

# Traditional Chinese Medicine for Stroke Treatment Inhibits Abnormal Amyloid Precursor Processing in Alzheimer's Disease

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**Research**

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# Abstract

*Ethnopharmacological relevance:* Traditional Chinese Medicine (TCM) has a long history in oriental countries for its therapeutic benefits with stroke therapy, and improves cognitive deficits in those patients, but the potential impact on Alzheimer's disease therapy remains unknown.

*Objective:* To address this issue, *in vitro*, we have examined the effects of four types of TCMs selected from the ginsenoside family of drugs that are currently used in clinical stroke therapy on APP processing, *DengZhanXiXin* (D1), *TongLuoJiuNao* (T2), *QingKaiLing* (Q3) and *HuangQinGan* (H4).

*Materials and methods:* APP, BACE1 and C99 stable transfected cells have been used to test the APP processing. The A $\beta$  production, BACE1, NEP and  $\gamma$ -secretase activity were assessed by ELISA, RT-PCR and Western blot analysis.

*Results:* In this study, D1 increases A $\beta$  production but reduces the ratio of A $\beta$ 42/A $\beta$ 40 by up-regulating BACE1 activity; T2 reduces A $\beta$  production and A $\beta$ 42/A $\beta$ 40 ratio by down-regulating BACE1 activity and modulating  $\gamma$ -secretase expression; Q3 reduces A $\beta$  production by down-regulating BACE1 activity; H4 does not change A $\beta$  production by the compromising effects on down-regulating BACE1 and NEP activity.

*Conclusion:* These studies suggest that these four anti-stroke TCMs show different mechanisms on APP processing, and have the potential to be used in A $\beta$  clearance; particularly, T2 with relatively simple components and evident effects in APP processing may be a promising candidate for the treatment of AD.

## 1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the progressive formation of insoluble amyloid plaques and vascular deposits consisting of the amyloid  $\beta$ -peptide (A $\beta$ ) in the brain. However, it is still not completely clear whether AD results from a primary abnormality in the amyloid precursor protein (APP) or from the deregulation of the inflammatory system (Weiner, *et al.*,2002;Newcombe, *et al.*,2018) although several lines of evidence implicate abnormal processing of APP, which is cleaved by two enzymes:  $\beta$ -secretase (BACE1) and  $\gamma$ -secretase to generate excessive A $\beta$ , as one of typical pathological features of AD (Selkoe, *et al.*,2016;Gowrishankar, *et al.*,2015)

AD and stroke are both common in elderly individuals, but the relation remains controversial. Recent studies showed that stroke patient might be more likely to experience memory decline (Tang, *et al.*,2018;Bernhardt, *et al.*,2018); additionally, it was reported that stroke increased the risk of Alzheimer's disease (Kuzma, *et al.*,2018). It is well known that stroke results a hypoxic environment in the brain. Interestingly, hypoxia is shown to facilitate AD pathogenesis by up-regulating BACE1 gene transcription; hypoxia markedly increased A $\beta$  deposition and neuritic plaque formation and potentiated the memory deficit in APP transgenic mice(Sun, *et al.*,2006;Nguyen, *et al.*,2018). Our study also showed that the death of brain cells caused by a stroke or head injury may cause generation of A $\beta$  by building up BACE1 protein

level (Tesco, *et al.*,2007), raising the prospect that therapeutic intervention with such process could lower the risk of AD caused by stroke.

