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A Novel Herbal Formula that may benefit Small Vessel Disease of the Brain

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Summary

Small vessels in the brain are affected by atherosclerotic changes in the general vascular system and extensive involvements could be the cause of early neurodegeneration. Early evidences indeed have been worked out on the co-existence of small white matter hyper intensities in the brain and early cognitive impairments. It is therefore recommended that once detected, early control is advisable. However, there is yet a total lack of preventive measures.

Traditional Chinese Medicine has a rich collection of medicinal herbs that have been used to counteract symptoms and syndromes resembling neurodegeneration: The research being described, started with the selection of two herbs believed to be suitable for small vessels disease viz. Salviae miltiorrhizae Radix et Rhizoma (Danshen) and Gastrodia elongate Blume (Tianma) and putting them on bioactivity platforms to confirm their neuro-regenerative abilities.

The platform studies included basic in-vitro tests to confirm their individual anti-inflammation, anti-oxidation and vascular protective effects; then working out the favourable ration of 2:1, Danshen: Tianma, to be adopted in the simple formula.

Using the Danshen-Tianma formula, the in-vitro tests on neuroprotection were repeated, followed by in-vivo experiments using special mouse models. The results well supported the neuroprotective effects f the formula.

In addition, one novel mouse model of forced cigarette smoking using an enclosed smoke chamber in an 8 weeks daily (hourly) treatment was used to testify the effects of the formula. The good results of intervention further supported the alleviating effects of the formula.

It is suggested that the Danshen-Tianma formula is now ready to be tested clinically on patients with early signs of small vessel disease.

Keywords: Alzheimer's disease; Chinese herbs; Danshen; Neuro-degeneration; Tianma.

Introduction

Research on neurodegeneration and Alzheimer's disease is currently emphasizing on the early detection of mild cognitive impairment and to delay structural changes in the brain. Functional magnetic resonance imaging (fMRI) on the brain of individuals suffering from early cognitive impairment has shown cerebral white matter hyperintensities which are considered manifestation of severe cerebral arteriosclerotic small vessel disease. Pathologically the white matter hyperintensities consist of partial loss of myelin, axons and oligodendroglial cells, with reactive astrocytic gliosis and sparsely distributed macrophages, all resulting from blockages of related small vessels [1-3].

White matter hyper intensities are usually subclinical but carry high risks of development to dementia. It is therefore recommended that once detected, measures designated for its control, should be started [4,5].

It has been observed that the Chinese elderlies might be more prone to such small vessel disease, making control of this condition among Chinese Communities more important [6].

Preventive and control measures normally include the control of hypertension and other essential manoeuvres for cardiovascular health. However, the actual effective value seems uncertain [3].

The outcome of small vessel disease of the brain involves firstly luminal narrowing of the arteriosclerotic small vessels causing ischaemia, secondly there is co-existing impaired cerebral vasomotor reactivity which normally maintains adequate blood flow through sophisticated smooth muscle relaxations of the vessuls. Other mechanisms that contribute towards cerebral ischaemia include breakage of the blood-brain-barrier leading to leakage of toxic substances, oxidative stress, inflammation and venous collagenosis [1].

Medications that may effectively counteract those pathophysiological changes are lacking. Available measures currently indicated for cerebral degenerations are only providing palliative benefits for well-developed cases. Before the appearance of a suitable remedy for the preceding small vessels disease of the brain, different groups are seriously looking for supplementary measures like the provision of special diets [7] that might help.

Traditional Chinese Medicine has a rich collection of medicinal herbs that have been used since ancient days for various groups of clinical syndromes, like those related to cardiovascular health and dementia like symptoms [8,9]. It would be convenient to select from the rich data bank a few that own both historical favour of effectiveness, as well as current day bench research data of beneficial bioactivities and clinical efficacies.

Creation of a Simple Herbal Formula that possesses both cardiovascular protection and Neurological Support
A two-herb formula consisting of *Salviae miltiorrhizae Radix et Rhizoma* (Danshen) and *Gastrodia elongate Blume* (Tianma) was chosen for research.

