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Original Article

NeuroDefend, a novel Chinese medicine, attenuates amyloid-β and tau pathology in experimental Alzheimer's disease models

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ABSTRACT

Alzheimer's disease (AD) is the most common age-related neurodegenerative disorder. Amyloid-β (Aβ) and hyper-phosphorylated tau accumulation are accountable for the progressive neuronal loss and cognitive impairments usually observed in AD. Currently, medications for AD offer moderate symptomatic relief but fail to cure the disease; hence development of effective and safe drugs is urgently needed for AD treatment. In this study, we investigated a Chinese medicine (CM) formulation named NeuroDefend (ND), for reducing amyloid β (Aβ) and tau pathology in transgenic AD mice models. Regular oral administration of ND improved cognitive function and memory in 3XTg-AD and 5XFAD mice. In addition, ND reduced beta-amyloid precursor protein (APP), APP C-terminal fragments (CTF-β/α), Aβ and 4G8 positive Aβ burden in 3XTg-AD and 5XFAD mice. Furthermore, ND efficiently reduced the levels of insoluble phospho-tau protein aggregates and AT8 positive phospho tau neuron load in 3XTg-AD mice. Hence, ND could be a promising candidate for the treatment of AD in humans.

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1. Introduction

Alzheimer's disease (AD) is an unfortunately common neurodegenerative disorder that is affecting the elderly worldwide. It is not clear whether cure is possible, and currently available treatments offer only moderate symptomatic relief. Effective, safe therapies and/or drugs are needed. The key hallmark features of AD are: loss of cholinergic neurons; tau-associated neurofibrillary tangles (NFTs) and accumulating amyloid β-peptide (Aβ) generates senile plaques (SP) derived from amyloid precursor protein (APP) [1].

Aβ and NFTs are well correlated with cognitive impairment [2]. Several critical research works carried out on a transgenic mice model conclude that there is a metabolic link between Aβ and tau [3]. Tackling Aβ and NFTs seems to be a better strategy most likely to succeed in drug discovery than directly tackling tau. Although SP and NFTs are hallmark symptoms of AD. Several microarray analysis and histopathological studies of the brains of AD patients, particularly examining SP and NFT-bearing neurons, show that behavioral impairments are closely associated with reactive astrocytosis and that the occurrence of reactive glial cells may be due to neuroinflammation [1,4,5]. Hence, it is also important to analyze the level of neuroinflammation in the brain [1]. Currently, AD drugs in the market are rudimentary and can only relieve certain symptoms but cannot cure the disease. As such, effective and safe drugs are the need for the complete cure of AD. Hence, disease-modifying therapies are important to address cognitive impairments and neuroinflammation of the disease for the quality of life in worldwide aged patients [4,5].

LaFerla and his group were the first to generate the 3XTg-AD mouse model which is generally used in drug screening and drug discovery. This transgenic mice models express mutated APP, tau protein (APPswe and TauP301L respectively), and mutated PS1 protein (PS1M146V), which develop both NFTs and amyloid plaques [6]. Interestingly, 3XTg-AD mice exhibited a phenotype of Aβ and NFTs pathology affecting synaptic plasticity that in turn impacts long-term potentiation and memory loss [7,8].

An ideal drug for AD would reduce both Aβ and NFTs; thus, current drug research focuses on these essential aspects. Traditional Chinese Medicine (TCM), an ancient and effective medicinal system extensively used in East Asia, uses this type of combinational approach, with multiple herbal extracts for addressing various aspects of a medical condition simultaneously. Today, TCM is used for the treatment of several neurological diseases including AD. TCM herbs are attracting wide attention for drug discovery and therapeutic approaches. In our previous work, we have shown that modified Huang-Lian-Jie-Du-Tang (HLJDT-M, i.e., HLJDT without Huangqin, US provisional patent No.61834437) has positive activity compared to the classic formula HLJDT in reducing the amyloid-β (Aβ) load in both *in vitro* and *in vivo* AD models [9–11]. Although HLJDT-M has a significant Aβ-reducing effect, it does not possess aggregated tau-reducing activity. Hence, there is a need to optimize HLJDT-M for AD treatment.

In order to optimize the HLJDT-M for targeting Aβ and NFTs, we developed a novel formulation termed as NeuroDefend, which was obtained by combining HLJDT-M with *Radix Salviae Miltiorrhizae*, *Rhizoma Corydalis* and *Uncaria Rhynchophylla* using key weight ratio derived from a uniform design program. The ratio of the 6 component herbs *Rhizoma coptidis*, *Radix scutellariae*, *Cortex phellodendri*, *Radix Salviae Miltiorrhizae*, *Rhizoma Corydalis* and *Uncaria Rhynchophylla* (Uncaria Hook) is 0.83: 1.66: 4.16: 2.91: 4.57: 6.24 for 1 kg lyophilized powder preparation. We have already filed the US provisional patent No.504824619 and US non provisional patent No.505236638 for ND in treating Alzheimer's disease

In this study, we investigated whether ND can ameliorate memory and promote the clearance of insoluble tau and Aβ in a 3XTg-AD mice models. Furthermore, we also evaluated the efficacy of ND on Aβ pathology in a transgenic 5XFAD mice model.

2. Materials and methods

2.1. Reagents and antibodies

All primary antibodies used in the study are given in detail (Table 1). Tau antibodies PHF1, CP13 and MC1 were the generous gift from Prof. Peter Davies at Albert Einstein College of Medicine. HRP-conjugated goat anti-mouse (115-035-003) and goat anti-rabbit (111-035-003) secondary antibodies were purchased from Jackson Immuno Research. PBS (P5368) purchased from Sigma, USA. Page Ruler Pertained Protein Ladder (26616), Cryomatrix (6769006) were purchased from Thermo Fisher Scientific. Vector ABC staining kit (PK-6100 Elite), DAB Peroxidase Substrate Kit (SK-4100) (VECTOR, Burlingame, USA). Alexa Fluor® 488 goat anti-mouse IgG (A-11001) and Alexa Fluor® 594 goat anti-rabbit IgG (A-11012) were purchased from Thermo Fisher Scientific. West Pico or West Femto chemiluminescent substrate kit (Thermo Fisher Scientific, USA).

2.2. Preparation and quality analysis of NeuroDefend

Initial testing of quality control of individual herbs of the ND formulations was performed by assessing the percentage of key marker of each herb. All 6 herbs of three different batches were acquired from the HKBU Chinese Medicine Clinic. Dry materials of the plants were ground into powder using a Waring blender. The extraction of ND was done as per the protocol indicated in our previous publication [11]. Voucher samples of each herb were deposited in the herbarium of the School of Chinese Medicine, Hong Kong Baptist University (HKBU).

Approximately 1 kg powder of ND was extracted in water and steeped in 1 L of 70% alcohol overnight. Extracted solutions were then filtered. This procedure was repeated three times. Solutions were pooled, and approximately 12 L of the pooled solution was concentrated by rotary evaporation under vacuum at 50 °C. All the formulations were finally lyophilized under vacuum (LABCONCO, Laboratory Construction Company, USA) and the resultant yield was powdered and mixed until homogenous. The powder thus obtained was stored at 4 °C. Ultrahigh-performance liquid chromatography with quadruple time-of-flight mass spectrometry (UHPLC-Q-TOF-MS) was used to identify and quantify the reference

Table 1 – Specifications of antibodies used in the present study.