Traditional Chinese Medicine (TCM) has a long history in stroke therapy and their therapeutic efficacy has been confirmed by clinical studies(Han, *et al.*,2017;Zhang, *et al.*,2020;Wei, *et al.*,2015;Liu, *et al.*,2016;Gong, *et al.*,1999). However, since stroke could increase the risk of AD, whether these TCM for treating stroke could have an effect on APP processing as well as A $\beta$  production remains unknown. There are four types of TCMs selected from the ginsenoside family used in clinical stroke therapy— *DengZhanXiXin* injection (D1), *TongLuoJiuNao* injection (T2), *QingKaiLing* injection (Q3) and *HuangQinGan* (H4) (Fig. 1). D1 is produced from *Erigeron breviscapus*, which has the ability in cold repelling, dehumidification, and pain relieving, according to TCM. Its main effective gradient scutellarin has been proved to improve cognition impairment in hypoxia mouse model through facilitating neuron stem cells proliferation and neuron differentiation(Wang, *et al.*,2017). The composition of T2 is *Panax notoginseng* and *Cape Jasmine*, the main active components of which are ginsenoside and geniposide. Q3 is a Chinese formula containing multiple ingredients including cholic acid, mother-of-pearl, hyodeoxycholic acid, Cape jasmine, buffalo horn, radix isatidis, baicalin and honeysuckle. It is extensively applied in acute stage of cerebrovascular disease including ischemia stroke due to its regulation of several signal pathway or target such as toll-like receptor, PI3K-Akt(Ma, *et al.*,2020;Cheng, *et al.*,2012). T4 is extracted from the root of *Scutellaria baicalensis Georgi*. It exerts the neuroprotective effect on ischemia-induced neuron damage through anti-oxidative and anti-apoptosis pathway(Sowndhararajan, *et al.*,2017). Therefore, in order to examine the effects of these four types of TCMs used in clinical stroke therapy on APP processing, *in vitro*, by using APP and C99 transfected cell as models, we found that anti-stroke TCMs modulated APP processing and A $\beta$  clearance pathways by different mechanisms. Among them, the effectiveness of T2 is the most evident on APP processing.

## 2. Materials And Methods

### 2.1. TCM medicine

D1 was purchased from Yunnan BioValley Pharmaceutical Co. Ltd., China (Coad No. by SFDA: Z53021569); T2 was provided from Tianjin Chase Sun Pharmaceutical Co. Ltd., China (Cat. No. 090602); Q3 was purchased from Shineway Pharmaceutical Group Ltd., China (Coad No. by SFDA: Z13020880); H4, Ginsenoside (Gin), Geniposide (Gen or GP) and PNS were purchased from National Institute for the Control of Pharmaceutical and Biological Products, China (Cat. No. 110842, Cat. No. 110703, Cat. No. 110749, Cat. No. 110870). *In vitro*, Gin and Gen were dissolved in DMEM to make 10ug/ml stock.

### 2.2. Cell culture and TCMs treatment

*In vitro*, 293 cells were obtained from ATCC and maintained in 10%FBS DMEM, APP stable transfected cell was maintained in 200ug/ml G418. pcDNA-BACE1 stable transfected cell was maintained in 200ug/ml Zeocin. C99 expression vector was constructed, simply C99 DNA fragment was ligated to APP signal peptide, cloned into pFLAG-CMV vector. Transfection was performed by lipofectamine2000

according to the manufactory's instruction. The toxic points of each drug to 293 cells were tested, doses that lower than the toxic point were used in the experiments (data not shown). TCMs were directly mixed with the medium containing 10% FBS DMEM and added to the cells. 72hrs after treatment, medium was collected for A $\beta$  ELISA assay and cells were kept at -80 °C until use.

### 2.3. A $\beta$ ELISA

*In vitro*, A $\beta$ 40 and A $\beta$ 42 were measured with an A $\beta$ 40 and A $\beta$ 42 ELISA kit (KHB3481 and KHB3544, Biosource, USA). The ELISA system has been extensively tested and no cross-reactivity between A $\beta$ 40 and A $\beta$ 42 was observed (data no shown). Data are presented as means  $\pm$  SD of 3 experiments. Control sample without drug treatment was regarded as 100% and the relative level shown in percentage of the control were calculated.

## 2.4. BACE1 and NEP activity

For BACE1 activity, cells were homogenized in lysis buffer (10 mM Tris-HCl, pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10% Glycerol, 0.5% Triton X-100). BACE substrate (Calbiochem, Germany) was dissolved in DMSO and mixed with reaction buffer (50 mM HAc, 100 mM NaCl; pH 4.1) to make final concentration to 2.5uM. An equal amount of protein was mixed with 100  $\mu$ l of substrate, and fluorescence intensity was measured with a microplate reader at an excitation wavelength of 430 nm and an emission wave length of 520 nm. Maximal enzymatic speeds were calculated and the samples without drug treatment were regarded as 100%, relative activity percentage was calculated.