Danshen has been a herb most frequently used for cardiovascular maintenance and protection [10]. It has been found to have bioactivities of anti-inflammation, anti-oxidation [11,12], vascular protection and cardiac protection [13-15].

Tianma is a remarkable medicinal herb widely used for the treatment of epilepsy, stroke, headache and vertigo. It has been found in various studies to be anti-inflammatory, anti-oxidative, anti Aβ activities, promoting better neural cell viability and maintains better vascular flow [16-19].

Further proof on the Validity of using Danshen and Tianma in a combined formula

Effects of Danshen and Tianma extracts on H_2O_2 induced oxidative stress model using different cell lines to illustrate their individual neuroprotective effects: -

PC12 cells survival

Different concentrations of H_2O_2 treatment on PC12 cells produced different percentages of cell morbidity. 75μM H_2O_2 concentration was found most convenient for cell culture experiments.

Tianma at 125, 250, 500 and 1,000 μg/ml significantly increased cell viabilities in a dose-dependent manner (Figure 1).

Danshen at 15.6, 31.25, 62.5 and 125μg/ml also significantly increased cell viabilities in a dose-dependent manner (Figure 1).

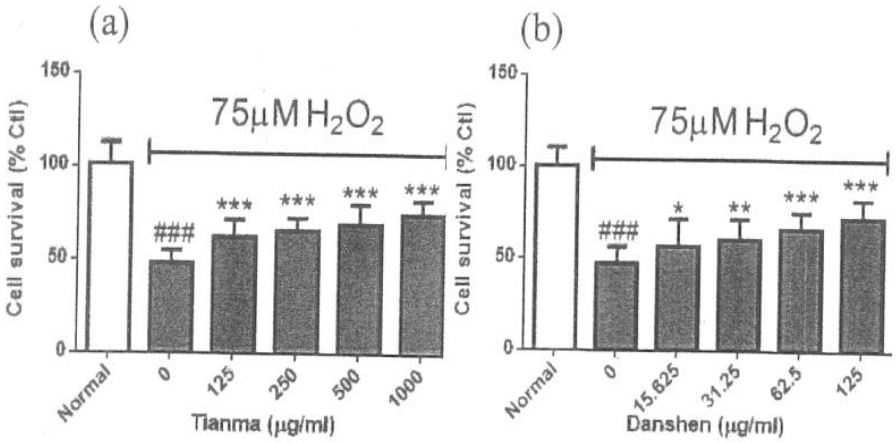

Figure 1: Effect of Tianma, Danshen and Nuzhenzi extracts against 75 μM H2O2-induced oxidative stress in PC12 cells. The cell survival after 24-hour co-treatment of (a) Tianma, (b) Danshen.

PC12 cells and Aβ-induced toxicity

Accumulation of Aβ in the brain is harmful against neuronal viability. 24 hours treatment of Aβ produces cytotoxicity on PC12 cells. When Tianma and Danshen extracts were used in co-cultures, cell survivals were found to increase with concentrations of 1,000 μg/ml (Figure 2). Similar increased survival results were obtained with 48 hours treatment.

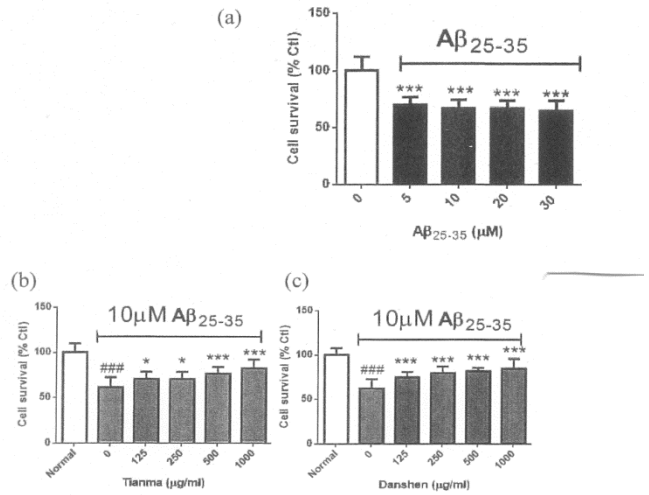

Figure 2: Effect of Tianma, Danshen and on Aβ_induced cytotoxicity on PC12 cells for 24 hours. (a) Aβ25-35 induced cytotoxicity on PC2 cells for 24 hours. The cell survival after 24 hours 10 μM Aβ25-35 co-treatment with different concentration of (b) Tianma, (c) Danshen.

