disease is proposed to be related with anemia. In line to this notion, low levels of hemoglobin and red blood cell have been reported in patients suffering from Alzheimer's disease (AD), a chronic neurodegenerative disease caused by β -amyloid peptide (A β) accumulation. Therefore, we would like to probe the neuroprotective functions of this ancient herbal formula in vitro.

Method: The neuroprotective effects of DBT in the A β -induced cell death were detected in cultured cortical neurons by multiple techniques, i.e. confocal and western blot.

Results: In the cultures, application of DBT reduced A β -induced apoptosis rate in a dose-dependent manner. In A β -treated cortical neurons, the expression ratio of Bcl2 to Bax was altered by DBT. In parallel, application of DBT markedly suppressed the A β -induced expressions of apoptotic markers, i.e. cleaved-caspase 3/9 and PARP.

Conclusion: Taken these results, DBT shows promising protective effects against A β -induced stress or insult in cultured neurons.

Keywords: Danggui Buxue Tang, Alzheimer's disease, Complementary and alternative medicine

Background

Neuron is the basic unit in nervous system. Its major functions are to receive incoming information and send signals to other neurons, muscles and glands. Neurons are delicate, and they are not capable of reproducing. Neurodegeneration is one type of brain diseases, resulting in progressive degeneration and/or neurons death. Alzheimer's disease (AD), classified as one type of neurodegeneration diseases, is an irreversible disorder by smashing memory and finally destroying the performance of simple tasks [1]. The hallmark of AD diagnosis

is the extracellular plaque deposition of β -amyloid peptide (A β) [2]. Slowing down the progression of brain deterioration in AD patients by herbal medicine is believed to be a possible treatment.

Traditional Chinese medicine has been used for thousands of years, which is also called complementary and alternative medicine. Indeed, brain problem is proposed to be related with anemia as believed in ancient theory [3, 4]. Anemia is generally caused by the dysfunction of erythropoietin (EPO) production, stimulating the proliferation and differentiation of erythroid precursor cells [5]. In line to this notion, low levels of hemoglobin and red blood cell have been reported to be correlated in AD patients [6, 7]. In parallel, Faux et al. (2014) found that low level of hemoglobin was associated with AD, as well

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as other cognitive impairments [8]. Astragali Radix (AR; root of *Astragalus memebanaceus* (Fisch.) Bunge var. *mongholicus* (Bunge) Hsiao) is one of herbal materials found predominantly in complementary and alternative medical decoction, and this herb is commonly used for enriching vital energy as well as stimulating EPO production [9, 10]. Astragaloside IV, one of major ingredients of AR, possesses neuroprotective effects during cerebral ischemia/reperfusion injury via anti-oxidation, anti-inflammation and anti-apoptosis [11–13]. Angelica Sinensis Radix (ASR; root of *Angelica sinensis* (Oliv.) Diel) is also called “female ginseng”: this herb protects cultured Neuro 2A cell against A β -triggered oxidative damage, i.e. reactive oxygen species (ROS), and which also rescues the membrane potential of mitochondrial transmembrane [14].

AR and ASR are two major herbs commonly used together to constitute herb-pair in complementary and alternative medical formulae [15, 16]. Danggui Buxue Tang (DBT), containing AR and ASR at the ratio of 5:1, is a simple herbal mixture, and this herbal mixture has been utilized for more than 800 years. DBT was recorded in *Neiwaishang Bianhuo Lun*, written by Li Dongyuan in AD 1247 [16, 17]. This ancient herbal formula is suggested to be consumed by patients who would like to improve the syndromes of anemia. The neuroprotective functions of AR and ASR were well demonstrated both in vitro and in vivo [18–21]. However, the neuroprotective function of DBT decoction has not been revealed yet. Hence, we are aiming to probe the neuroprotective effects of this ancient herbal decoction in cell models, as well as to reveal the underlying signaling pathway involved.

Materials and methods

Preparation of DBT decoction

The herbal materials of *A. memebanaceus* var. *mongholicus* (AR) and *A. sinensis* (ASR) were obtained and morphologically identified by Dr. Tina TX Dong (one of the author) in 2013. The voucher specimens of AR (# 02–10–04) and ASR (# 02–09–01) were recorded and displayed in Centre for Chinese Medicine of HKUST. To generate DBT formula, 5 parts of AR and 1 part of ASR were mixed well and boiled in water for twice, according to previous reports [16, 17]. The water extract of DBT was dried by lyophilization and re-suspended in water at 100 mg/mL final concentration. Before application, DBT was diluted to the required concentration in the cultured medium. The treatment of DBT in the A β -treated cultures was about 48 h, unless otherwise specified.

Fingerprint of DBT decoction

An Agilent 1200 series system (Agilent, Santa Clara, CA) was utilized here to determine the chemical compositions of DBT decoction. The Agilent 1200 series system was equipped with degasser, binary pump, auto-sampler, and

thermo-stated column compartment. Agilent, Eclipse Plus, C₁₈ column (4.6 × 250 mm, 5 μ m) with acetonitrile (as Solvent A) and 0.01% formic acid (as Solvent B). The detailed HPLC condition was described before [10].

Primary cell culture

The experimental procedures were approved by The Animal Experimentation Ethics Committee of the Hong Kong University of Science and Technology (No. 17–283 for Animal Ethics Approval) and under the guidelines of “Principles of Laboratory Animal Care” (NIH publication No. DH/HA&P/8/2/3). Primary cultured cortical neurons were cultured as described previously with slightly modifications [18, 22]. Cortex was dissected by trypsin from embryonic day 18 rats by 100 mg/kg of Ketamine and 10 mg/kg of Xylazine treatment. The dissociated cortical neurons were cultured in neural basal medium with B27 and 0.5 mM GlutaMax at 37 °C incubator supplying with 5% CO₂. Cells were cultured for 14 days before treatments. Reagents for cell cultures were purchased from Invitrogen Technologies (Carlsbad, CA).