NEP enzyme activity was measured by quenched fluorogenic substrate as described previously (Li, *et al.*,1995). Cells were homogenized in 100 mM MES pH6.5 buffer containing 200uM PMSF and proteinase inhibitor mix (Sigma, USA). The resultant homogenate was directly used in NEP activity assay. The hydrolysis of the fluorogenic substrate peptides Mca-RPPGFSAFK(Dnp)-OH (R&D systems) in 100 mM MES buffer, pH6.5 at concentration of 5uM was measured by following the increase in fluorescence (excitation at 342 nm and emission at 420 nm) that occurred upon peptide bond cleavage. Fluorescence was read for 1 hour, 5 min/reading. The max velocity of NEP activity was calculated by the first 20 min. Samples without drug treatment were regarded as 100%, relative activity percentage were then calculated.

## 2.5. Western blot

50ug of the total protein was separated on SDS-PAGE, then transferred to PDVF membrane by semi-dry transfer equipment. The blot was probed by anti-BACE1 antibody(Yang, *et al.*,2003); anti-APH1 polyclonal antibody (kind gift from Dr. Yuming Li, Memorial Sloan-Kettering Cancer Center, New York); anti-Nicastrin polyclonal antibody (kind gift from Dr. Gang Yu, University of Texas Southwestern Medical Center); anti-PS1 N-terminus polyclonal antibody (kind gift from Dr. Sangram Sisodia, University of Chicago); anti- $\beta$ -actin antibody (Sigma, USA).

### 2.6. RT-PCR

Total RNA was extracted drug treated 293 cells by RNeasy mini kit from Qiagen. For BACE1, forward primer is 5' AGGGAGCATGATCATTGGAG, backward primer is 5' CGTGGATGACTGTGAGATGG to amplify a 475 bp fragment. For human NEP RT-PCR, primers 5' GGA CTC GAC TGG AGA TCA GC and 5' CCA AGT CGA GGT TGG TCA AT were used to amplify region from 83–686 nt from NEP mRNA. For  $\beta$ -actin RT-PCR, the primers are: forward 5'- GGACTTCGAGCAAGAGATGG, backward 5' GAAGCATTTGCGGTGGAG to amplify the  $\beta$ -actin mRNA coding region from 633 to 1125.

## 2.7. Statistical analysis

All data are reported as mean  $\pm$  SEM. Paired or unpaired Student's t-test (for two group means) or one or two-way analysis of variance (ANOVA) with post-hoc Tukey test, Scheffé's test, or Bonferroni test, as appropriate, were conducted by using Prism. The significance level for the two-sided analyses was set at  $p \leq 0.05$ .

## 3. Results

### 3.1. TCMs for Stroke have different effect on A $\beta$ production

To determine the effect of anti-stroke TCMs on A $\beta$  production, 293 cell with stable transfected APP695 was treated with different dose of four types of TCMs. High total A $\beta$  secretion has been reported in 293 human embryonic kidney cells stably transfected with APP695 that carries the Swedish APP mutation. The Swedish mutation makes APP a better substrate for BACE1 thus increases the production of both secreted A $\beta$ 40 and A $\beta$ 42 in the medium, which allow an accurate quantification by our sandwich ELISA (Fig. 2A). Therefore, this system allowed us to assess the potential effect of how TCMs affect A $\beta$  production.

After 72 hours' treatments with different dosages of D1, T2, Q3 and H4, we found that T2 and Q3 reduced A $\beta$ 40 and A $\beta$ 42 level, quantitatively T2 reduced 5–27% of A $\beta$ 40, 30–50% of A $\beta$ 42, while Q3 reduced 0–20% of A $\beta$ 40, 0–19% of A $\beta$ 42. In contrast, D1 increased secreted A $\beta$ 40 and A $\beta$ 42 production in a dose-dependent manner, quantitatively it raised A $\beta$ 40 for about 18–37%, A $\beta$ 42 for 10–32%. H4 did not significantly change the A $\beta$ 40 and A $\beta$ 42 level (Fig. 2BC). Moreover, interesting finding is the ratio of A $\beta$ 42/ A $\beta$ 40 under D1 and T2 is significantly reduced (Fig. 2D).