Oxidative stress is considered an important pathway in the pathogenesis of neural degeneration. Oxidative stress cell models are therefore widely accepted as useful tests for the screening of neuroprotective agents [20]. Our individual tests on Danshen and Tianma separately, have given support for the selection of these two herbs as being suitable for inhibition of neural degeneration. Although, Danshen is traditionally used for cardiovascular protection, some studies have demonstrated the neuroprotective effects of some chemical components of Danshen in ischaemic stroke and cerebral degeneration [21].

Danshen is to be combined with Tianma in a twin-herb formula with the intension of achieving synergy. The ratio of the two herbs needs to be worked out, then the combined formula would need to be tested for the expected vascular and neuroprotective effects.

To work out a favourable ratio between Danshen and Tianma for further testing, a standard anti-oxidant study platform using PC12 and UMR-106 cells was utilized. To follow a neuro-protective pathway, not only the anti-oxidant ability of the two herbs, but also their anti-amyloid induced toxicity effects was tested. Seven different ratios of Danshen to Tianma (4:1, 3:1, 2:1, 1:1, 1:2, 1:3, 1:4) were tested. The ratio of 2:1 Danshen - Tiama was found to be giving the best results of anti-oxidant and anti-toxicity effects [22,23].

Experimental Studies of Danshen – Tianma (D.T.) Formula on its effects related to Neuro-regeneration - *In-Vitro* Experiments on Neuroprotection, Inhibition of Acetylcholinesterase (AChE) Activities on PC12 Cells PC12 cells were seeded in 6-well plates overnight then cultured with DT formula at different concentrations against positive controls using Donepezil. Standard procedures of measuring acetylcholinesterase activities indicated DT significantly inhibited AChE activities and was just inferior to Donepezil (Figure 3).

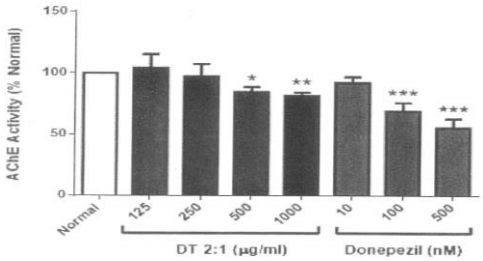

Figure 3: DT formula possessed inhibition on AChE activities in PC12 cells fter 24-hour treatment. The AChE activities were assessed by using Abcam's Acetylcholinesterase Assay Kit. Donepezil was used as positive control.

Aβ-induced apoptosis

The anti-apoptotic effect of the DT formula was highlighted by flow cytometry with Annexin V-FITC and PI staining. DT intervention significantly decreased early and late apoptosis populations. (Figure 4).