Cell viability assay

MTT assay was used for analysis cell viability (Sigma-Aldrich, St. Louis, MO). In brief, cells were seeded in 96-well plate and then performed drug treatment for 48 h. After that, MTT solution was added into the cultures and incubated for 2 h, the purple crystal production was dissolved by DMSO solvent and detected at 570 nm absorbance. In crystal violet assay, cells were seeded and cultured in 6-well plate. Colonies were fixed with an ice-cold methanol-acetic acid solution (3:1) for 30 min at –20 °C and stained with 1% crystal violet for 30 min at room temperature and visualized.

Western blot

The protein expressions of Bcl2, Bax, cleaved-PARP, cleaved-caspase 3/9 and β -actin were revealed by western blot. Cultures were seeded onto 6-well plate. After drug treatment, the cultures were harvested in high salt lysis buffer (1 M NaCl, 10 mM HEPES, pH 7.5, 1 mM EDTA, 0.5% Triton X-100). Before conducting SDS-PAGE analysis, equal amount of protein level were adjusted with 2X lysis buffer (0.125 M HCl, pH 6.8, 4% SDS, 20% glycerol, 2% 2-mercaptoethanol and 0.02% bromophenol blue). The membranes were incubated with anti-Bcl2, Bax, cleaved-PARP and cleaved-caspase 3/9 (CST, Danvers, MA) at 1: 1000 dilutions, respectively, and anti- β -actin (Abcam, Cambridge, MA) at 1: 10,000 dilutions were kept for over 12 h at cold room. Horseradish peroxidase (HRP)-conjugated secondary antibodies were employed at 1: 5000 dilutions for 3 h at room temperature, the immune-complexes were visualized by the enhanced ECL method (Amersham Biosciences, Marlborough, MA).

Caspase-3/9 activity assay

All processes were instructed following the protocol of caspase-3/9 assay kit (Biovision, Milpitas, CA). Olympus Fluoview FV1000 laser scanning confocal system (Olympus America Inc., Center Valley, PA) at 400 nm excitation and 505 nm emission was employed here for photosensitive reaction measurement. The fold of changes was calculated relative to the untreated cells.

PI and Annexin V assay

Cells were plated and cultured in 24-well plates. After drug treatment for 48 h and labeled with Annexin V-FITC/PI apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ) as followed manufacturer's instructions. In brief, 100 μ L of binding buffer containing annexin-V/FITC and propidium iodide (PI) were used for covering cells for 15 min at room temperature in the dark condition and then detected by the Olympus Fluoview FV1000 laser scanning confocal system.

Statistical analysis and other assays

Protein levels were measured by Bradford's method (Hercules, CA). Statistical tests have been done by using one-way analysis of variance. Data were expressed as Mean \pm SEM, where $n = 3-4$. Statistically significant changes were classified as significant (*) where $p < 0.05$, more significant (**) where $p < 0.01$ and highly significant (***) where $p < 0.001$ as compared with control group. Statistically significant changes were classified as significant (^) where $p < 0.05$, more significant (^^) where $p < 0.01$ and highly significant (^^^) where $p < 0.001$ as compared with A β group.

Results

DBT attenuates A β -induced cell death

Prior to the biochemical analysis of any herbal extract, it is indispensable to perform chemical composition analysis. The HPLC chromatogram of DBT water extract was shown here (Fig. 1). The accurate amounts of each major chemicals were calibrated by a calibration curve, which was obtained from a series of different concentration of the chemical markers. The calibration curve of ferulic acid was $y = 21.134x + 19.607$; calycosin was $y = 10.189x - 10.129$; formononetin was $y = 13.602x + 12.705$. After calibrated by the standard curve, 1 g qualified DBT water extract contained 809.44 μ g of ferulic acid, 693.19 μ g of calycosin and 164.58 μ g of formononetin. The cyto-protective functions of DBT against the A β -induced cell death in primary cultured neurons were analyzed. Application of A β in cultured cortical neurons provoked cell death obviously in a dose-dependent manner (Fig. 2a). From the results, the death rate was $\sim 60\%$ under the challenging of 20 μ M of A β (Fig. 2a). The treatment of DBT reduced the A β -induced cell death in a concentration-dependent