### 3.2. TCMs for Stroke modulate APP processing—BACE1

Since we found that these four anti-stroke TCMs showed different effect on A $\beta$  production, our next question is that whether treating with these TCMs could indeed regulated the APP processing pathway therefore regulate A $\beta$  production.

Two key enzymes are involved in APP processing:  $\beta$ -secretase (BACE1) (Vassar, *et al.*,1999;Selkoe,2003;Tanzi, *et al.*,2005) and  $\gamma$ -secretase (Selkoe,2003;Tanzi, *et al.*,2005). BACE1 is well-known to be the first step of producing A $\beta$  peptide. Recently, using BACE1 activity assay, we reported that BACE1 activity increased in AD brain (Yang, *et al.*,2003;Li, *et al.*,2004) and MCI CSF (Zhong, *et al.*,2007).

Then, we firstly tested whether these TCMs could possible regulate the BACE1 activity in APP transfected cells. Interestingly, though we only found T2 and Q3 could lower the A $\beta$  production in APP transfected cells above, three of the four TCMs, including T2, Q3 and H4 significantly decreased BACE1 activity, quantitatively T2 decreased by about 25%, Q3 decreased by about 25% and H4 decreased by about 34%. In contrast, BACE1 activity increased after D1 treatment, quantitatively increased by about 14%, which is consistent with the result of increasing A $\beta$  level above (Fig. 3A).

Since T2, Q3 and H4 reduced BACE1 activity while D1 up-regulated BACE1 activity, we further found, however, no mRNA level changed (data not shown). Due to confirm that these three TCMs could regulate BACE1 at its protein level, BACE1 stable transfected cell, whose expresses stable high level of BACE1 protein has been used.

By using BACE1 stable transfected cell, again, BACE1 activity has been evaluated. Which is consistent with earlier results in APP transfected cells, the activity of BACE1 was quantitatively increased by about 13%; in contrast, T2 decreased by about 26%, Q3 was by about 27% and H4 by about 34% (Fig. 3B). Then, Western Blot analysis of BACE1 level is consistent with the activity assay: D1 increased BACE1 protein level, while the others decreased BACE1 expression (Fig. 3C). This indicates that D1 could elevate BACE1 activity by increasing BACE1 protein level, which eventually leads to the accumulation of A $\beta$  production.

In addition, one of the possibilities of TCMs' certain gradients could have direct effects of inhibiting BACE1 activity. We directly mixed TCMs within the BACE1 activity assay reactions. The concentration of the TCMs is the same as the highest concentration used in the cell treatment. However, we did not find any direct regulation of BACE1 activity by mixing the drugs to the reaction (Fig. 3D). These results indicate that these four anti-stroke TCMs could regulate BACE1 at its protein level differently.

### **3.3. TCMs for Stroke modulate APP processing— $\gamma$ -secretase**

Pathogenic A $\beta$  peptides are generated from APP by sequential cleavages by BACE1 and  $\gamma$ -secretases: APP is first cleavage by BACE1 to produce a short C-terminal fragment (CTF) C99, C99 is then further processed by  $\gamma$ -secretase to produce A $\beta$  peptide (Selkoe,2003;Tanzi, *et al.*,2005) (Figure 4A). For the above experiment, we got a first impression of how the TCMs affect A $\beta$  production. To further isolate which step during the APP processing that the TCMs is regulating, a C99 transfected cell, which only contains  $\gamma$ -secretase processing site, has been used to examine the effect of TCMs on A $\beta$  production. There is no significant change of A $\beta$ 40 and A $\beta$ 42 level of cells treated with D1, Q3 and H4; however, significant decreased A $\beta$ 40 and A $\beta$ 42 levels were found in cells treated with T2 (Fig. 4BC), which quantitatively decreased the A $\beta$ 40 from 23–41%, A $\beta$ 42 from 22–27%, suggesting T2 might regulate the expression or activity of  $\gamma$ -secretase.