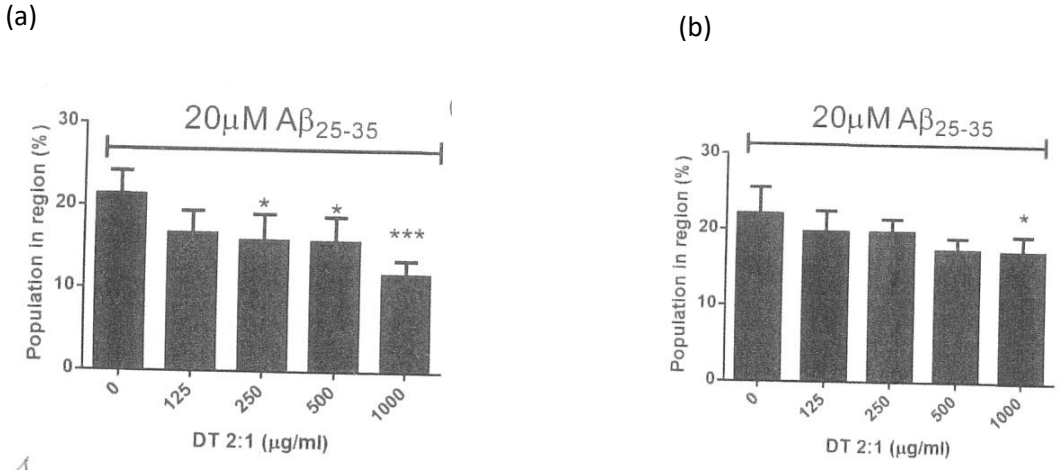

Figure 4: Anti-apoptotic effect of DT formula against Aβ-induced toxicity in PC12 cells using flow cytometry. (a) early apoptosis and (b) late apoptosis.

Anti-oxidative effects of DT formula against OGD/R induced ROS

After 16 hours ischaemia and 24 hours reperfusion, OGD/R induced a significant reduction in cell viability by 57%. DT intervention provided dose-dependent protection on cell viability, as well as increases in ROS activities (Figure 5).

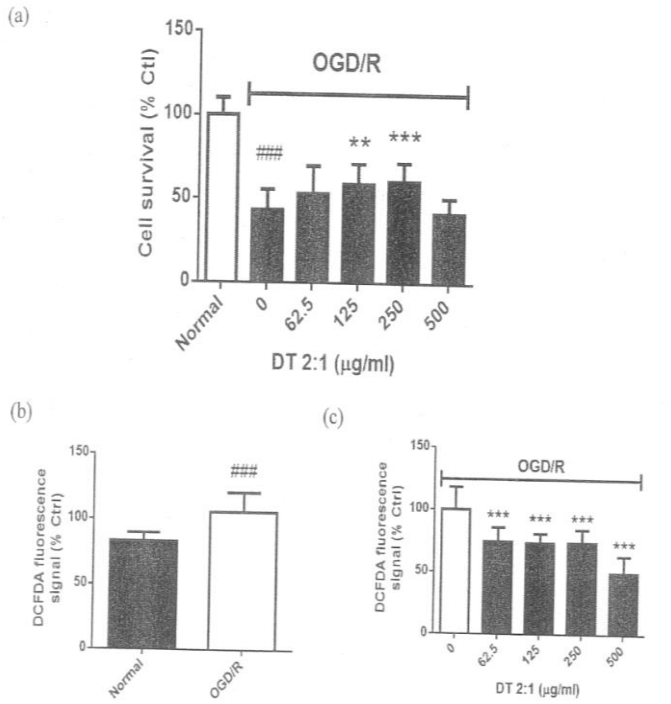

Figure 5: OGD/R-induced oxidative stress on PC12 cells survival after DT formula treatment with the presence or absence of 16-hour ischemia and followed by 24-hour reperfusion on PC12 cells. (a) Cell viability was measured by MTT assays. ROS production (b) induced by OGD/R and (c) after DT formula treatment on PC12 cells.

In-vivo Experiments on Neuroprotection

Protective Effects of DT formula on ischaemic stroke using middle cerebral artery occlusion (MCAO) rat model. The rats were randomized into 4 MCAO operated groups of low dose DT; high dose DT; Herbal control using Ginkgo biloba and sham operation.

Assessments included Cerebral blood flow measurement; neurological scoring after recovery; and brain infarct evaluations. MCAO-induced neurological deficits were significantly attenuated with low and high doses of DT which compared well with Ginkgo biloba (Figure 6).