Antibody (clone)	Region specificity	Antigen	Source	Use and Dilution
AT8 monoclonal to phospho Tau biotinated	Human, mouse and rat phospho Tau	Epitopes matching to residues neighboring Ser 202, Thr 205 phosphorylated sites	Thermoscientific Waltham, MA, USA	IHC 1:500
HT7 monoclonal to total Tau biotinated	Human specific	Human Tau between residue 159 and 163	Thermoscientific Waltham, MA, USA	IHC 1:500
PHF-1 monoclonal to phospho Tau	Human, mouse and rat phospho Tau	Epitopes matching to residues neighboring Ser396 and Ser404 phosphorylated sites	Prof. Peter Davies Albert Einstein College of Medicine, Manhasset, NY, USA	WB 1:1000
Mouse monoclonal to β-actin (C4)	β-actin	Bird gizzard actin	Santa Cruz, Dallas, TX, USA	WB: 1:10000
CP13 monoclonal to phospho Tau	Human, mouse and rat phospho Tau	Epitopes matching to residues neighboring Ser 202 phosphorylated sites	Prof. Peter Davies Albert Einstein College of Medicine, Manhasset, NY, USA	WB 1:1000
MC1 monoclonal to phospho Tau	Human, mouse and rat phospho Tau	Epitopes matching to residues neighboring Ser 312–322 phosphorylated sites	Prof. Peter Davies Albert Einstein College of Medicine, Manhasset, NY, USA	WB 1:1000
AT8 monoclonal to phospho Tau	Human, mouse and rat phospho Tau	Epitopes matching to residues neighboring Ser 202, Thr 205 phosphorylated sites	Thermoscientific Waltham, MA, USA	WB 1:1000
Rabbit polyclonal to APP CT695 (CT695)	Human, mouse and rat FL-APP and CTFs	C-terminus 22 amino acid residues of β-APP peptide	Thermoscientific, Waltham, MA, USA	Western blotting (WB) 1:1000;
Biotinylated mouse monoclonal to human Aβ17-24 (4G8)	hAβ	Amino acids residues 17–24 of hAβ peptide	Biolegend, Dedham, MA, USA	IHC 1:500
Rabbit polyclonal to phosphorylated APP (Thr668)	Human Phosphorylated APP at Thr668	Phospho epitopes matching to residues neighboring Thr668 of human APP695	Cell signaling, Danvers, MA, USA	WB 1:1000

compounds of ND using both positive and negative mode electrospray ionization (ESI) MS as described below. Lyophilized powder of ND was mixed and dissolved in methanol and centrifuged at 10,000 rpm. The supernatant was filtered for the analysis of active compounds in ND. Agilent 1290 (Agilent Technologies) UHPLC system, equipped with a binary pump, a thermostatic column compartment and an auto sampler, were used for the chromatographic analysis. The injection volume of the samples was 2 μl for MS analyses. C18 column (1.7 um 2.1 × 100 mm) (Acquity UPLC BEH) was used to separate the samples at 40 °C. 0.1% formic acid (A) in water and 0.1% formic acid in ACN (B) were used as mobile phases with a constant flow rate at 0.1 ml/min. MS and MS/MS data was acquired by Agilent 6540 Q-TOF mass spectrometer (Agilent Technologies) equipped with a jet stream ESI source in positive ion mode. Data analysis was done by Mass Hunter (Agilent Technologies) B.03 software and for all mass peaks, the mass spectral data were analyzed at a range of 100–1700 *m/z*.

2.3. Animals

All animal experiments for the project were approved by the Committee on the Use of Human and Animal Subjects in Teaching and Research (HASC), Hong Kong Baptist University and by the Committee on the Use of Live Animals for Teaching and Research (CULATR-3399-14) at the University of Hong Kong for all animal behavioral studies. All methods and experiments were performed in accordance with the approved guidelines and regulations. Animal license to conduct the experiments were approved by the Department of Health, Hong Kong under the animal license no. (17-64) in DH/SHS/8/2/6 Pt.1.

The 5XFAD and 3XTg-AD mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and housed in the animal unit. The colony of animals was maintained in our animal unit. 5XFAD transgenic mice possessing overexpression of both mutant human PS1 harboring L286V, M146L mutations and human APP (695) with the Florida (I716V), Swedish (K670N, M671L) and London (V717I) with Familial Alzheimer's disease (FAD) mutations were also used in this study. 5XFAD transgenic male mice were crossed with C57 female mice and the resulting transgenic offspring were used after confirming transgene by PCR according to the supplier's protocol. 3XTg-AD mice show both Aβ and tau-associated NFTs, and the amyloid deposition precedes tangle formation, with increased synaptic dysfunction starting from 6 to 12 months of age. At 4 months of age the 3XTg-AD mice show [6] long-term memory retention deficits in the Morris water maze and accumulation of intra-neuronal Aβ; at 13 months, the tau-associated NFTs become visible. The 3XTg-AD mice harbor three human mutant genes, namely APP with the Swedish mutation, presenilin-1-KI, and TauP301L, on a mixed C57B6/129SVJ background [6]. The 3XTg-AD mice show various symptoms of AD and are universally considered to be the best model for drug discovery and development of therapeutic strategies for AD. 5XFAD mice at the age of 2 months show $A\beta_{42}$ deposition (and gliosis) in the cerebral hemisphere; at 4–5 months, this deposition accumulates into a large plaque burden with impaired memory in the Morris water test [12,13].

ND at three different doses (1.9, 3.8 and 7.6 g/kg) was dissolved in 1 L of distilled water and mixed in 1 kg feed powder. The feed mixture was incubated at 55 °C to evaporate the excessive water content. The feed admixture was fed to the ND treatment groups regularly, the assessment of body weight and feed consumption was monitored every week till the end of the study. The 5XFAD mice were fed every day with ND feed admixture at 2 months of age and lasted for 3 months up to 5 months of age and 3XTg-AD mice were fed every day with ND feed admixture at 6 months of age and the treatment period lasted for 8 months till the mice reached 14 months of age with a low dose (1.9 g/kg/d), a middle dose (3.8 g/kg/d), a high dose of herbal extracts in feed admixture (7.6 g/kg/d), or vehicle for 8 months before being sacrificed. Each group consisted of 10 animals per group and both male and female were equally used in the 5XFAD mice study and only female mice were used for 3XTg mice model. Mice were maintained under a 12-hour light/12-hour dark cycles, a pathogen-free environment with food and water providing ad libitum.

2.4. Open field test

Open field test was performed as described previously [11,14] to assess locomotor and exploratory behavior of the mice. The apparatus consists of a square plexiglass box (25 × 25 cm). Firstly, a camera is mounted onto top of the plexiglass box to view the area as well as the animal movement, and later markings were made at the base of the box which is required in setting-up the animal tracking software. All mice used in the experiment were placed at the same place, namely a corner, and then permitted to move freely; the locomotor and exploratory behavior activity of each mice were recorded for 5 min. The parameters such as velocity, time spent in movement, distance walked and time spent in central/marginal areas were noted and analyzed using Ethovision video tracking system (Version 3.0, Noldus Information Technology, Leesburg, VA, USA).

2.5. Morris water maze test (MWM)

The MWM test was performed as per protocol described by our group [9,11]. The MWM test is performed in a 1 m diameter circular pool made of white metal and filled with water. Transparent plexiglass platform of 9 cm diameter and 29 cm height was placed in the pool at a fixed position. Experimental mice were taken to the behavior room for acclimatizing and training. The path of swimming was monitored by an automated video tracking system (Ethovision software, Noldus). Visible platform trials were done to assess sensorimotor and/or motivational deficits that could affect the performance of mice during the learning task in MWM. The visible platform training (4 trials per day) was done with a flag attached to the platform, and the platform position was changed for each trial during the training session. The hidden-platform training comprised 4 trials per day for 6 days; each trial consisted of four 60-second trials with a 30-min interval. In the hidden platform sessions, the platform location remained constant and the entry point for placing the mice in the tank was changed randomly between the training days. To assess memory, we removed the platform from the tank to conduct a

probe trial and allowed the mice to search for the platform for 60 s. The time spent in the quadrant where the platform had been removed was assessed as a measure of the level of memory. The percentage of time spent in each quadrant and distance taken to reach the target quadrant of the pool during probe trial were recorded and assessed using an Ethovision video tracking system (Version 3.0, Noldus Information Technology, Leesburg, VA, USA).

2.6. Contextual fear conditioning test

Fear conditioning test was performed as per protocol [15] in a 30 × 24 × 21 cm chamber positioned in a 110 × 50 × 60 cm plastic cabinet. The mice were placed in a chamber during the training and allowed to explore the chamber for 2 min. After 2 min, the mice were exposed to the audio tone for 28 s (conditional stimulus: 5 kHz, 70 dB) followed by a 2-seconds foot shock (unconditional stimulus: 0.5 mA) from the metal grid floor. The intensity of the foot shock was from a preliminary test with other experimental mice which were not involved in the experiments to determine the intensity of foot shock that stimulates a response. The total experimental time was 5 min with each session lasting 30 s. A camera was used in the chamber to record the movements of mice. "Freezing" was assessed by calculating the length of any period with no movement longer than 1 s in the chamber. On the 2nd day, memory deficits of the mice were assessed by both cued conditioning and contextual tests. The contextual fear session consisted of 5 min without any shock or audible tone. Mice were placed in the conditioning chamber for the contextual session, and the duration of freezing was recorded as an index of contextual memory assessment. The tone-associated and cued conditioning tests were performed on the experimental mice after an interval of 3 h. When the mice were returned to the experimental chamber to experience the different context, the floor and the wall of the chamber was covered by a white plastic board and a black plastic was inserted onto the walls. The mice were allowed to explore the experimental chamber for 5 min in absence of audio tone and then followed by 5 times 30-sec audio tones repeated every 30 s apart during the session. The freezing time during and between the audio tones were recorded as an index of cued memory assessment. The recorded video data was analyzed and assessed using video tracking software equipment (Ugo Basile, Italy).