### **3.4 .TCMs for Stroke modulate clearance pathway—NEP**

Previously in this study, we found that there was a discrepancy result of H4 treated cells, which decreased BACE1 activity but without significant lower of A $\beta$  production, suggesting H4 could affect the downstream process after the A $\beta$  is produced. One of such downstream process could be NEP, an A $\beta$  degradation enzyme (Iwata, *et al.*,2000;Leissring, *et al.*,2003). NEP activity assay (Miners, *et al.*,2006) was used to measure the NEP activity in APP transfected cell after treated with four anti-stroke TCMs. Under H4 treatment, NEP activity was significantly decreased (Fig. 5A). Together with above results, this indicates that H4 could down-regulate BACE1 activity to reduce A $\beta$  production, however, by reducing NEP activity the extracellular A $\beta$  concentration could be increased in contrast. Therefore, the reason why A $\beta$  level does not change a lot under H4 is defect in NEP activity could compromise its ability to decrease BACE1 activity.

Also, there is a decreased tendency in NEP activity in D1 treated cells, quantitatively about 15%, while no significant change in T2 and Q3 treated cells (Fig. 5A). To further analyze how TCMs regulate NEP activity, we performed RT-PCR to exam if NEP mRNA level could have changed. Again, in H4 treated cell, the level of NEP mRNA was reduced (Fig. 5B), indicating H4 could down-regulate NEP transcription so that to reduce NEP activity.

### *3.5. T2 affects APP processing, and Ginsenoside is the active gradient to reduce BACE1 level and activity*

We found that T2 and Q3 have consistent result in reducing BACE1 activity and protein level, as well as A $\beta$  production. Two gradients are reported to be the major components of T2, Ginsenoside (Gin) and Geniposide (GP), interestingly, Gin, the main gradient of *Panax notoginseng saponins* (PNS) is also a component in Q3. However, besides Gin, Q3 also includes multiple other TCM recipes including radix istidis, radix scutellariae, flos lonicerae, deoxycholic acid, cornu bubali and the hydrolysis fluid of nacre, which makes its function relatively complicated and difficult to isolate. Compared to Q3, T2 is relatively simple. Therefore, we focused on Gin and Gen and tried to find out if either gradient is responsible for the reduced A $\beta$  level in T2 treated cells.

Purified Gin, the cell can be treated as much as 1ug/ml without obvious toxicity (data not shown); for Gen, no obvious toxicity could be found at concentration up to 0.25ug/ml (data not shown). In APP stable transfected cells, a different dosage of Gin and Gen was administrated. For cells treated with Gin, we found that A $\beta$ 40 level decreased from 34–50% in a dose-dependent manner. In contrast, A $\beta$ 40 level in Gen treated cells increased from 4–16% in a dose-dependent manner (Fig. 6A).

In order to find out whether Gin also affects BACE1 activity as T2, we next examined the BACE1 activity in these Gin treated cells. Interestingly, we found that Gin reduced the enzymatic activity from 12–35% in a dose-dependent manner, while Gen did not show any reduction effect in BACE1 activity (Fig. 6B). This result confirmed that Gin reduced A $\beta$  production through down-regulating BACE1 enzymatic activity. Meanwhile, Gen, with respect to BACE1 regulation, it plays a contrary role to Gin, which shows no effect on BACE1 enzymatic activity and increases A $\beta$  level. Also, there is no significant change in BACE1 mRNA level in Gin treated cells (data not shown).

To further confirm that Gin could modulate BACE1 protein level, we again treated BACE1 stable transfected cell line with Gin. Similarly, after treating with Gin, BACE1 activity from BACE1 stable transfected cell decreased from 21–45% (Fig. 6C). Western blot showed that BACE1 protein level was significantly reduced in a dose-dependent manner (Fig. 6D). These results are consistent with T2 treated BACE1 stable transfected cell, indicating Gin is the active component in T2 that reduces BACE protein level and activity.