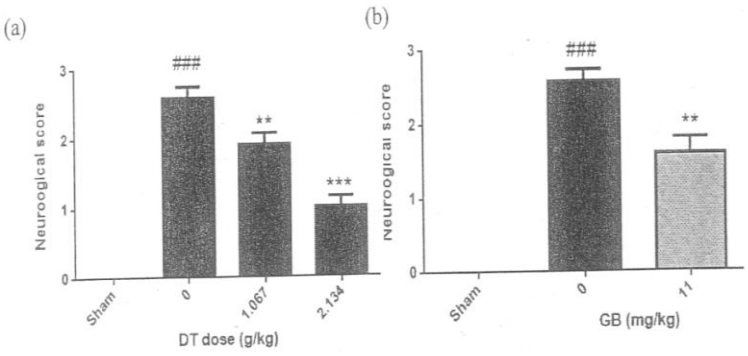

Figure 6: DT intervention attenuated neurological deficit in MCAO rats.
Neurological deficit on MCAO-operated rat was assessed by scoring system 24 hours after reperfusion with (a) DT formula and (b) Ginkgo biloba extract treatment.

After neurological assessments, the rats were sacrificed and their brains were dissected out. Coronal slices were cut from the brain to evaluate the average infarct volume. DT at low and high doses exhibited significant dose-dependent reduction in the MCAO-induced brain infarct volumes (Figure 7).

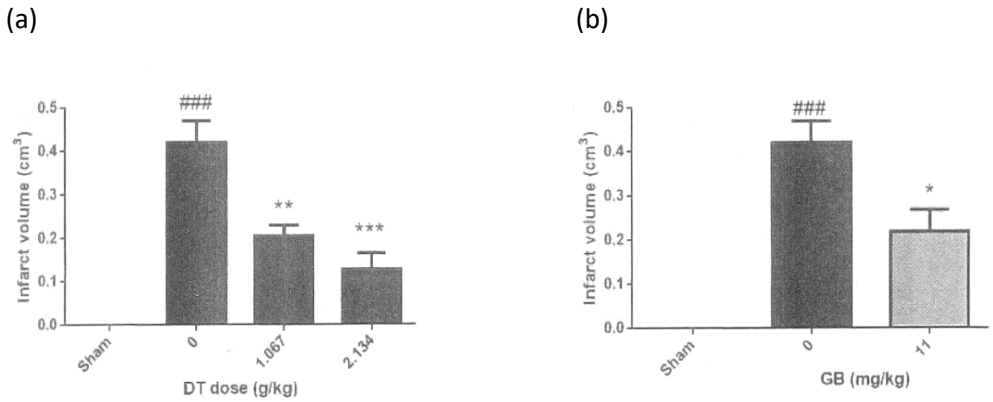

Figure 7: DT intervention attenuated brain infarct volume in MCAO rats. Quantified brain infarct volume in MCAO-operated rats with (a) DT intervention and (b) Ginkgo biloba extract treatment.

Molecular Studies on MCAO induced infarcts

MCAO significantly down regulated the SOD and GPX activities. But rats subjected to DT low and high doses, showed increased SOD and GPX activities compared with controls (Figure 8).

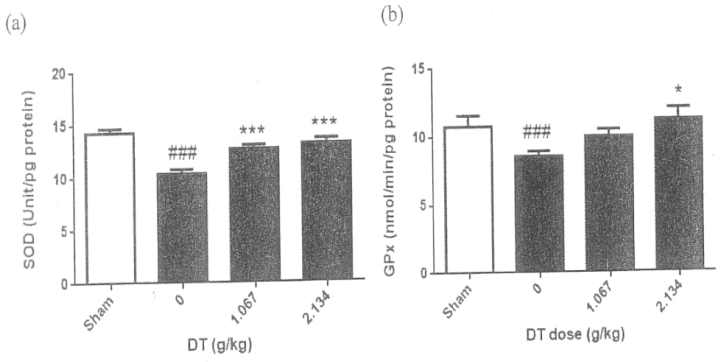

Figure 8: DT intervention enhanced anti-oxidative enzymes activities (a) SOD, (b) GPx in MCAO rats.

Neuro-inflammation (TNFα and IL6 production)

MCAO induced cerebral ischaemic increased TNFα and IL6 concentrations in the rat plasma. DT administration significantly lowered their contents (Figure 9).