2.7. Tau extraction

The extracts of sarkosyl-soluble and -insoluble tau species from 3XTg-AD mice brain were prepared as described previously [16]. Briefly: For post-nuclear brain lysate preparation, 3XTg-AD mice brain was homogenized in 12 volumes of RIPA buffer with a tablet of a cocktail of protease inhibitors and phosphatase inhibitors (Roche). The homogenates were kept on ice for further processing. The homogenates (2 ml) were centrifuged at 30,000 g for 20 min to separate proteins into soluble (S1, supernatant) and insoluble (pellet) fractions. The supernatant containing soluble non-pathological tau proteins was removed. The RIPA-soluble fraction was incubated with 1% sarkosyl for 1 h or 2 h at room temperature. After ultracentrifugation at high speed (100000 g) for 60 min, sarkosyl-insoluble proteins in the pellet fraction were collected. The sarkosyl-soluble protein in supernatant was designated as the S2 fraction. The pellet fraction was re-suspended in 50 μl of RIPA and labeled as the P2 fraction (sarkosyl-insoluble tau) [17].

2.8. Western blot analysis

Immunoblot analysis was performed as per the protocol described by us [11,18]. Cell lysates or brain lysates (equal to 5–10 μg of total protein) were separated on 8% or 10%–15% double layered SDS–PAGE gels and the proteins were blotted onto PVDF membranes to detect the protein level of PHF-1 (phosphorylated tau), AT8, CP13, MC1 and β-actin (Table 1). The blotted membranes were blocked with 0.1% Tween 20 in 5% skimmed milk; the blots were probed with primary antibodies overnight at 4 °C with shaking. After overnight incubation, the blots were washed in TBST and incubated with corresponding horseradish peroxidase-conjugated secondary antibodies at room temperature. Goat anti-mouse IgG was used (1:10,000) when the designated primary antibody was PHF-1 (phosphorylated tau), AT8, CP13, MC1, and βactin; goat anti-rabbit IgG (1:10,000) were used when the designated primary antibody was full length APP (FL-APP) and CTFs. One or two hours after incubation with secondary antibody, the immunoblots were incubated with West Pico or West Femto chemiluminescent substrate kit (ThermoScientific, USA) for 5 min and developed using X-ray film (Kodak) in the dark room. The developed films were scanned, and the percentage of band relative intensity was analyzed using Image J software (NIH Image).

2.9. Immunohistochemistry (IHC)

Paraformaldehyde-fixed, frozen brains of 3XTg-AD mice were sectioned in three different regions; slices of around 30 μm thick were mounted on coated slides, and processed for IHC as described by us [18]. Three different sets of brain slice sections in each region were prepared for immunostaining analysis. For tau immunostaining, AT8 (phospho-Tau) was assessed separately. Amyloid deposits in the mice were confirmed with immunostaining using 4G8 amyloid-specific antibodies (Millipore). For staining, 4G8 or AT8 antibodies were used to detect the localization of these antigens in the brain slices. The brain slice sections of each anatomic region of interest were captured by a camera fitted on the microscope, and image analysis was done using Image J analysis (NIH) software. To perform the DAB staining in the brain slices, endogenous peroxidase activity was quenched by incubating the brain slices with 1% H_2O_2 for 10 min. After H_2O_2 incubation the brain slices were washed with 0.4% Triton-X100 in PBS. Then the processed brain slices were blocked with 2% BSA for 30 min at room temperature. The blocked brain slice sections were incubated with primary antibody biotinylated AT8 and 4G8 for 12 h overnight at 4 °C. The primary antibody-incubated brain slices were washed with PBS and incubated with ABC kit (VECTOR, Burlingame, USA) for 30–60 min at room temperature. To stain the brain slice sections with DAB, brain slices were incubated for 5–10 min in DAB reagent [11]. The brain slice sections were placed on slides, dried in an oven at 55 °C, and mounted with Leica mounting medium. Images of the

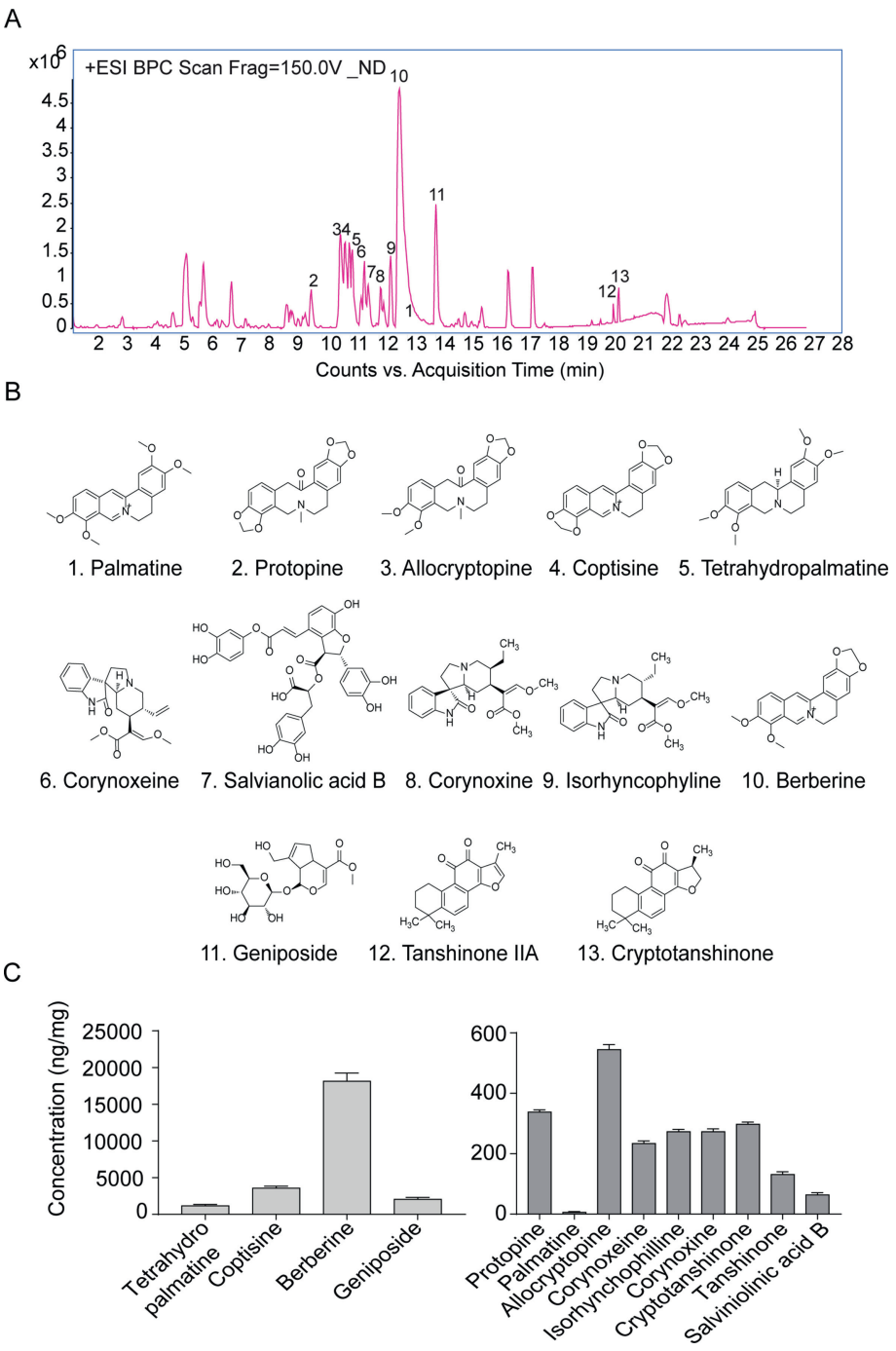


Fig. 1 – Herbal preparation and qualitative analysis of ND. (A) LC-ESI-Q/TOF chromatograms (TIC) of ND, (B) Peaks: 1. Palmatine, 2. Protopine, 3. Allocryptopine, 4. Coptisine, 5. Tetrahydropalmatine, 6. Corynoxeine, 7. Salvianolic acid B, 8. Corynoxine, 9. Isorhyncophyline, 10. Berberine, 11. Geniposide, 12. Tanshinone 2A, 13. Cryptotanshinone. (C) Bioactive components in ND determined by LC-ESI-Q/TOF chromatogram. The quantitative analysis for the major components and the minor components of ND.

brain slices were assessed using Image J analysis (NIH) software.

