Dian-Xian-Qing granule attenuates learning and memory deficits by reducing tau hyperphosphorylation in amyloid1-42-induced mice

Yue Qi[1], Dong Jia[1*], Kai Kang[2], Hong Jiang[1], Min Qiao[1], Xiao-Hu Liu[1], Shao-Jie Xiang[1], Jing-He Zhu[1], Wen-Yan Qin[1], Li-Zhao[2], Ya-Bin Wang[2]

[1]Department of Pharmacology, the Second Hospital Affiliated to Liaoning Chinese, Medical University, Shenyang 110034, PR China

[2]College of Pharmacy, Liaoning University of Traditional Chinese Medicine, Shenyang 110034, PR China

Abstract

Our previous study demonstrated that DXQ has neuro protective effects in the AD mouse model. However, the exact mechanism through which DXQ improves amyloid beta (Aβ)-induced memory impairment by inhibiting the expression of hyper-phosphorylated tau protein is unknown. Aβ1-42 was slowly administered via the intra cerebro-ventricular (ICV) route in a volume of 3 μL (≈410 pmmol/ mouse) to mouse. The mice were administered DXQ (3.12, 6.24 or 12.48 g/kg) or vehicle starting from the second day after Aβ1-42 injection to the end of the experiment. Behavioural test was performed from on day 21. On day 22 after the ICV administration of Aβ1-42, the mice were sacrificed for biochemical analysis. DXQ (3.12-12.48 g/kg) significantly improved learning and memory impairments assessed by Y maze test. Western blotting revealed that DXQ decreased tau phosphorylation protein levels at Thr181 and Ser 404 in the hippocampus and increased the phosphorylation levels of phosphatidylinositol-3-kinase (PI3K), threonine/serine protein kinase B (Akt) and glycogen synthase kinase-3β (GSK-3β). These results suggest that DXQ rescues learing and memory in Aβ1-42-induced mice via the PI3K/Akt-dependent GSK-3β signalling pathway.

Keywords: Alzheimer's disease, Tau, Glycogen Synthase Kinase-3β, Phosphatidylinositol-3-kinase, Threonineserine Protein Kinase B.

Accepted on 26 April, 2017

Introduction

Alzheimer's disease (AD) is a neurological condition characterized by progressive cognitive deterioration and memory loss. Extracellular amyloid beta (Aβ) deposits are assumed to be initial pathological markers of AD [1,2]. It has been proposed that the deposition of Aβ in AD lead to the formation of senile plaques (SPs) and subsequently to intraneuronal neurofibrillary tangles (NFTs) composed of aggregates of hyperphosphorylated tau [3].

Tau is a microtubule-associated protein predominately expressed in neurons, which stabilizes microtubules under physiological conditions, and as such regulates axonal stability and cell morphology [4]. Under pathological conditions such as AD, tau is abnormally hyperphosphorylated leading to a decrease in its affinity for microtubules, resulting in cognitive impairments [5]. Steady status of tau phosphorylation is maintained by tau protein kinases and phosphatases. Numerous studies have demonstrated that many kinases and phosphatases are involved in regulation of tau phosphorylation, such as protein phosphatase 1, protein phosphatase 2, mitogen-activated protein kinase, cyclin-dependent kinase 5, protein kinase C, and glycogen synthase kinase-3β (GSK-3β) [6].

As a major tau kinase, GSK-3β is a downstream target of the phosphatidylinositol-3-kinase (PI3K)/threonine/serine protein kinase B (Akt) signalling pathway, and involved in most of the hyperphosphorylated serine/threonine sites in tau both *in vivo* and *in vitro* [7]. Therefore, the PI3K/Akt/GSK-3β signalling pathway is considered as an important target for the diagnosis and treatment of AD characterised by tau pathology.

Dian-Xian-Qing granule (DXQ) consists of Herba Acori Tatarinowii Rhizoma (Acori tatarinnowii Schtt, Shichangpu: Anhui, China), herba Paeoniae Radix Alba (Paeonia lacti flora Pall,Baishao:Anhui,China), herba Polygoni Multiflori Radix (Polygonum multi florum Thumb, Heshouwu: Zhejiang, China) and herba Anemarrhenae Rhizoma (Anemarrhena asphodeloides Bge, Zhimu:Neimenggu, China) and so on, which are selected based on the theory of traditional Chinese medicine by senior experienced physicians.