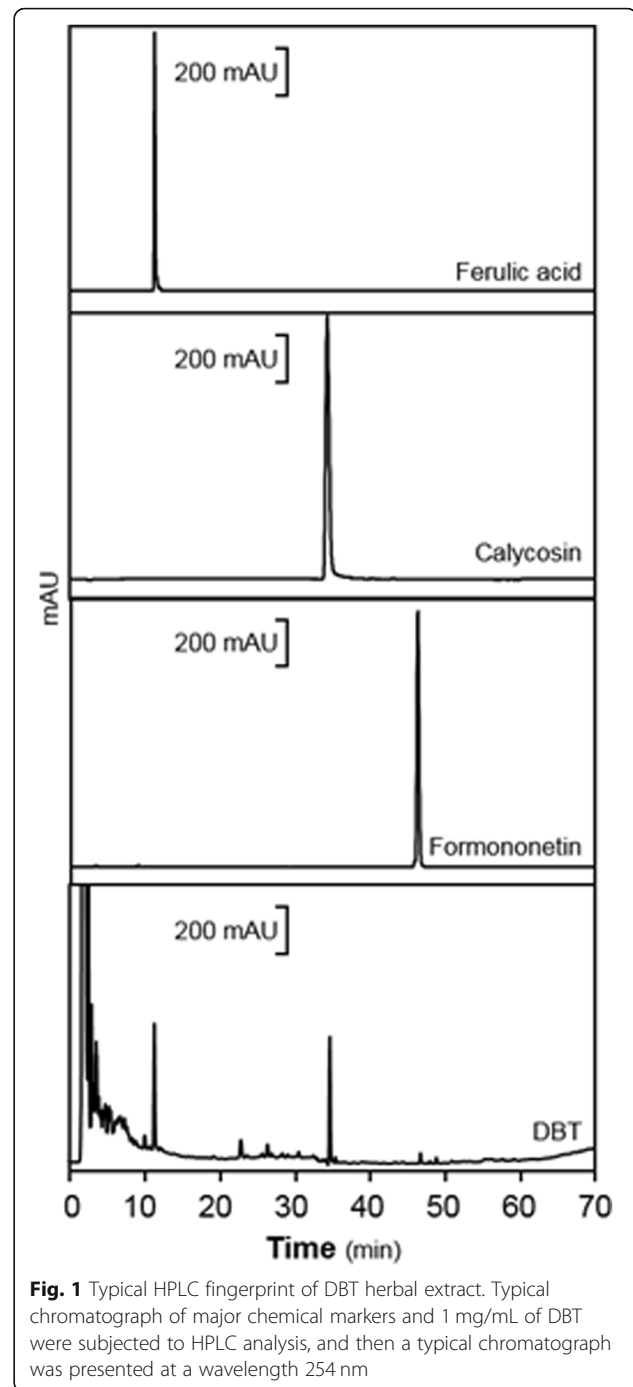
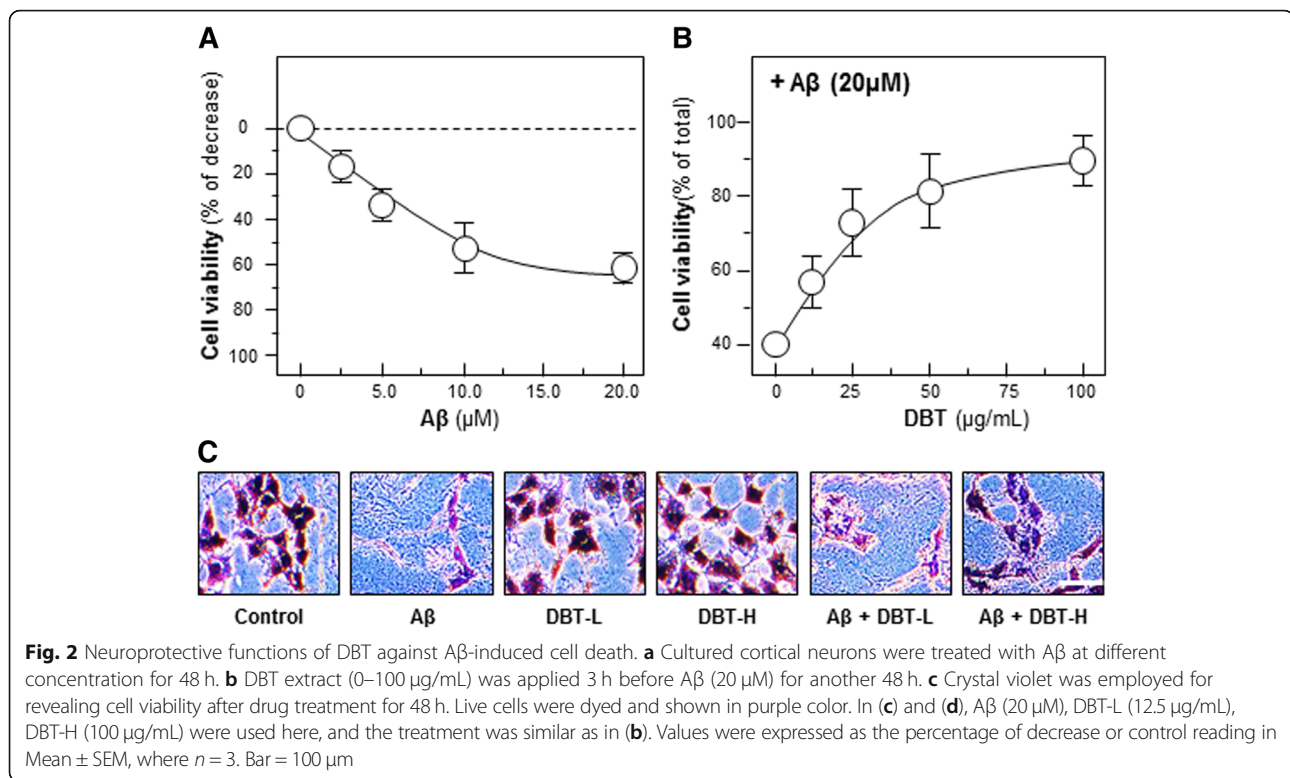


Fig. 1 Typical HPLC fingerprint of DBT herbal extract. Typical chromatograph of major chemical markers and 1 mg/mL of DBT were subjected to HPLC analysis, and then a typical chromatograph was presented at a wavelength 254 nm

manner (Fig. 2b). Higher dose of DBT significantly protected cells from the injury: the maximum protection was able to recover cell number up to over 80% at 0.1 mg/mL (Fig. 2b). We also utilized crystal violet to determine cell viability, directly. The death cells lose their adherence, and finally resulted in reducing amount of crystal violet staining in cultures [23]. The DBT-treated cells remained healthy even after the challenge of A β (Fig. 2c). The sensitive apoptotic markers, Annexin V- and PI, were measured



by fluorescent microscope. The A β -treated cells had a higher apoptotic rate of PI and Annexin V, as compared to the blank (Fig. 3). The ratio of Annexin V(+)/PI(+) of blank was 19.5%, positive treatment was 43.7%, lower concentration of DBT application (DBT-L: 12.5 μ g/mL) was 19.6%, higher concentration of DBT challenging (DBT-H: 100 μ g/mL) was 11.4%, the cotreatment of herbal decoction and A β were 41.6 and 21.8%, respectively (Fig. 3). The treatment of DBT suppressed the A β -induced cell apoptosis, dramatically (Fig. 3).