2.10. ELISA

The whole brain homogenates of the ND-treated and vehicle AD mice were subjected to three sequential extractions using Tris buffered saline (TBS)-, TBS with 1.0% SDS (TBS-SDS)- and formic acid-soluble fraction. These extractions were subjected to ultra-centrifugation at 100,000 $\times g$ for 1 h by the method described previously [9,11,18]. SDS soluble fractions were subjected to ELISA for detection of detergent soluble Aβ and for immunoblot analysis. Formic acid fractions were subjected to ELISA for insoluble $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$.

2.11. Sub-chronic toxicity

As the first step of the chronic toxicity study of ND, we assessed the toxicity of ND as per OECD guidelines for testing drugs toxicity [OECD 407, EC B.7]. The high doses of ND extract were 10–25% of the corresponding LD_{50}, while the medium and low doses of QYS were two-fold diluted. After the observation period, all mice in the ND-treated and control groups were sacrificed, and gross necropsy and histopathological studies were performed [19,20]. The tissues of brain, liver, kidneys, heart and spleen from each mouse in the sub-chronic toxicity study were preserved in 10% neutral buffered formalin for histopathological examination. The preserved tissues were molded in paraffin and cut into 5 μm-thick sections; sections were stained with haematoxylin and eosin (HE), according to the standard method. Histopathological observations were made using a light microscope (Nikon E600; magnification, ×20).

2.12. Pharmacokinetics study

A non-compartmental pharmacokinetic analysis was performed on the plasma and brain concentrations for each mouse using DAS 3.0 pharmacokinetic program (BioGuider Co., Shanghai, China). Pharmacokinetic parameters determined were, namely time to reach Cmax (Tmax), maximum concentration (Cmax), area under the concentration–time curve (AUC0-t, AUC0-∞) using a linear trapezoidal method from time 0 to the last time observed concentration time

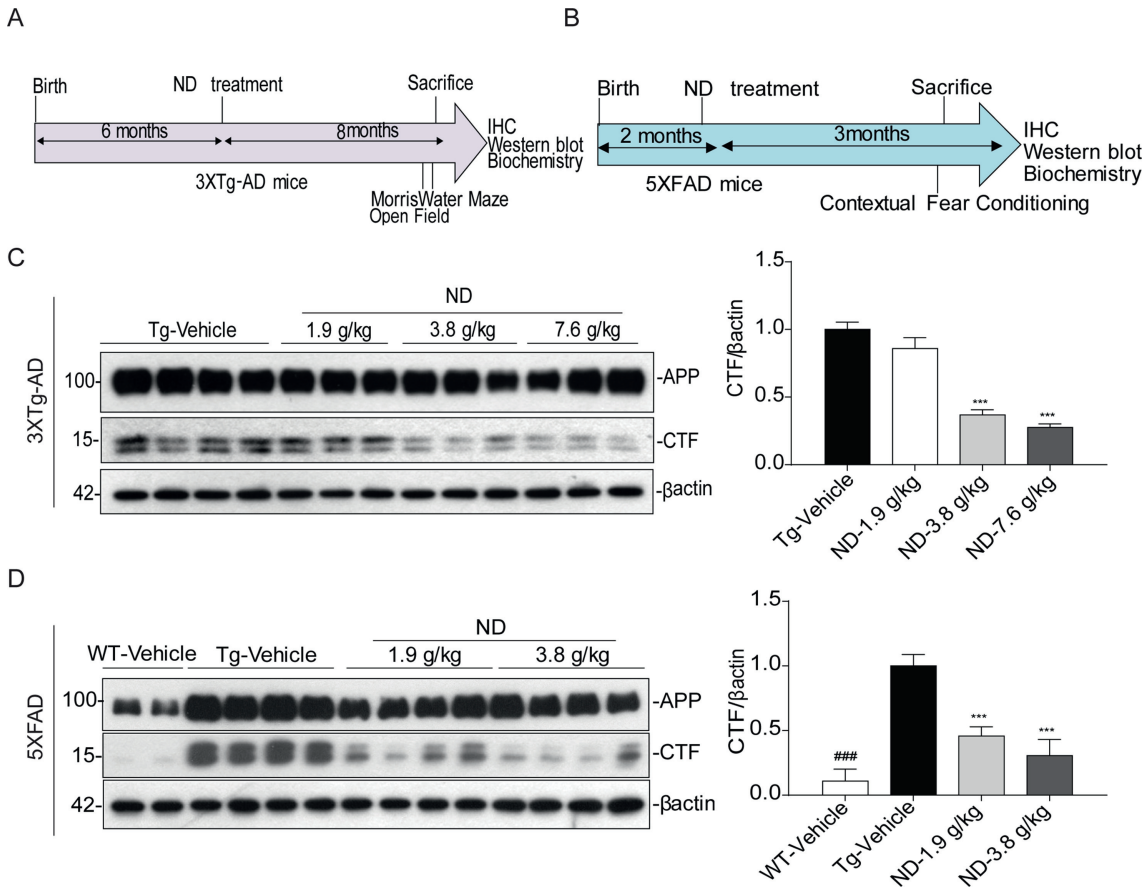


Fig. 2 – ND reduces the levels of APP metabolites, Aβ pathology in 3XTg-AD and 5XFAD mice. Chronic treatment effects of ND on the levels of APP metabolites in 3XTg-AD mice. (A) Timeline for ND treatment and behavior experiments schedule in 3XTg-AD (1.9, 3.8, 7.6 g/kg) and (B) 5XFAD (1.9, 3.8 g/kg) mice. (C) The levels of FL-APP and CTFs (CTFα and CTFβ) presented in immunoblot representing treatment effects of ND and its quantification on the ratio of CTFs against βactin in the SDS brain lysates of 3XTg-AD and (D) 5XFAD mice treated with ND or Tg-vehicle. Data represent mean ± SEM. N = 10 in each group, the statistical significance are denoted as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ treatment group when compared with vehicle-treated 3XTg-AD mice.