Our previous study demonstrated that DXQ exerts neuroprotective effects *in vivo* in an Alzheimer's disease model [8]. This present study examines the effect of DXQ on learning and memory impairments induced by an injection of $A\beta_{1-42}$ in a mouse model through Y maze test. Furthermore, its effects on hyperphosphorylated tau proteins (Thr 181 and Ser 404) and PI3K/Akt-dependent GSK-3β signalling pathway were investigated.

Materials and Methods

Reagents and instruments

Amyloid$_{1-42}$ and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma-Aldrich. The total protein extraction kit was purchased from Nanjing keyGEN Biotech. Co., Ltd, and a Page-Ruler prestained protein ladder (10-170 kDa) were purchased from Thermo Fisher Scientific Chemical Reagent Co., Ltd. The BCA protein assay kit, SDS-PAGE gel preparation kit, SDS-PAGE sample loading buffer (5X), HRP-labelled goat anti-rabbit IgG (H+L), HRP-labelled goat anti-mouse IgG (H+L), and BeyoECL Plus (ECL-like Western reagent) were obtained from the Beyotime Institute of Biotechnology. Antibodies against Ser 404, Thr 181, PI3K, p-PI3K (P85), AKT, p-AKT (Ser 473), GSK-3β, p-GSK-3β (Ser 9) and β-actin were purchased from Santa Cruz Biotechnology, Ltd. Antibodies against Tau-5 were obtained from Invitrogen Biotechnology, Ltd.

Preparation of drugs

The husks of DXQ (Shichangpu, Baishao, Heshouwu and Zhimu, et al.) were provided by Liaoning Fudong Pharmaceutical Co., Ltd (Benxi, China), and were identified by Pro. Xianmin You, Liaoning traditional Chinese medicine. The crushed husks of DXQ were soaked in eight volumes (w/v) of water at room temperature for 2 h, extracted at 80 for 6 h, drug residues were extracted 2 times and every time with 2 hour by water dicocting method. The filters were combined, and extraction solution to concentration income, some relative density 1.2 (60) dextrin in dry and granulating decompression. Donepezil with purity higher than 98% was obtained from Eisai, Ltd. All other chemicals were of analytical grade.

Experimental animals

Equal numbers of adult male and female ICR mice (n=48, 8 weeks of age) weighing 20-22 g were obtained from the Beijing HFK Bioscience Co., Ltd, and used in the study. The animals were housed in a polyacrylic cage with four mice per cage and maintained under standard housing conditions (22°C ± 2°C and humidity 50% ± 5%) with a 12 h light/12 h dark cycle. Food and water were available *ad libitum*. All efforts were made to minimize the number of animals and their suffering throughout the experiments. All animal studies were performed in strict accordance with the P.R. China legislation on the use and care of laboratory animals and the guidelines established by the Institute for Experimental Animals at Liaoning University of Traditional Chinese Medicine (131/2010; approved on November 1, 2011).

Animal model and Aβ$_{1-42}$ administration

Aβ$_{1-42}$ was dissolved in sterile physiological saline and aggregated by incubation at 37°C for five days prior to injection according to a previous report [9]. The mice were divided into six groups; there were eight mice in each group. The mice were anaesthetized with chloral hydrate (300 mg/kg i.p.) and placed on a stereotaxic apparatus (David Kopf Instruments) using a mouse adaptor (Kopf 921), and the skull was then exposed. Aβ$_{1-42}$ was injected via the ICV route slowly in a volume of 3 μL (≈410 pmol/mouse) on day 1, as described by Chi [10]. The coordinates from the bregma were the following: AP, -0.5 mm, ML, -1.0 mm, and DV, -3 mm [11]. The control animals were injected with equivalent volumes of sterile physiological saline. The mice were orally administered DXQ (3.12, 6.24 or 12.48 g/kg), donepezil (1.3 mg/kg) or vehicle by gavage starting from the second day after Aβ$_{1-42}$ injection to the end of the experiment. The mice were then treated once daily or 1 h before the behavioural tests.

Behavioural analysis

The Y-maze test was performed twenty-one days after Aβ injection. The maze had three arms, and the dimensions of each arm were 40 × 12 × 10 cm (length × height × width). Each mouse was placed at the terminus of one arm and allowed to move freely through the maze for 5 min. The total number of arm entries (N) and the sequence of entries were recorded. Alternation behaviour was defined as entries into all three arms on consecutive occasions. The alternation rate (%) was calculated as follows: alternation behaviour (%)=number of alternations/(N-2) × 100.