The aforementioned results showed that DBT could protect cell death against A β challenge.

DBT protects cells from apoptosis

Apoptosis is a programmed cell death, and inhibitors of apoptosis, e.g. Bcl2, and inducers of apoptosis, e.g. Bax, have been reported and identified [24]. The protein ratio of Bcl2 to Bax gives an index to the cell survival or death, after an apoptotic stimulus [25]. Here, we utilized western blot to reveal the protein expressions of two apoptotic markers in cultured cortical neurons, i.e. Bcl2 (~27 kDa) and Bax (~21 kDa) (Fig. 4). The application of A β significantly up-regulated the expression of Bax by over 3-fold and down-regulated Bcl2 by over 50%, as compared to the negative control (Fig. 4). Thus, A β markedly increased the ratio of Bax/Bcl2. Application of DBT in the cultures was capable to reverse the expressions of A β -induced cell apoptotic markers in a concentration-

dependent manner (Fig. 4). More importantly, DBT decreased the ratio of Bax/Bcl2 obviously, as compared to the A β -treated group (Fig. 4), which suggested that this herbal decoction possessed neuroprotective effects in AD cell model.

Caspases are a group of protease enzymes acting as important roles in manipulating cell apoptosis [26]. For example, the activation of caspases triggers degradation of cellular components in a programmed manner, which results in cell death [26]. In cultured cortical neurons, the amounts of cleaved-caspases 3, 9 and PARP were much higher in the A β -treated group, as compared to the negative control (Fig. 5). Challenging of DBT, on the other hand, suppressed the expressions of these cleaved-caspases in concentration-dependent manners (Fig. 5). The presence of high dose of DBT (0.1 mg/mL) in cultured cortical neurons decreased the levels of cleaved-PARP, caspase 3/9 at ~25%, ~50% and ~40% respectively, indicating the neuroprotective functions of DBT decoction (Fig. 5). Fluorescence signal, directly reflecting the amount of caspase activity in the cells, was also utilized and measured here [27]. In cultured cortical neurons, we found that the activities of caspases 3 and 9 were strongly activated in A β -treated group, as compared to others (Fig. 6). However, lower fluorescent signals were observed in DBT-treated group, and these results were fully in line with the western blotting results.

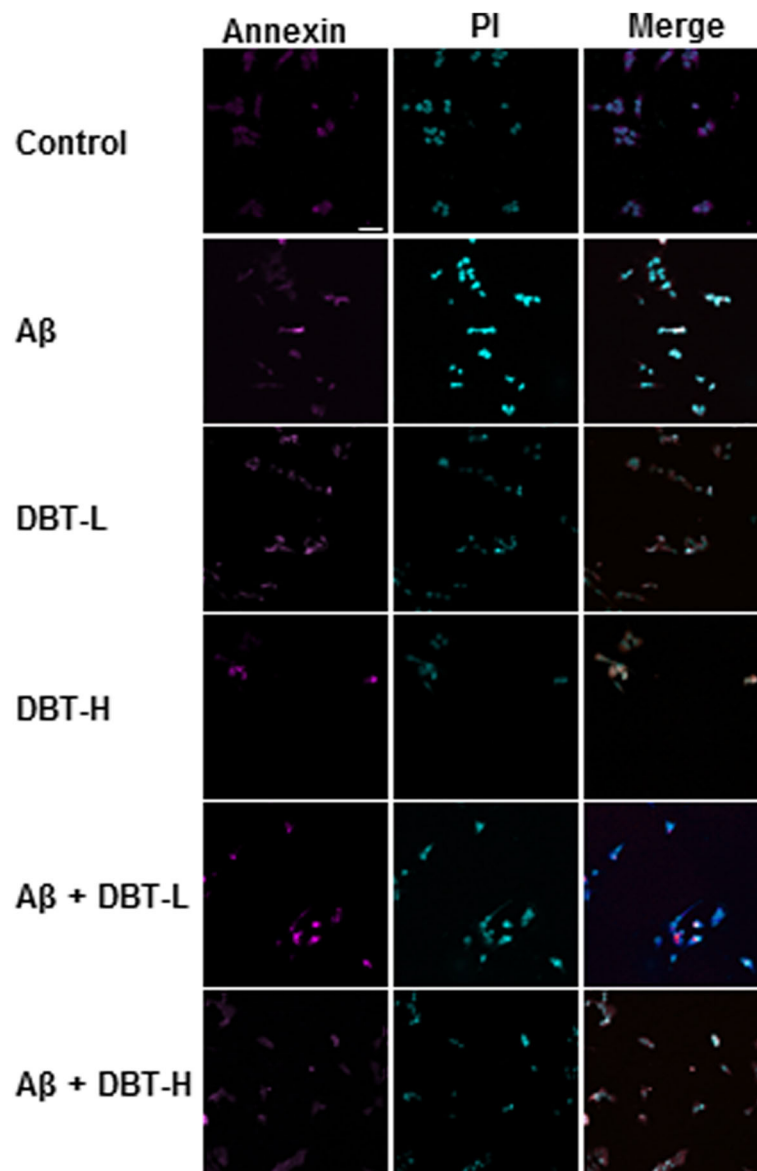


Fig. 3 DBT inhibits Annexin V and PI rates. Representative images of Annexin V-FITC and PI fluorescence staining were shown for the cell apoptosis after drug treatment for 48 h (A β : 20 μ M; DBT-L: 12.5 μ g/mL; DBT-H: 100 μ g/mL). Annexin V was visualized by purple signal, and PI was shown in blue color. Scale bar = 50 μ M

Synergistic effects of AR and ASR

To reveal that synergistic effect of AR and ASR in the pharmacological properties of DBT, the specific apoptosis markers were determined here. One hundred μ g of ASR significantly inhibited the expressions of Bax, cl-PARP and cl-caspase 9 in cultured neurons, as compared to the control. On the other hand, 100 μ g of AR was capable to suppress the expressions of cl-PARP, cl-caspase 3/9 activities (Fig. 7). However, the application of DBT dramatically declined Bax/Bcl2 ratio and other apoptosis marker expression levels, and the rate of decline was much stronger than the sum of single application of ASR and AR (Fig. 7).