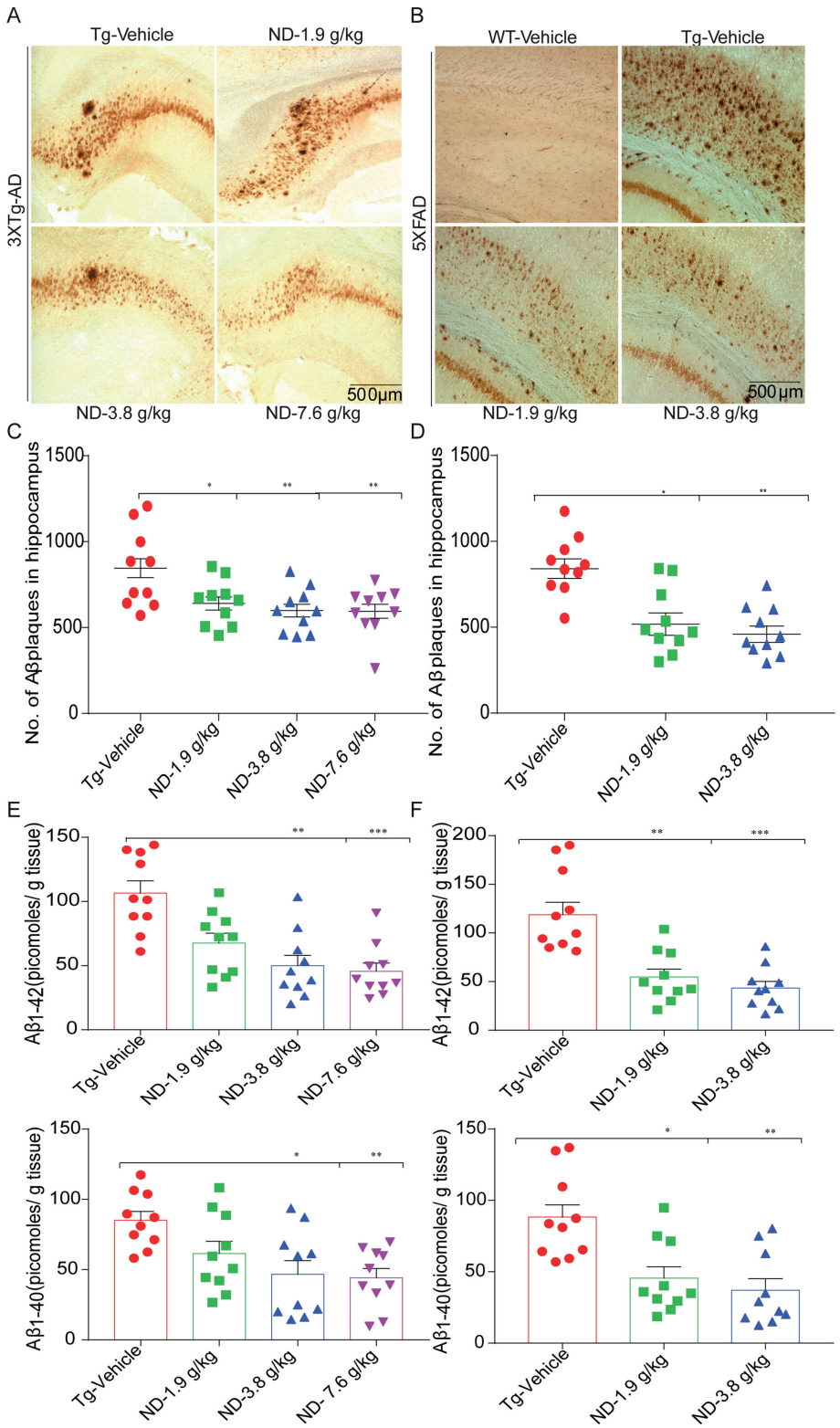
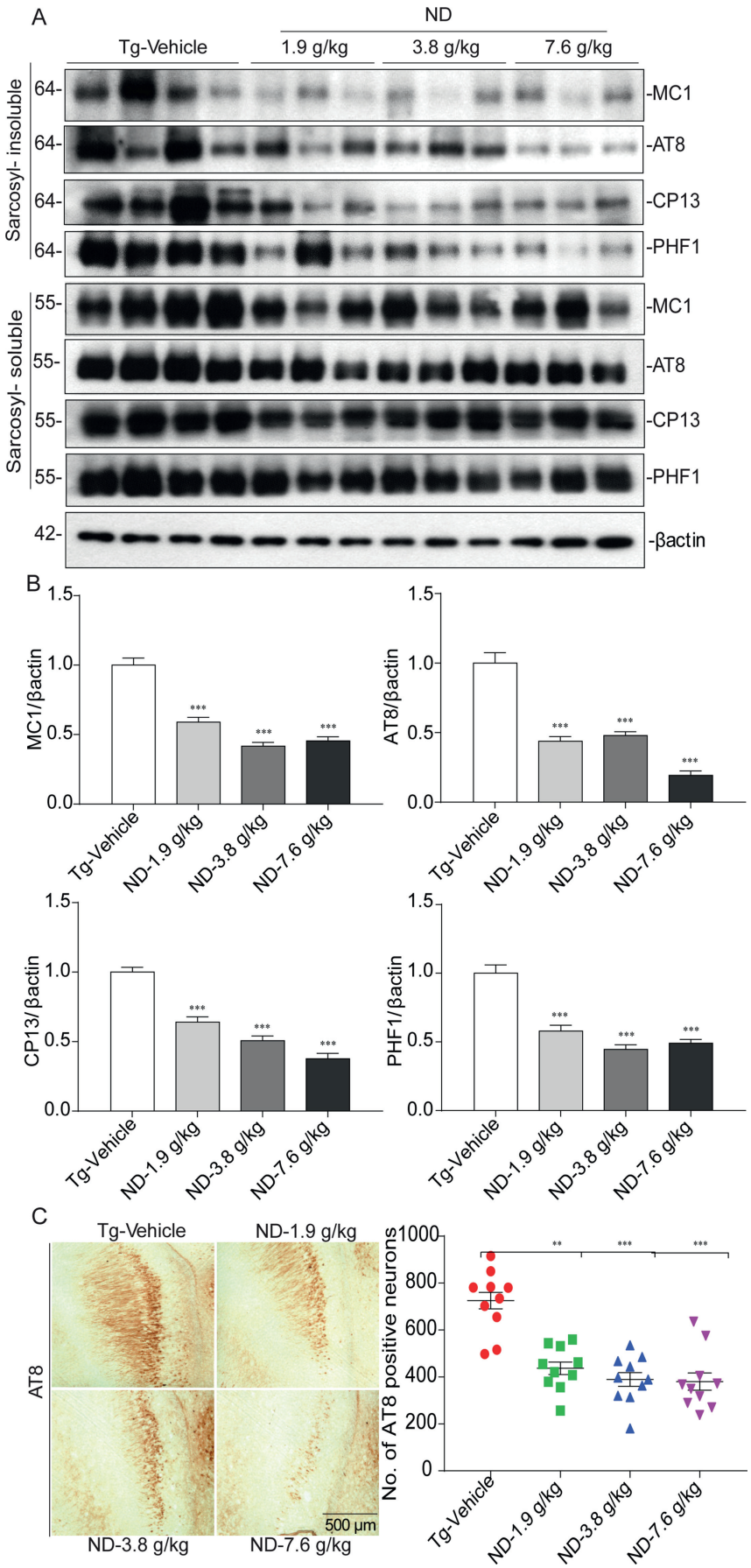


Fig. 3 – ND treatment mitigates Aβ pathology and reduces Aβ plaques in 3XTg-AD mice. ND treatment reduces 4G8 positive neurons in 3XTg-AD mice. Representative figures of coronal sections from Tg-vehicle and ND treated (A) 3XTg-AD and (B) 5XFAD mice. Quantification of 4G8 positive neurons in hippocampus in the brain sections of (C) 3XTg-AD and (D) 5XFAD mice. (E) Chronic treatment effects of ND on the levels of Aβ1-42 and Aβ1-40 in the formic acid soluble brain lysates of 3XTg-AD mice and its quantification. (F) Chronic treatment effects of ND on the levels of Aβ1-42 and Aβ1-40 in the formic acid soluble brain lysates of 5XFAD mice and its quantification. Data represent mean ± SEM. N = 10 in each group, $^{**}p < 0.01$; $^{***}p < 0.001$.


A
ND
Tg-Vehicle
1.9 g/kg
3.8 g/kg
7.6 g/kg
Sarcosyl- insoluble
Sarcosyl- soluble
64-
55-
42-
-MC1
-AT8
-CP13
-PHF1
-βactin
B
MC1/βactin
AT8/βactin
CP13/βactin
PHF1/βactin
1.5
1.0
0.5
0.0
***
Tg-Vehicle
ND-1.9 g/kg
ND-3.8 g/kg
ND-7.6 g/kg
C
AT8
Tg-Vehicle
ND-1.9 g/kg
ND-3.8 g/kg
ND-7.6 g/kg
500 μm
No. of AT8 positive neurons
1000
800
600
400
200
0
**
***

point, oral clearance (CL/F) and mean residence time (MRT0-t and MRT0-∞), the area under the curve (AUC), the area under the first moment curve (AUMC), the mean residence time (MRT), the variance of residence time (VRT), the terminal elimination half-life (t1/2), the clearance (Cl), the volume of distribution at terminal phase (Vz). Pharmacokinetics parameters were calculated for QYS aglycone. The LC-ESI Triple-Quad/TOF (an Agilent 6410 triple-quadrapole mass spectrometer) or LC-MS-Q-TOF method was applied to analyze the concentrations of key markers in the plasma and brain samples.