Western blotting analysis

Twenty-two days after the ICV Aβ$_{1-42}$ injection, the mice were sacrificed (Figure 1). Brain tissues were rinsed twice with cold PBS, disrupted with 400 μL of cold hypotonic buffer (10 mM HEPES pH 7.9, 10 mM KCL, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 M PMSF) for 15 min on ice and incubated for an additional 5 min after the addition of 25 μL of 10% NP-40. The samples were then sonicated for 15 s and centrifuged at 12,000 × g and 4°C for 10 min. The protein levels were determined using the Bradford assay (Beyotime). Protein extracts (50 μg) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and then electrophoretically transferred to polyvinylidene difluoride membranes (Millipore). After incubation at room temperature with 5% non-fat milk in PBS for 2 h, the membranes were incubated overnight at 4°C with the indicated primary antibodies against Tau-5 (Invitrogen), Ser 404, Thr 181, PI3K, p-PI3K (P85), AKT, p-AKT (Ser 473), GSK-3β, p-GSK-3β (Ser 9) and β-actin (Santa Cruz). The membranes were then incubated with secondary anti-mouse IgG or anti-rabbit IgG antibodies (Santa Cruz) for 2 h at room temperature. Protein bands were developed using electrochemiluminescent western blot reagents (Cwbiotech). The intensity was quantified by densitometry using Quantity One 4.6.2 software (Bio-Rad) and corrected with the corresponding β-actin level. The results are expressed as percentages of the control or Aβ levels.

Statistical analysis

The data are expressed as the means ± SD. The statistical significance was determined through one-way or two-way ANOVA followed by Fisher's LSD multiple comparisons test. A p value less than 0.05 was considered to indicate statistical significance. The data were analysed using SPSS 17.0.

Figures and Legends

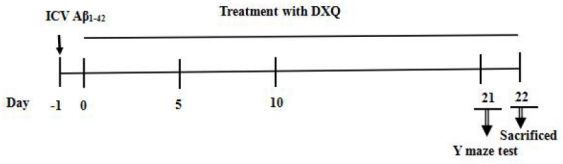

Figure 1. Experimental protocol of DXQ.

Results

In Y-maze test, the Aβ group exhibited significantly reduced spontaneous alternation behaviour than the control group ($p<0.01$). DXQ (3.12-12.48 g/kg) attenuated the impairment of spontaneous alternation behaviour (Figure 2a). The group administered donepezil (1.3 mg/kg) was used as a positive control. The total number of arm entries was not significantly different among the groups (Figure 2b).

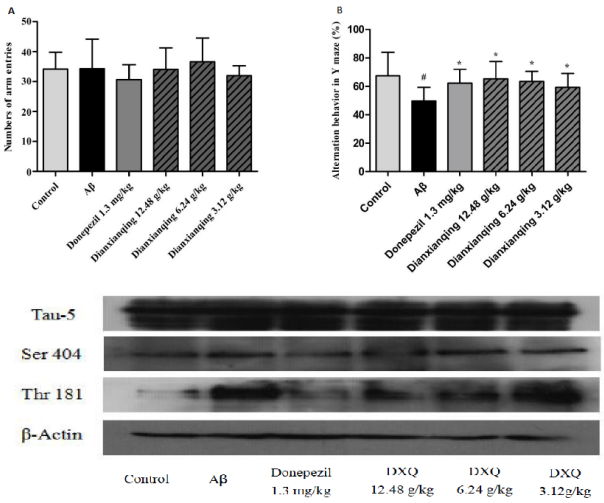

Figure 2. Effects of DXQ on learning and memory deficits induced by ICV Aβ in the Y maze test, (A): The total number of arm entries from Y-maze test, (B): Alternation (%) from Y-maze test. All of the results are expressed as the means ± SD. n = 8 animals; #p<0.05 vs. control; *p<0.05 *vs.* Aβ.

To test whether $A\beta_{1-42}$ induces tau level in mice, we assessed the production of phosphorylated tau and measured the expression of phosphorylated tau through western blot analysis. The results indicate that the level of phosphorylated tau at the Thr-181 and Ser-404 sites in the hippocampus was significantly higher in the Aβ group than in the control group (Thr-181, $p<0.05$; Ser-404, $p<0.05$, Figure 3), but was significantly lower in the group treated with DXQ group ($p<0.05$, Figure 3).