Discussion

It is estimated that each year about 200,000 new cases of people younger than 65 years are suffering from AD, and the number is increasing, suggesting the younger onset of AD population. One of the targets for AD treatment is to prevent neuronal cell death. Being a neuroprotective agent, estrogen and its similar chemicals have drawn an attention, and they have been proven to enhance cognitive impairments both in vitro and in vivo [28]. Flavonoids, sharing the basic chemical structures with estrogen, also called phytoestrogen, are proposed to have neuroprotection, and which therefore are believed to pave a direction

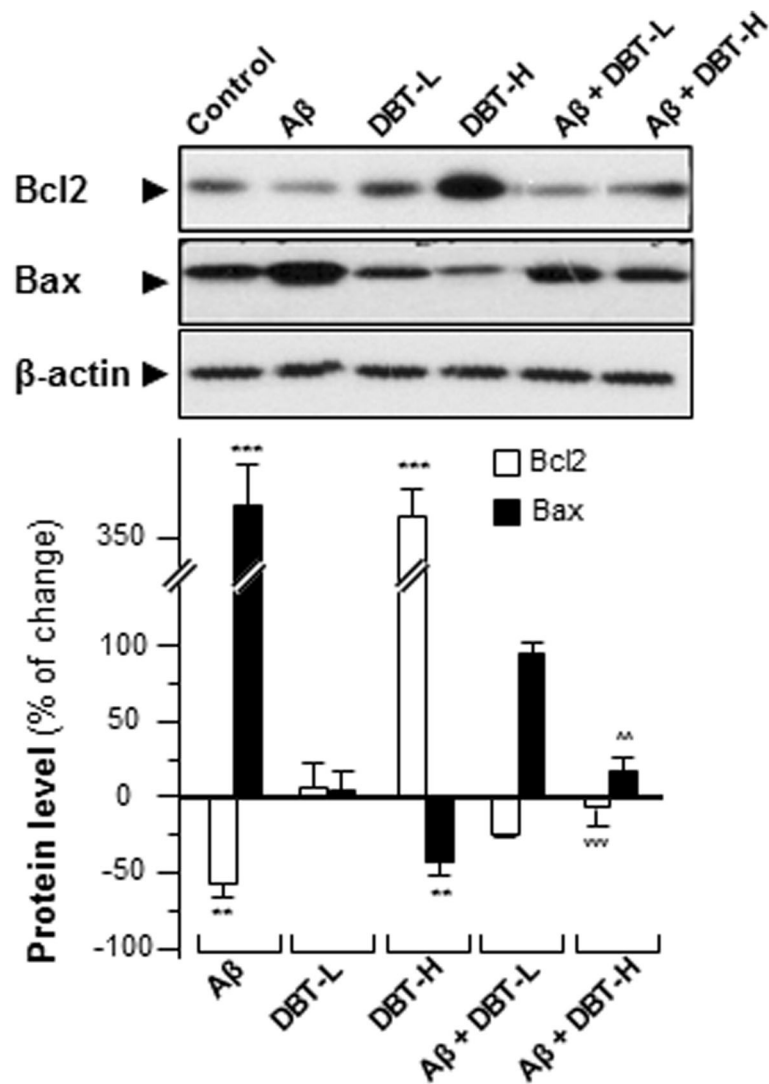


Fig. 4 DBT regulates expressions of Bcl2 and Bax. Cultured cortical neurons were co-treated with various concentration of DBT (DBT-L: 12.5 $\mu\text{g}/\text{mL}$; DBT-H: 100 $\mu\text{g}/\text{mL}$) and A β (20 μM) for 48 h. The protein expression levels of Bcl2 (~ 27 kDa) and Bax (~ 21 kDa) were detected by immunoblot analysis using specific antibodies, and β -actin (~ 42 kDa) served as an internal control. Quantification of target protein expression was calculated by a densitometer. Values were expressed as the percentage of change as compared to control reading, in Mean \pm SEM, where $n = 3$. Statistically significant changes were classified as more significant (**) where $p < 0.01$ and highly significant (***) where $p < 0.001$ as compared with control group. More significant (^^) where $p < 0.01$ and highly significant (^^^ where $p < 0.001$ are compared with A β group

for new drug development [19, 28, 29]. In line to this notion, DBT contains high amounts of flavonoids, and these flavonoids are classified as phytoestrogens [18, 19, 29]. Indeed, the flavonoids isolated from AR, i.e. calycosin and/or formononetin, are believed as an imperative and practical component found within this ancient herbal decoction [18]. To exert their biological functions in brain, flavonoids could cross the blood-brain barrier (BBB) before being delivered to the brain. BBB selectively restricts the passage of vast majority of small polar molecules and macromolecules from circulatory system to the brain [30]. Flavonoids, e.g. hesperetin, naringenin and their glucuronidated conjugates, have been shown

to have good permeability across the BBB in vitro [31]. The epicatechin glucuronide was found in rat brain tissue after oral administration of epicatechin [32]. Collectively, the possible BBB permeable flavonoids are promising candidates for neuroprotective drug development. Indeed, the glucuronidated flavonoids are found abundantly in DBT [16, 17]. However, the therapeutic effect of flavonoids, so far, is very limited, and longtime consumption of flavonoid has been reported to trigger mutation and generation of free radicals [33].