2.13. *In vitro studies*

N2a cells were cultured in DMEM supplemented with 10% FBS. The cells were transiently transfected with human APPSwe/Ind (APP 695 Swedish/Indiana mutation) (30145, Addgene) or P301L-Tau (46908, Addgene) plasmids with empty vectors as the control, and then treated with 8 major active compounds namely berberine, coptisine tetrahydropalmatine, corynoxeine, corynoxine, protopine, allocryptopine and geniposide (10 μm) of ND for 24 h and the cells were extracted using the RIPA buffer and the cell lysates were run by Western blot to identify the protein expressions of different targets namely APP,CTFs, PHF1 and β-actin.

2.14. *Statistical analysis*

All the analyzed data were presented in histograms as mean ± S.E.M. The statistical analysis of data was assessed using one-way ANOVA or two-way ANOVA followed by post hoc comparison of the mean ± S.E.M using Tukey multiple comparison test and Bonferroni's or Dunnett's T3 methods. A probability value of $P < 0.05$ was considered to be statistically significant. GraphPad Prism 6 was used to perform the graphical presentation and statistical tests (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. *Herbal preparation and qualitative analysis of NeuroDefend*

Qualitative analysis of ND by LC-QTOF/MS was carried out at HKBU. The total ion current (TIC) chromatograms corresponding to positive ions of ND is shown in (Fig. 1A). The most abundant thirteen peaks in the chromatogram were identified and confirmed in comparison with an external standard marker depicting in high-resolution MS and MS/MS fragmentation. Quantitative analysis of ND formulation was performed, and the levels of major and minor bioactive components are given in (Fig. 1B and C). The major components of ND are: berberine, geniposide, tetrahydropalmatine, coptisine and minor components protopine, palmatine, allocryptopine, corynoxeine, corynoxine, isorhynchophylline, salvianolic acid B, Tanshinone 2A, and cryptotanshinone.

3.2. *Sub-chronic toxicity and pharmacokinetics of ND*

In preliminary experiments, we investigated the toxicity of ND in wild-type C57BL/6 mice. The oral LD_{50} of ND for these mice was around 76 g/kg, respectively (Data not shown). Since AD therapy requires long-term and repeated treatment, the *in vivo* biocompatibility of ND is critical for the safety evaluation. Therefore, we used three different doses of ND namely 1.9 g/kg, 3.8 g/kg and 7.6 g/kg for sub-chronic toxicity in wild-type SD rats. These studies revealed that ND did not show any adverse effect during the chronic toxicity testing (Supplementary Fig. 1C and D), and the doses given were well tolerated by the animals according to histopathological parameters (Supplementary Fig. 1E). In addition, we evaluated the brain permeability of the ND in ICR mice. We performed pharmacokinetics by LCMS and found brain bioavailability of major bioactive components of ND in the ICR mice brain and plasma (Supplementary Figs. 2A and 3B). Pharmacokinetic parameters are given in (Supplementary Tables 1A and 1B) for understanding the pharmacological effect of ND in treating AD.

3.3. *ND reduces the levels of APP metabolites, Aβ pathology in 3XTg-AD and 5XFAD mice*

We orally administered ND (1.9, 3.8, 7.6 g/kg/d) in the form of feed admixture to both 3XTg-AD and 5XFAD mice models. During the treatment period, the animal body weight and the food intake were assessed every week (Supplementary Fig. 1A and 1B). Time line for ND treatment and schedule of behavior experiments is shown in (Fig. 2A and B) for 3XTg-AD and 5XFAD mice models. We further investigated the anti-Aβ effect on APP metabolites in the 3XTg-AD and 5XFAD mouse model to assess the long-term effects of ND (1.9, 3.8, 7.6 g/kg/day). Specifically, we elucidated the levels of APP metabolites in the brain homogenate using immunoblot. ND treatment significantly and dose-dependently reduced the levels CTFs in SDS brain homogenates (Fig. 2C and D) when compared to the Tg vehicle. These *in vivo* data show that ND have the potential to reduce Aβ pathology in the 3XTg-AD and 5XFAD mouse model.

To further confirm the above results, we investigated the long-term effect on Aβ reduction of ND (1.9, 3.8, 7.6 g/kg/day) *in vivo* 3XTg-AD and (1.9, 3.8 g/kg/day) 5XFAD mouse models. We performed Immunohistochemical analyses of 30 μm-thick brain slices from anterior, medial and posterior hippocampus regions of the brain by estimating the number of 4G8-positive neurons. ND reduced hippocampal Aβ-plaque burden in brain slices significantly and dose-dependently

Fig. 4 – ND reduces the level of insoluble phosphorylated and misfolded tau in 3XTg-AD mice. Chronic treatment of (A) ND reduced the insoluble phospho and pathological tau levels in the sarcosyl insoluble brain lysates of 3XTg-AD mice. (B)The densitometric analysis of the insoluble tau levels namely AT8, MC1, CP13 and PHF1 in 3XTg-AD mice brain lysates of ND treatment. (C) ND treatment reduces AT8 positive neurons in 3XTg-AD mice, the representative figures of coronal sections from Tg-vehicle and ND treated mice and its quantification of AT8 positive cells in the brain sections of 3XTg-AD mice. Data represent mean ± SEM, N = 10, $^{**}p < 0.01$; $^{***}p < 0.001$.