We examined the expression of PI3K, Akt and GSK-3β to investigate the potential involvement of the PI3K/Akt pathway in the DXQ-induced protection against phosphorylated tau. Aβ injection significantly decreased the phosphorylation levels of PI3K ($p<0.05$, Figure 4a), Akt ($p<0.05$, Figure 4b) and GSK-3β ($p<0.05$, Figure 4c). Treatment with DXQ at doses of 12.48 g/kg and 6.24 g/kg significantly increased the phosphorylation levels of PI3K ($p<0.05$, Figure 4a), Akt ($p<0.05$, Figure 4b) and GSK-3β ($p<0.05$, Figure 4c).

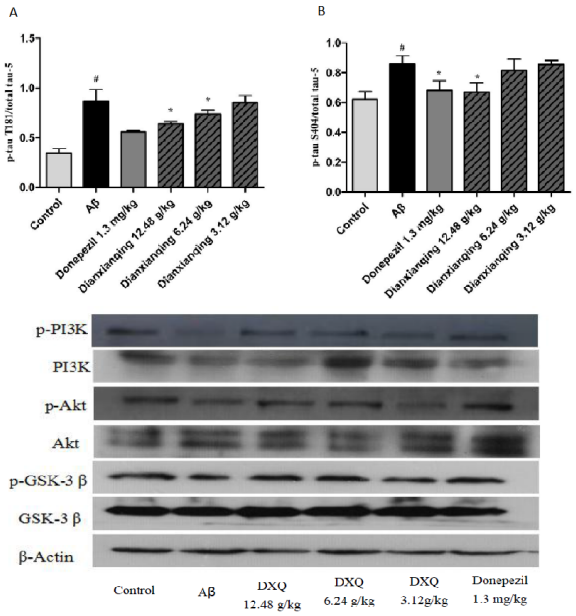

Figure 3. Effects of DXQ on the expression of Thr 181, Ser 404 and Tau-5 in the hippocampus. DXQ (12.48 g/kg and 6.24 g/kg) significantly decrease Thr 181 (A), Ser 404 (B), as determined by western blotting. All of the results are expressed as the means ± SD. n=3 animals; #p<0.05 *vs.* control; *p<0.05 *vs.* Aβ.

Discussion

The brains of patients suffering from Alzheimer's disease have three classical pathological hallmarks: amyloid-beta plaques, tau tangles, and neuronal loss. Abnormal A β deposition in the brain are the primary influences driving AD pathogenesis [12,13], during which the aggregation of A β and the subsequent formation of amyloid plaques results in a cascade of cellular events that ultimately leads to neuronal loss. It is this series of events that has been recognized as key to the neuropathogenesis of AD [14].

Abnormal fibres composed of hyperphosphorylated tau protein can form NFTs in neuron cell bodies or other cell types of the brain [15]. DXQ treatment was reported to be effective in reversing the deficits in memory and learning using Aβ 25-35-induced AD model mice [8], but the mechanism that how DXQ improves Aβ-induced memory impairment via inhibiting tau hyperphosphorylation in vivo is not elucidated. In our present study, we demonstrated that the DXQ is effective in providing protection against learning and memory deficits and in inhibiting tau hyperphosphorylation in the hippocampus. The possible mechanism is through the inhibition of the PI3K/Akt-dependent GSK-3 β signalling pathway.

In this study, intracerebroventricular injection of $A\beta_{1-42}$ in mice was used to investigate behavioural changes of DXQ with Y maze test. The results indicated that ICV injection of $A\beta_{1-42}$ caused an impairment of learning and memory performance, DXQ have protective effects on learning and memory impairment in the AD-like model.