The working mechanism of A β -induced neuronal cell death remains controversial. Free radical's production and oxidative stress have been proposed to be ones of the

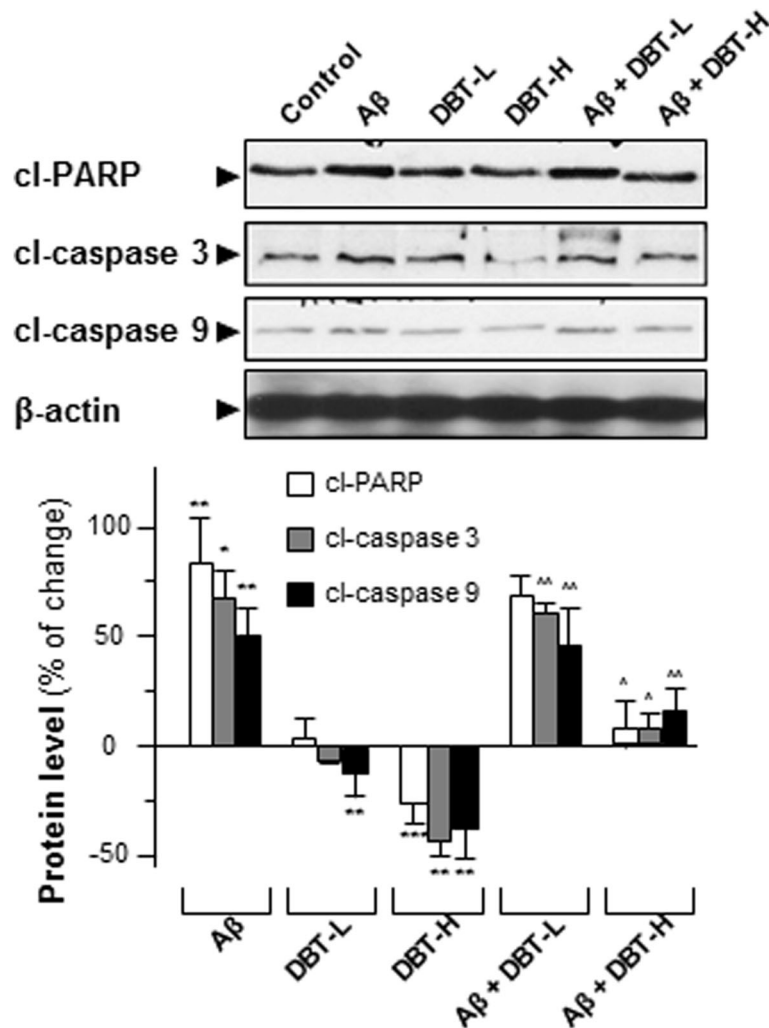
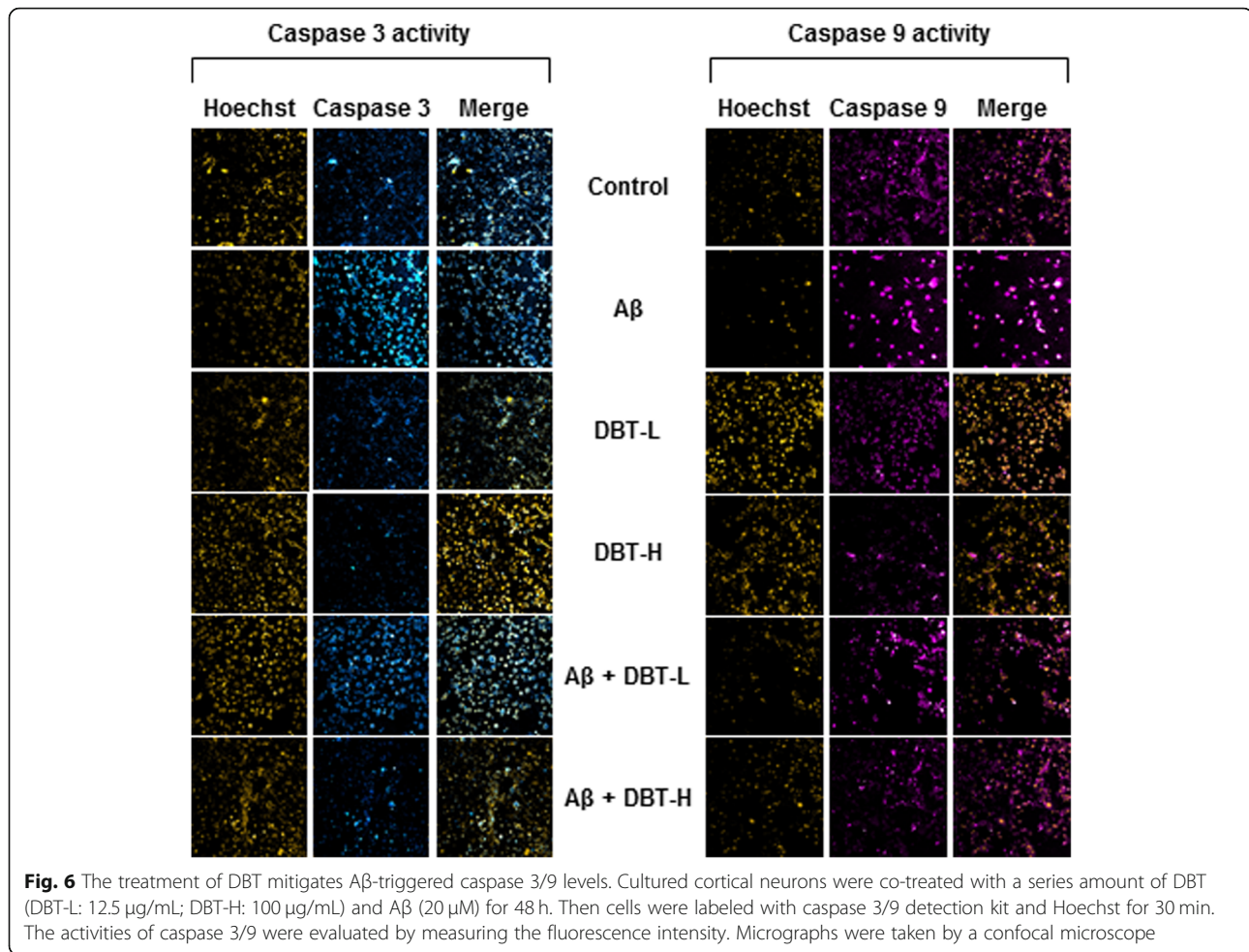