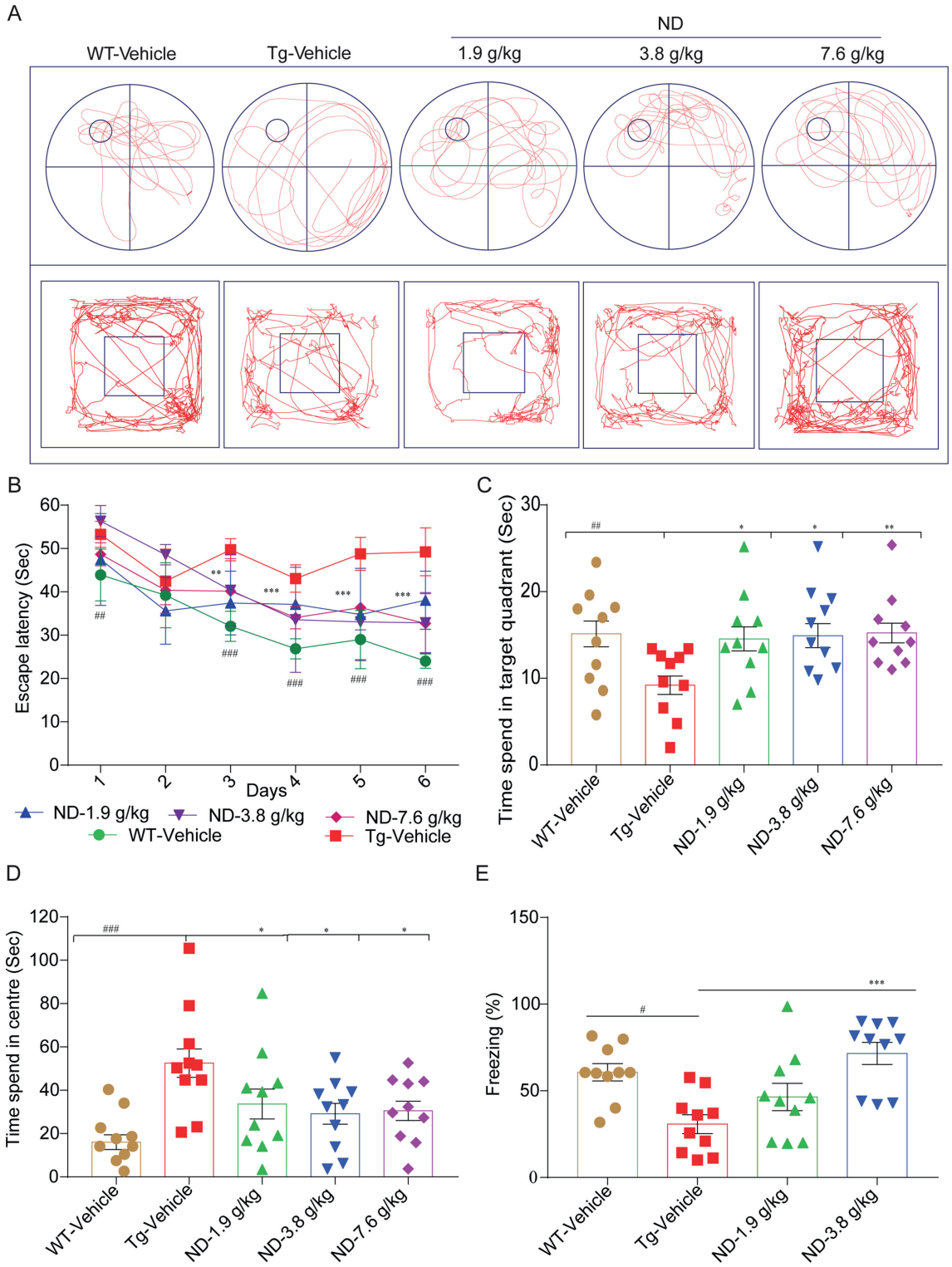


Fig. 5 – ND treatment ameliorates spatial learning, memory in 3XTg-AD and 5XFAD mice. In Morris water maze test followed by visible platform training, 3XTg-AD mice were trained for another 6 days to learn the location of a hidden platform. (A) The pictures depict animal's behavior of ND treatment groups on probe trial and open field by video tracking software ETHOVISION, Nodulus. The learning memory was quantified in comparison of Tg-Vehicle and (B) ND treated 3XTg-AD mice. Each point represents the mean values of 4 trials per day, (N = 10). $^{###}p < 0.001$ (WT treated with vehicle vs. Tg treated with vehicle); $^{***}p < 0.001$ (Tg treated with ND vs. Tg treated with vehicle). The symbols denote differences between the given groups over all trial days. In the probe trial, the (C) ND, treated mice stayed longer in the target quadrant than the vehicle-treated Tg mice. $^{###}p < 0.001$ (WT treated with vehicle vs. Tg treated with vehicle); $^{***}p < 0.001$ (Tg treated with ND vs. Tg treated with vehicle), (N = 10). Memory and learning improved in ND treatment groups of 3XTg-AD mice compared to

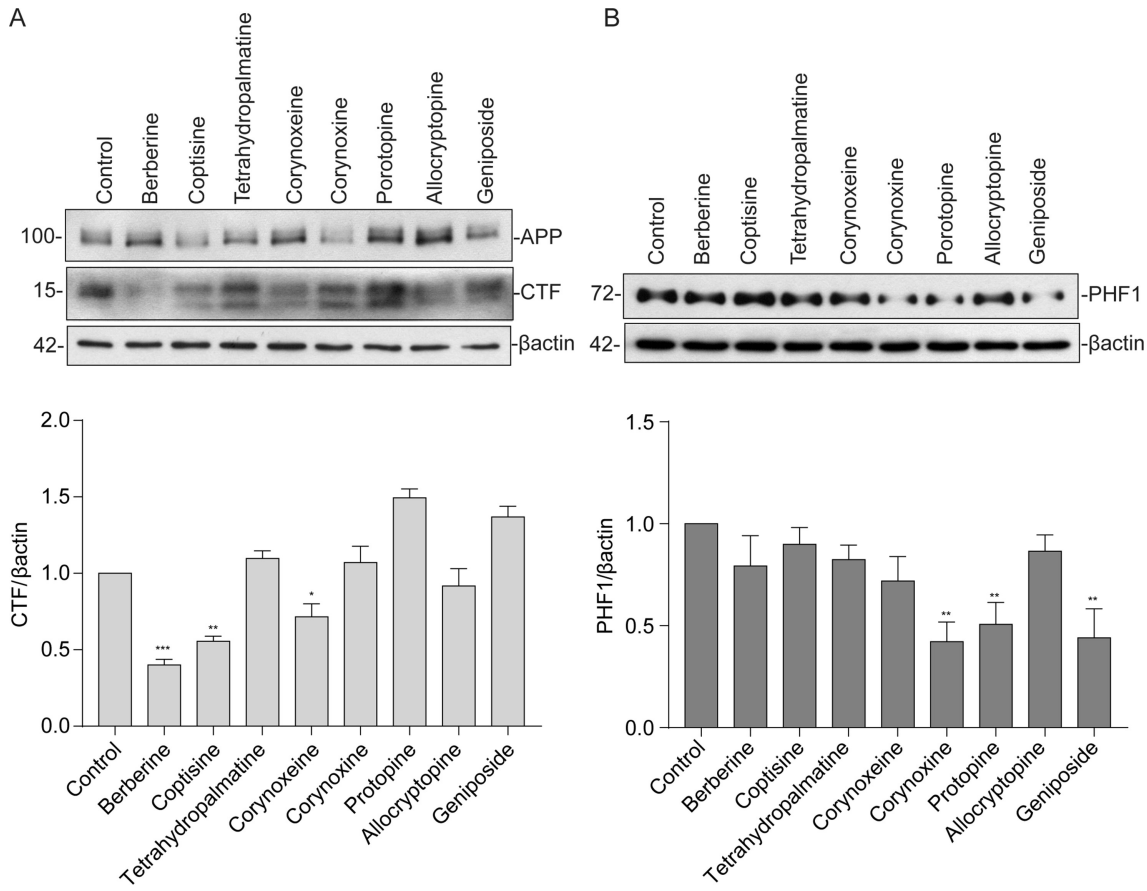


Fig. 6 – ND active compounds reduces the levels of APP metabolites and phospho-tau in Neuronal cells. (A) N2a cells overexpressing APP695, were treated with 8 major active compounds of ND, among the active compounds berberine, coptisine and corynoxeine significantly reduced the levels of CTFs in the N2a-APP cells and its quantification. (B) N2a cells expressing P301L-Tau, were treated with 8 major active compounds of ND, among the active compounds corynoxine, protopine and geniposide significantly reduced the levels of PHF1 in the N2a-P301L cells and its quantification.

when compared to the Tg-Vehicle group in 3XTg-AD (Fig. 3A and C) and 5XFAD (Fig. 3B and D) mouse models. Differential extraction of brain tissues by SDS and formic acid is a protocol generally used to quantify the detergent-soluble and formic acid-soluble Aβ species, respectively, in AD mouse brain. In addition, we evaluated the $A\beta_{1-42}$ and $A\beta_{1-40}$ burden in the formic acid-soluble fraction of 3XTg-AD and 5XFAD brain homogenate by ELISA. ND decreased the levels of $A\beta_{1-42}$ and $A\beta_{1-40}$ (Fig. 3E and F) significantly and dose-dependently compared to the Tg-Vehicle group. These *in vivo* data suggest that ND have the potential to reduce Aβ pathology and hippocampal Aβ plaque burden in a dose-dependent manner.

3.4. *ND treatment mitigates tau pathology and reduces phospho-tau in 3XTg-AD mice*

To assess tau pathology in the brains of 3XTg-AD mice, we performed Western blot analyses for the different fractions of brain homogenate. The insoluble tau in the 3XTg mice brain was separated by differential extraction into soluble and insoluble tau fractions and were designated as sarkosyl-soluble and sarkosyl-insoluble tau, respectively. Phosphorylated tau species in both the soluble and insoluble fraction were detected by AT8, CP13, and PHF-1 antibodies. The misfolded tau species was detected using MC1 antibody. The phosphorylated and misfolded tau in the sarkosyl-insoluble fraction was significantly reduced in ND-treated groups when compared to the vehicle-treated group (Fig. 4A and B). However, there were no significant differences in the sarkosyl-soluble fraction of ND-treated groups when compared to the Tg-vehicle-treated group. Notably, these effects are concomitant with a large reduction of insoluble tau levels, suggesting that ND can reduce abnormal tau aggregation in 3XTg mice.

Further we used AT8 monoclonal antibodies to assess the insoluble phosphor-tau load in the brain. The epitope of AT8 is located outside the region of internal repeats of

the Tg vehicle, the data of animal's behavior was quantified by video tracking software ETHOVISION, Nodulus. (D) Exploratory behavior and locomotor activity of 3XTg-AD mice treated with ND improved in open field experiment and was quantified with Ethovision software, Nodulus. (E) Memory improved in ND treated mice compared to the Tg vehicle and ND ameliorates hippocampal-dependent memory in 5XFAD mice by contextual fear conditioning.

microtubule binding domains (RT1-4) and requires the phosphorylation of Ser96/Ser404. Immunostaining AT8-positive neurons in the brains of 3XTg-AD mice revealed the insoluble phospho-tau load. ND reduced the hippocampal phospho-tau load significantly and dose-dependently (Fig. 4C) when compared to the Tg-Vehicle group. These *in vivo* data suggest that ND have the potential to reduce hippocampal insoluble tau load and phospho-tau burden in the brains of 3XTg-AD mice. Altogether, these results demonstrate that ND have the potential to reduce tau pathology in 3XTg-AD mice.