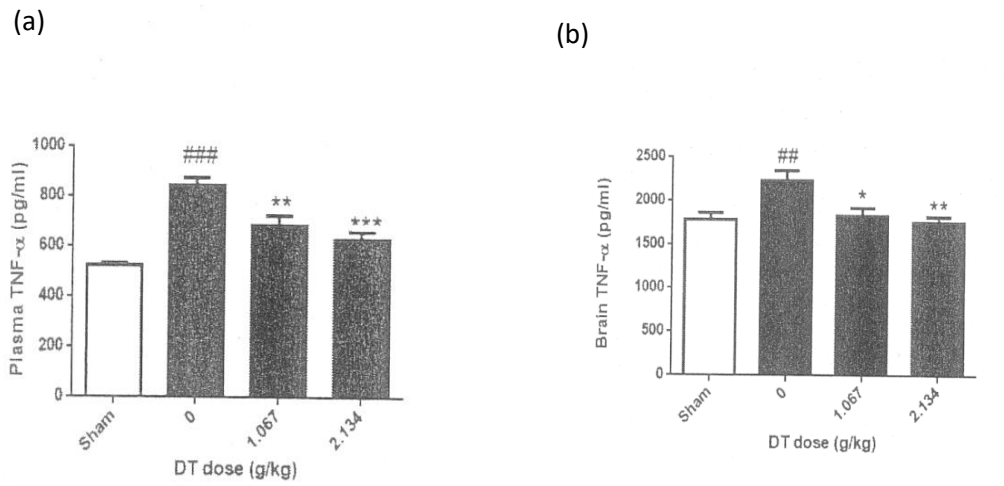

Figure 9 (i): The effect of DT on TNFα level in MCAO-operated rat. The level of TNFα after (a) DT treatment and (b) brain homogenate.

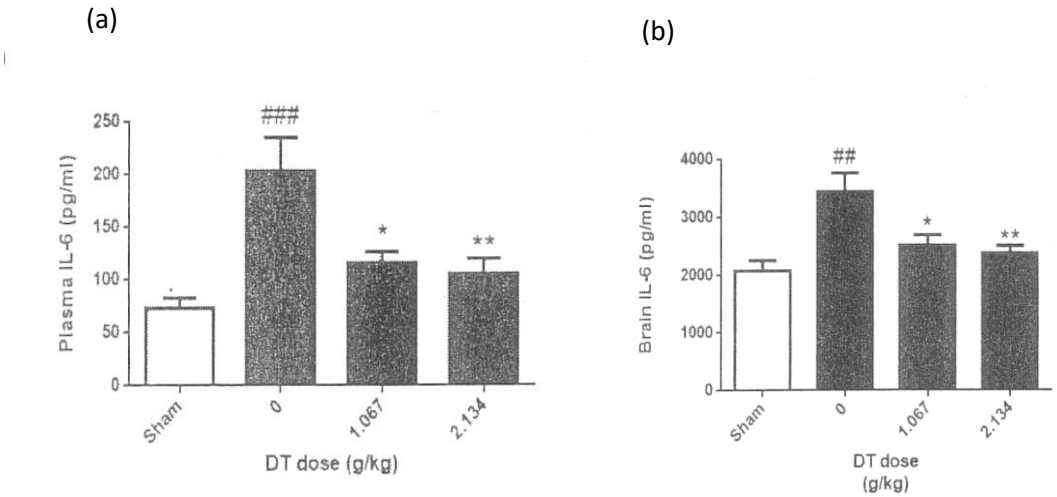

Figure 9 (ii): The effect of DT or GB treatment on IL-6 level in MCAO-operated rat. The level of IL-6 after (a) DT treatment and (b) brain homogenate.

Neuroprotective effects of DT formula on 5xFAD transgenic Alzheimer disease mouse model

In order to provide a rapid and severe development of amyloid pathology the high expression Tg6799 of the transgenic model was selected. Apart from the observation on the development of cerebral amyloid plagues and gliosis in the two months old 5xFAD mice, massive Aβ accumulation, synaptic markers reduction, neuron loss and memory impairment in behavioural experiments were included in the study [24].