Fig. 5 DBT attenuates the A β -induced apoptosis markers. Cultured cortical neurons were co-treated with different amounts of DBT (DBT-L: 12.5 μ g/mL; DBT-H: 100 μ g/mL) and A β (20 μ M) or directly treated with A β or DBT alone for 2 days. The translational levels of cleaved-PARP (cl-PARP at ~ 85 kDa), cleaved-caspase 3 (cl-caspase 3 at ~ 17 kDa) and cleaved-caspase 9 (cl-caspase 9 at ~ 37 kDa) were detected by immunoblot analysis by specific antibodies. Beta-actin (~ 42 kDa) served as an internal control. Quantification of target protein expression was calculated by a densitometer. Values were expressed as the percentage of change as compared to control reading, in Mean \pm SEM, where $n = 3$. Statistically significant changes were classified as significant (*) where $p < 0.05$, more significant (**) where $p < 0.01$ and highly significant (***) where $p < 0.001$ as compared with control group. Significant (Δ) where $p < 0.05$ and more significant ($\Delta\Delta$) where $p < 0.005$ are compared with A β group

causes. It is proposed that the A β -induced neurotoxicity is triggered by destabilization of intracellular Ca $^{2+}$ and/or oxidative stress [2]. The apoptotic marker, Bcl-2, protects neurons against oxidative stress directly, and which maintains cellular Ca $^{2+}$ hemostasis [34]. Therefore, Bcl-2 and the related anti-death proteins are acting as key regulators in monitoring programmed cell death in neurons. Therefore, the disruptions of these proteins could lead to an increased neuronal vulnerability. Here, we have shown the neuroprotection of DBT against A β -induced cell death in cultured cortical neurons. The treatment of this ancient herbal decoction in cultures could dramatically suppress the amounts of Bax and cleaved-PARP, cleaved-caspase 3/9, as induced by A β , indicating that the DBT was capable

of attenuating A β -induced apoptosis. Moreover, the inhibitions of apoptotic markers, triggered by DBT, were much stronger than its herbal ingredients, suggesting that the boiling two herbs together at the ratio of 5:1 should be required. Furthermore, the in vivo data indicated that the formulation of AR and ASR at a 5:1 ratio was the most effective herbal decoction in triggering immune responses, enhancing formation of capillaries, up-regulating myocardial mitochondrial functions, as compared to AR or ASR herbal extract [17]. Higher pharmacological values of DBT than its ingredients could be explained by several possibilities. First, the AR-derived active components, i.e. saponins, may be able to enhance the pharmacological properties of active components from ASR. Second, the

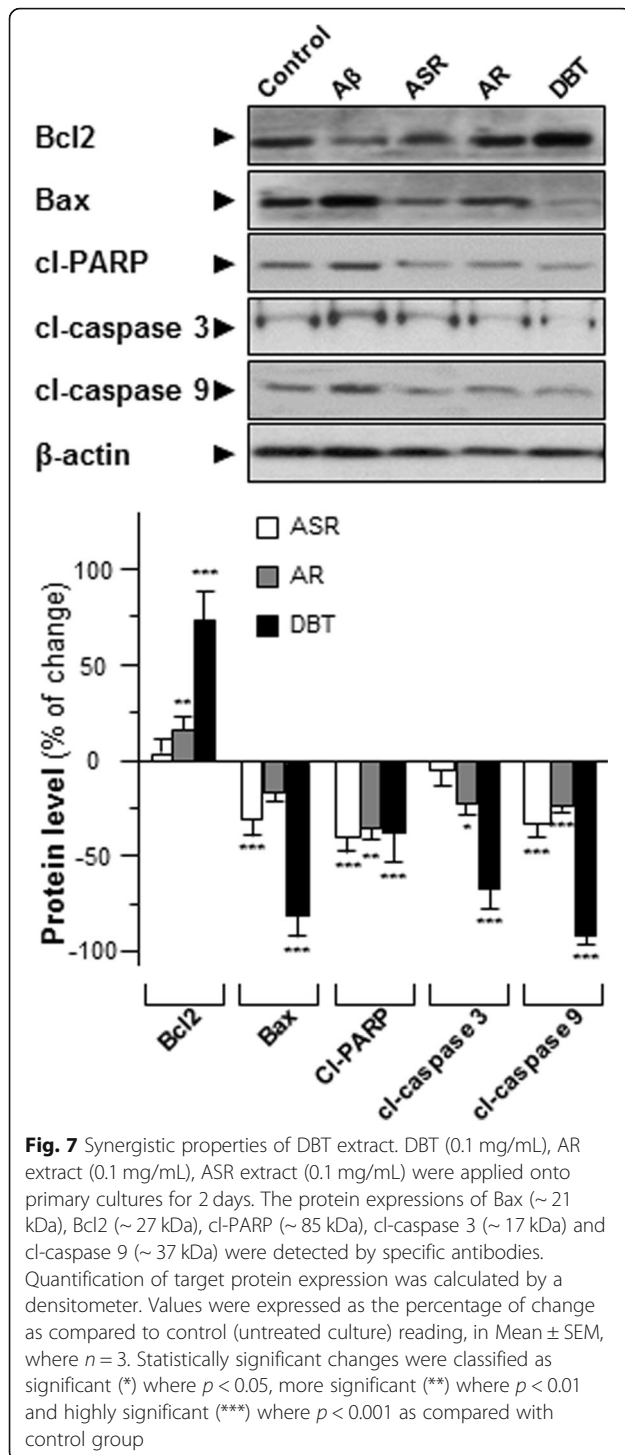


stability of active components could be prolonged by having a cocktail of DBT decoction [17]. Nevertheless, the pharmacological effects and the solubilities of active components within DBT are much stronger than that from AR or ASR extract.