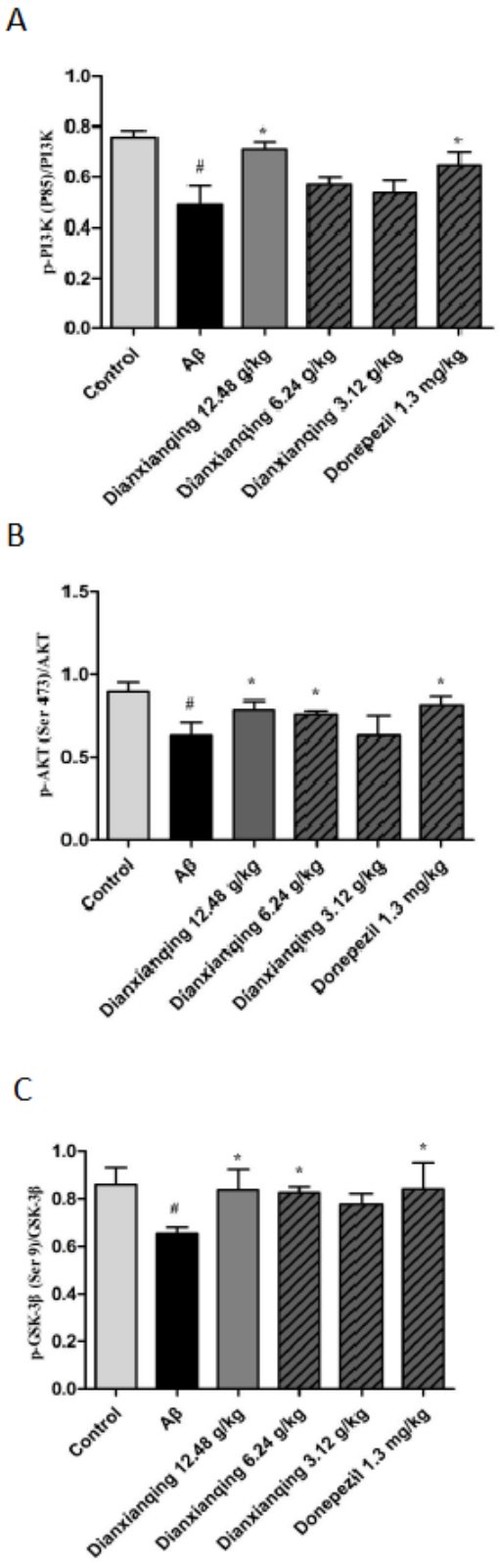

Figure 4. Effects of DXQ on the expression of p-PI3K, p-AKT, and p-GSK-3β in the hippocampus. DXQ (12.48 g/kg and 6.24 g/kg) significantly increase p-PI3K (A), p-AKT (B) and p-GSK-3β(C), protein expression level, as determined by western blotting. All of the results are expressed as the means ± SD. n=3 animals; $^{\#}p<0.05$ *vs.* control; $^{*}p<0.05$ *vs.* Aβ.

Tau is a major microtubuleassociated protein that plays a large role in the outgrowth of neuronal processes and the development of neuronal polarity. Studies have shown that the density and distribution of neurofirillary tangles in the brain correlates with the severity of dementia [16], with the tau protein abnormally phosphorylated in the brains of AD patients. The total phosphorylation sites on tau protein, serines (53% of phosphorylation sites of tau) and threonines (41%) are main [17]. In this study, two individual tau phosphorylation sites (Thr-181and Ser-404) were investigated. Western blot analysis showed that hyperphosphorylation occurred at the two sites of tau expressed in the Aβ -induced mice, but significantly decreased in the DXQ group mice at doses of 6.24 and 12.48 g/kg. These results indicate that DXQ is effective in providing protection against hyper-phosphorylated tau induced by Aβ.

Hyperphosphorylation of tau is mainly regulated by glycogen synthase kinase 3 β (GSK 3β), and GSK-3β plays a central role in the pathogenesis of Alzheimer's disease [18]. GSK-3β activation is regulated by serine and tyrosine phosphorylation. GSK-3β activity is increased by Tyr216 (active form) phosphorylation but decreased by Ser9 (inactive enzyme) phosphorylation, GSK-3β is regulated by multiple mechanisms, including the PI3K/Akt pathway. Hyperactivation of the PI3K/AKT/ GSK-3β pathway has a central function in neuronal survival, synaptic plasticity, cell apoptosis, cell adhesion and division. Our data showed that Aβ decreases PI3K p85 and Akt Ser473 phosphorylation. We found that DXQ significantly increases GSK-3β (Ser9) and PI3k (p85) and Akt (Ser473) phosphorylation level. These results suggest that the Aβ-induced tau hyperphosphorylation was attenuated by DXQ through the PI3k/Akt/GSK-3β cascade.