3.5. *ND treatment ameliorates spatial learning and memory in 3XTg-AD and 5XFAD mice*

The 3XTg mouse model shows late onset of symptoms, which is more similar to humans. Hence, we used this model to evaluate the long-term effect of ND on the amelioration of cognitive deficits. Further, to evaluate the long-term effect of ND on the amelioration of cognitive deficits in 3XTg-AD mice, we performed the Morris water maze experiment. Acquisition of spatial learning and memory were evaluated in the ND-treated 3XTg-AD mice. The mice were trained for 6 days in the Morris water maze to analyze the escape latencies of the ND-treated 3XTg-AD mice after the training period. During the 6 days of hidden platform training, all trained mice in the groups learned to identify the platform and as training progressed the escape latencies of mice decreased significantly except the Tg-vehicle treated group. The escape latencies of ND-treated groups during the 6-day training were significantly shorter than those of transgenic (Tg) vehicle-treated group (Fig. 5A and B). During the same 6-day training the wild type-vehicle (WT) groups exhibited significantly shorter distance travelled than those of Tg-vehicle group in learning. To test the memory in ND-treated and Tg-vehicle treatment groups, we performed probe trial 24 h after the 6th day of training. On the probe trial day, the ND-treated groups (Fig. 5C) spent longer time in the quadrant which had the platform earlier, as compared to the Tg-vehicle treated group. We conclude that memory and learning improved in the ND treatment groups compared to the Tg-vehicle group (Fig. 5A).

The exploratory behavior and locomotor activity of untreated 3XTg-AD mice and ND treated 3XTg-AD mice were assessed with open field test. No significant differences were observed between the vehicle and three ND groups in time spent in the center (Fig. 5D). ND did not cause any notable harmful side effects, so we concluded that ND preparations were well tolerated during the treatment period. These results indicate that all doses of ND did not affect the locomotor activity and exploratory behaviors compared to the Tg-vehicle group (Fig. 5A).

Further we investigated the amelioration of cognitive deficits of ND in 5XFAD mouse model because this model showed early aggressive Aβ accumulation. The hippocampal and amygdala-dependent memory deficit were tested by contextual fear conditioning. ND treated animals significantly froze more than vehicle-treated 5XFAD mice in freezing behavior and in the auditory cue test (Fig. 5E). Hence, ND ameliorates hippocampal and amygdala-dependent memory deficit in 5XFAD mice.

3.6. *ND active compounds reduces the levels of APP metabolites and phospho-tau in neuronal cells*

Our animal studies in 3XTg-AD and 5XFAD have demonstrated the efficacy of ND in memory, anti Aβ effect and anti-tau effect. We further validated the efficacy of active compounds of ND on neuronal cells. To test the Anti-AD effect of the active compounds, we tested the 8 major active compounds namely berberine, coptisine tetrahydropalmatine, corynoxeine, corynoxine, protopine, allocryptopine and geniposide on APP turnover and tau degradation in N2a cells. Firstly, in N2a cells overexpressing APP695, among the active compounds berberine, coptisine and corynoxeine significantly reduced the levels of CTFs in the N2a-APP cells (Fig. 6A). Further we evaluated anti-tau effect of the active compounds in the N2a cells expressing P301L-Tau, among the active compounds corynoxine, protopine and geniposide significantly reduced the levels of PHF1 in the N2a-P301L cells (Fig. 6B). Together, these results demonstrated that active compounds of ND promote anti-Aβ effect and anti-tau effect *in vitro*.

4. Discussion

Despite tremendous efforts in developing a disease-modifying drug for AD, none has been identified. Here, we demonstrate that ND for the treatment of AD because it has both the tau-reducing effect and anti-Aβ effect in the 3XTg-AD and anti-Aβ effect in the 5XFAD mouse model. The actual therapeutic value of the Chinese medicine ND in terms of its anti-Aβ effect, anti-tau effect and effect on cognitive deficits has not been validated so far. Therefore, we used 5XFAD mice model for the short-term treatment paradigm and 3XTg-AD mice model for the long-term treatment strategy to verify the NeuroDefend efficacy in animal models.

From our results, it is notable that ND is better in improving memory deficits in comparison to HLJDT-M in both 3XTg-AD and 5XFAD mice models, as ND is a combination of HLJDT-M with three other herbs added (i.e., Danshen, Gouteng and Yanhusuo). This improved anti-AD effect could be partly explained by studies performed using individual herbs [21]. Studies have shown that Danshen has significant cognition and memory improving capabilities in reducing intracerebroventricular $A\beta_{25-35}$-induced memory impairment in rat models. In this study, Danshen improved cognitive and memory functions by down-regulating APP and PS1 mRNA and protein levels, and simultaneously up-regulating IDE (Aβ degrading enzyme) mRNA and protein levels [22]. An *in-vitro* study showed active alkaloids (coptisine, berberine, palmitine, dehydrocorydaline and jatrorrhizine) from Corydalis yanhusuo can significantly inhibit acetylcholine esterase activity. Hence, addition of Yanhusuo may have significantly contributed to its neuroprotective activity in our ND formulation [23,24]. In the present study, we have shown evidence that specific formulation of the traditional Chinese medicine ND significantly improves spatial memory, learning and memory in 3XTg-AD and 5XFAD mice. Our results are consistent with those of our previous studies of HLJDT and HLJDT-M [11]. In this study, we have observed that ND reduced

insoluble tau levels in the brain homogenates of 3XTg-AD mice and decreased AT8 positive phosphor-tau in the brain slices of 3XTg-AD mice. An abnormal shift of normal brain tau from soluble tau to insoluble tau plays a mechanistic role in the onset and progression of AD [4]. Here, we have shown that the tested ND formulation facilitated the reduction of insoluble tau and phospho-tau in 3XTg-AD mice model, further confirming the capability of this formulation to delay the disease progression of AD. Hence, addition of Yanhusuo may have significantly contributed to its anti-tau activity in our ND formulation [23,24].

The brain homogenates of 3XTg-AD mice showed a significant decrease in the CTFs, the APP metabolite for Aβ formation, which clearly indicates the reduction of pathological Aβ aggregates and is verified in ND-treated 5XFAD mice model. In addition, we have evaluated the senile plaque formation in the brain slices of 3XTg-AD and 5XFAD mice by estimating the 4G8 positive neurons. ND treatment significantly reduced the Aβ plaques in both models 3XTg-AD and 5XFAD mice. These results are consistent with our previous studies of HLJDT and HLJDT-M [9–11]. The ability of ND to arbitrate a decrease in insoluble Aβ and plaques may elucidate its capacity to delay the onset of AD in both the mice models. Our previous study has shown Sal B from *Salvia miltiorrhiza Bunge* is a BACE1 inhibitor which may have contributed for the reduction of Aβ in ND treated mice [25]. The active compounds of ND have proved its efficacy to promote anti-Aβ effect and anti-tau effect in the cell model studies. Active compounds berberine, coptisine corynoxeine, corynoxine, protopine and geniposide have significantly reduced the levels of CTFs and phosphor-tau in the N2a cells (Fig. 6) which clearly illustrates its efficacy in AD.

In conclusion, our results suggest that specific variations of our NeuroDefend formula can improve spatial memory, learning and memory retention in 3XTg-AD and 5XFAD mice. ND formulation has shown an outstanding and promising therapeutic effect, including anti- Aβ effect, anti-tau effect and memory improvement in AD mice models. Our study reveals that ND treatment by oral feed admixture reduced tau pathology and Aβ pathology and enhances memory function in AD animal models. Since ND formulation is composed of several active compounds, their mechanism of action for AD therapy will be studied in the future. In the future, ND could be developed as a health food supplement, or its raw materials could be used as medicine to prevent or treat AD. This study contributes to the development of novel TCM formula for the treatment of AD.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jfda.2019.09.004.

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