The AR-derived calycosin, the most abundant flavonoid found within DBT decoction, was shown to induce nerve growth factor, glial-derived neurotrophic factor and brain-derived neurotrophic factor in cultured cortical neurons, and these inductions were in time- and dose-dependent manners [27]. This gene activation could be revealed similarly by the treatment of DBT [27]. By employing specific inhibitor of estrogen receptor, the activation of estrogen receptor was reported to be responsible for the calycosin-induced neurotrophic factor expressions [27]. In parallel, the *in vivo* rat middle cerebral artery occlusion model indicated that the treatment of calycosin could induce neuroprotection by up-regulating endogenous antioxidant and stabilizing the cells' membrane structures [35]. Besides, mechanistic studies have proposed that calycosin is the key player in DBT function, as to achieve the highest pharmaceutical values in terms of estrogenic, osteogenic,

erythropoietic and cardiovascular functions [10]. On other hand, AR-derived formononetin, and other isoflavonoid are highly enriched in DBT, which could exhibit anti-oxidation functions by suppressing H₂O₂-induced ROS formation, reducing Aβ aggregation; activating estrogen receptor and Akt phosphorylation, as well as suppressing the ratio of Bax/Bcl-2 [16]. The above-mentioned evidences indicated that AR could be a valuable herb in DBT for possible neuroprotective activities.

Ferulic acid is an antioxidant naturally presented in ASR. Long-term administration of ferulic acid protected against cognitive impairment-induced learning and memory deficits *in vivo*. Ferulic acid prevented peroxy radical-triggered cell death in hippocampus as well as suppressed protein oxidation in response to hydroxyl and peroxy radical generators, lipid peroxidation and ROS formation [21]. Moreover, ferulic acid protected against the free radical-mediated conformational changes in synaptosomal membrane proteins [21]. On the other hand, administration of Z-ligustilide, the primary lipophilic component in ASR, could diminish mortality, neurobehavioral deficits, brain edema, BBB permeability and cerebral vasospasm in AD



rat model [20]. In parallel, Z-ligustilide was able to reduce apoptotic cells in the brain injury site by down regulating cleaved-caspase 3 and p53 expressions [20]. These data therefore suggested that ASR might be effective in AD patients.

The importance of “Blood” sheds light on the treatment of AD or other cognitive impairment [3, 4].

Various cognitive impairments are associated with micro-nutrient, especially iodine and iron [7]. In rats, iron deficiency induces brain mitochondrial damage and other alterations [5]. On the other hand, erythropoiesis involves a close interaction of iron and EPO. Iron is the fuel to produce new red blood cells. In addition to induce erythroblast differentiation, EPO stimulates endothelial proliferation, increases the expression of synaptophysin, enlarges capillary density, and decreases A β level in vivo, suggesting that EPO possesses therapeutic functions in mitigating AD syndromes [8]. Interestingly, our previous studies have shown that DBT decoction stimulated EPO expression via the HIF pathway [9, 16, 36]. Up to now, we do not know how the ancestors make up the formulation of DBT. Recent investigations in neurodegenerative disease models have provided glimpses that DBT decoction may be a fruitful therapeutic strategy for mitigating AD syndromes. However, the effect of DBT in AD animal model should be done as to support the possible usage of DBT in AD treatment.

Conclusion

In cortical neuron cultures, DBT was capable of reducing the rate of A β -induced apoptosis in a dose-dependent manner; the effective concentration was revealed to be at 100 μ g/mL. In the A β -treated cortical neurons, the expression ratio of Bcl2 to Bax was altered by DBT in a concentration-dependent manner. In parallel, DBT application could markedly suppress the A β -induced expressions of apoptosis markers, i.e. cleaved-caspase 3/9 and PARP. Taken these results, DBT sheds light in protecting the brain against stress or insult, but animal study is needed for further confirmation.

Abbreviations

AD: Alzheimer’s disease; AR: Astragali Radix; ASR: Angelicae Sinensis Radix; A β : β -Amyloid; BBB: Blood brain barrier; DBT: Danggui Buxue Tang; ECL: Enhanced chemiluminescence; EPO: Erythropoietin; HRP: Horseradish peroxidase; PBS: Phosphate-buffered saline; PI: Propidium Iodide; ROS: Reactive oxygen species

Acknowledgements

Not applicable.

Funding

Supported by Research Fund of Zunyi Medical University for the Doctoral Program (F-937), NNSF of China (81202907), NNSF of China (21562053) and NNSF of Guangdong (2018A030307074), China.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

GG and YZ designed and performed experiments. YTL, TTXD and HYW contributed reagents/materials/analysis. YZ and KKWT wrote the main manuscript text. BQ was responsible for manuscript revision. All authors read and approved the final version of manuscript.

Ethics approval

The animal procedures were approved by The Animal Experimentation Ethics Committee of the Hong Kong University of Science and Technology (No. 17–283 for Animal Ethics Approval) and under the guidelines of “Principles of Laboratory Animal Care” (NIH publication No. DH/HA&P/8/2/3).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Received: 29 August 2017 Accepted: 17 December 2018

Published online: 08 January 2019

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