In conclusion, we provide the first demonstration that DXQ is effective in providing protection against Aβ-induced cognitive deficits and in inhibiting tau hyperphosphorylation in the hippocampus. The possible mechanism is through the inhibition of the PI3K/Akt-dependent GSK-3β signalling pathway. These data are of great interest for the proposal of using DXQ for treating AD.

Acknowledgements

The authors acknowledge the funding provided by the National Twelfth-Five-Year Plan in R&D of Important New Medicines (2012ZX09102201-005), the Program for Liaoning scientific Research (2004226010-6) and Doctor Funding of Liaoning province (201601385).

References

1. Qi Y, Zou LB, Wang LH, et al. Xanthoceraside inhibits pro-inflammatory cytokine expression in Aβ25-35/IFN-γ-stimulated microglia through the TLR2 receptor, MyD88, nuclear factor- κB, and mitogen-activated protein kinase signaling pathways. J Pharmacol Sci. 2013;122:305-17.
2. Krause DL, Müller N. Neuroinflammation, microglia and implications for anti-inflammatory treatment in Alzheimer's disease. Int J Alzheimers. Dis 2010.

3. Zumkehr J, Rodriguez-Ortiz CJ, Cheng D, et al. Ceftriaxone ameliorates tau pathology and cognitive decline via restoration of glial glutamate transporter in a mouse model of Alzheimer's disease. Neurobiol Aging. 2015;36:2260-71.
4. Avila J, Lucas JJ, Perez M, et al. Role of tau protein in both physiological and pathological conditions. Physiol Rev. 2004;84:361-84.
5. Matthew Barron, Jane Gartlon , Dawson, LA, et al. Atkinson, Marie-Christine Pardon. A state of delirium: Deciphering the effect of in fl ammation on tau athology in Alzheimer's disease. Exp Gerontol. 2016.
6. Wang Y, Yang R, Gu J, et al. Cross talk between PI3K-AKT-GSK-3Î² and PP2A pathways determines tau hyperphosphorylation. Neurobiol Aging. 2015;36:188-200.
7. Huang Y, Kang K,Qi Y, et al. Effects of Dianxianqing Granule on the Learning and Memory in Mice Induced byp-amyloid(A ß25-35). J Liaoning univ TCM. 2016;18:29-32.
8. Jana M, Palencia CA, Pahan K. Fibrillar amyloid-beta peptides activate microglia via TLR2: implications for Alzheimer's disease. J Immunol. 2008;181:7254-62.
9. Chi TY, Wang LH, Ji XF, et al. Protective effects of xanthoceraside on learning and memory impairment induced by intracerebroventricular injections of Aß1-42 in mice. J Shenyang Pharm Univ. 2010;27:314-20.
10. Laursen SE, Belknap JK. Intracerebroventricular injections in mice. Some methodological refinements. J Pharmacol Methods. 1986;16:355-7.
11. Pimplikar SW. Reassessing the amyloid cascade hypothesis of Alzheimer's disease. Int J Biochem Cell Biol. 2009;41:1261-8.
12. Huang Y, Mucke L. Alzheimer mechanisms and therapeutic strategies. Cell. 2012;148:1204-22.
13. Barage SH, Sonawane KD. Amyloid cascade hypothesis: Pathogenesis and therapeutic strategies in Alzheimer's disease. Neuropeptides. 2015;52:1-18.
14. Fu HJ, Rodriguez GA, Herman M, et al. Tau pathology induces excitatory neuron loss, grid cell dysfunction, and spatial memory deficits reminiscent of early Alzheimer's disease. Neuron. 2017;93:533-41
15. Simic G, Leko MB, Wray S, et al. Monoaminergic neuropathology in Alzheimer's disease. Prog Neurobiol. 2017;151:101-38.
16. Martin L, Page G, Terro F. Tau phosphorylation and neuronal apoptosis induced by the blockade of PP2A, preferentially involve GSK3ß, Neurochem Int. 2011;59:235-50.
17. Jin G, Wang LH, Ji XF, et al. Xanthoceraside rescues learning and memory deficits through attenuating beta-amyloid deposition and tau hyperphosphorylation in APP mice. Neurosci Lett. 2014;573:58-63.

***Correspondence to**

Dong Jia

Department of Pharmacology,

The Second Hospital Affiliated to Liaoning Chinese

Medical University, Huanggu District, Shenyang, China

Tel: +86-24-31207389; Fax: +86-24-31207389

E-mail: Jiadg2003@126.com