The effects of DT on improvement of spatial memory and learning ability were assessed using Morris Water Maze study. DT treatment was started with 4 month old mice, lasting 8 weeks. At the end of 8 weeks, body weights of the rat were not affected indicating the lack of toxicity. Learning and spatial memory assessments using the Morris Water Maze showed improvement with the DT groups. Study on the Aβ deposition demonstrated diminished values. The inflammatory factors TNFα and IL6 also appeared to be inhibited by D&T.

Small vessel Disease and DT intervention

A novel animal model of small vessels disease of the brain

was created using male balb/c mice with body weight of 20 gm. These mice were kept in specially designed closed environment ventilated with commercial cigarette smoke which was introduced 1 hour daily, 6 days per week for a total duration of 8 weeks.

The mice were sacrificed on completion of forced, passive smoking and studied for vascular and inflammatory changes in the brain. Cerebral vessels were visualized using intravascular perfusion of black pen ink introduced through the heart of the mouse. Images from the superior view of the transverse planes of the brains, were captured. DT was found to increase the superior vessel density which had been significantly affected by the passive smoking.

Chronic smoking leads to vasculitis, reduction of cerebral small vessels density and accumulation of pro-inflammatory cytokines [25,26]. The plasma TNFα and IL6 levels of the passive smoking mice were analyzed and found to be down regulated by the DT formula.

Discussion

Neurodegeneration leading to Alzheimer Disease is a big public health problem as aging and longevity are world-wide realities. The treatment options available for established Alzheimer Disease are short of reliable efficacy and progressive neural degeneration remains yet uncontrollable. Prevention of the degenerative process at the early stage of its detection would be an important challenge that could bring better outcome than conventional treatment which is usually too late.

Herbal remedy in Traditional Chinese Machine has enjoyed centuries of practical trust when it is used to prevent or alleviate symptoms. When we look for medicinal herbs that would help with neurological support we found two appropriate choices viz. Tianma and Danshen. The former has been one of the most frequently prescribed herb for the treatment of brain related symptoms and syndrome. The latter is better known as a cardiovascular tonic but is chosen partly because of the obvious fact that neuro-regeneration relys on vascular facilitation, and some recent laboratory work has also demonstrated its neuroprotective effects.

Using standard cell cultures for the study of neural regeneration, we have proven separately that Tianma and Danshen both possess neuroprotective ability.

A favourable ratio of 2:1 was worked out for the relative concentration of Danshen and Tiama to form a combined formula with synergistic effects. Indeed, the DT formula was shown under various platforms to be more potent that Tianma alone.

The DT formula was subsequently tested on *in vitro* platforms as before, using Ginkgo biloba as a positive control. DT formula was found effective protecting against cellular apoptosis, and controlling inflammation via its antioxidant abilities. More specifically, D.T. attenuated Acetylcholinesterase effects which should benefit neurological transmission.

In vivo experiments further confirmed the biological effects of the DT formula. Three pathological states were tested, viz. cerebral ischaemia produced by middle cerebral artery occlusion; a Alzheimer rat model and a small vessel disease model using forced passive cigarette smoking. Cerebral ischaemic rats recovered memory and performed better after DT treatment. Histomorphological studies also showed less ischaemic damages.

For the Alzheimer rat, spatial memory and learning ability were found better with the DT group. Amyloid deposition was less and inflammation was better controlled.

The rats were put into enclosed chambers fumed with cigarette smoke one hour per day for 8 weeks to produce a state of small vessel disease. Histomorphologically, cerebral vessels were narrowed or obliterated. The degree of Vascular Narrowing was lesser among the DT group. The associated inflammatory changes were also better controlled.

An overall analysis of the experimental results of the DT formula suggests that it should help to sustain cellular viability, provide antioxidant effects, control amyloid deposition and provide positive behavioural improvements in the life rat suffering from stroke, ischaemia, or neurodegeneration.

The two herbs of the DT formula are well-known edible herbs without known toxicity. It could become a favourable medicinal supplement for the prevention of neurodegeneration.

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