What's more, since T2 also decreased A $\beta$  level in C99 transfected cell, suggesting T2 could regulate  $\gamma$ -secretase pathway. Western blot of three  $\gamma$ -secretase components including Nicastrin, PS1 and APH1 showed PS1 full length protein (PS1-FL) did not show any change. However, at higher concentration and shorter exposure, PS1 N-terminal fragment (PS1-NTF) did show some decrease. Similar to PS1-NTF, APH1 and Nicastrin also showed some reduction (Fig. 6E). These results indicated that  $\gamma$ -secretase could show reducing function at high concentration of Ginsenoside through a reduced level of its major components. These suggest that the major gradient, Gin plays the main role in APP processing in terms of BACE1 and  $\gamma$ -secretase regulation.

## 4. Discussion

AD is the most common form of progressive dementia in the elderly, lines of evidence showed that stroke patient might be more likely to experience memory decline (Brainin, *et al.*,2015) as well as stroke could increase the risk of AD (Kuzma, *et al.*,2018). Here four TCMs examined in this research have well been reported in treating stroke patients clinically. D1 is produced from *Erigeron breviscapus*, a TCM in cold repelling, dehumidification, and pain relieving according to the theory of TCM. It remarkable effects on the improvement of neuron function damage of patients with acute cerebral infarction as well as drug combination therapy (Chao Zeng,2015). Vascular dementia patients who treated with D1 showed improving MMSE grading as well as blood dynamics of brain vessel (Wei-Jie Bai,2006); T2 is produced from *Panax notoginseng* and *Cape Jasmine*, the main gradients of which are Gin (a main gradient of PNS) and Gen. T2 improves the consciousness recovery and the cognitive function after ischemia stroke, especially show the remarkable effect in the memory repair of vascular dementia patients with cerebral infarction in 200 cases of double-blinded, multi-center comparison clinical trial (unpublished results). *In vivo*, T2 can improve spatial learning and memory in 10-month age of APP V717I Tg mice (Yang, *et al.*,2014); Q3 is produced from a variety of herbs, such as cholic acid, mother-of-pearl, hyodeoxycholic acid, medlar, buffalo horn, radix isatidis, baicalin, honeysuckle. It offers the neuroprotection effect in acute ischemia patient by inhibiting the adhesion of white blood cell and endothelia cell, relieving the damage of vascular endothelial cell as well as inhibiting the process of inflammation (Pengtao Li,2009; Lingming Sun,2016). In addition, Q3 injection can significantly improve the mental retardation degree, muscle strength and walking faculty of vascular dementia patients ; H4 is baicalin, extracted from the root of *Scutellaria baicalensis Georgi*. It attenuates cerebral ischemia via anti-oxidative and anti-apoptotic pathways (Cao, *et al.*,2011). Accompanied with jasminoidin and cholic acid, it further improved the cognitive function in ibotenic acid-induced rat models.

These TCMs are used in different aspects of stroke accompanied with different mechanisms in molecular levels. Then it is not surprise that their effect on APP pathway could be different: For D1, it increases A $\beta$  level through accumulating more BACE1 protein and enzymatic activity inside the cell, but the ratio of A $\beta$ 42/A $\beta$ 40 decreases; for H4, it lowers BACE1 protein activity, as well as NEP, an important enzyme in A $\beta$  clearance. These effects totally compensate and no different change occurred in A $\beta$  production; Q3 reduces A $\beta$  level by down-regulating BACE1 activity and expression; T2 shows the most active effect in reducing A $\beta$  level and A $\beta$ 42/A $\beta$ 40 ratio *in vitro*, partially through down-regulating BACE1 level and activity. Further, it has been proved that Gin, rather than Gen plays a main role in BACE1 modulation. The mechanism of how Gin affects BACE1 protein level remains to be investigated. Since Gin was found to have an effect to stimulate glucose uptake (Li, *et al.*,2018;Zhou, *et al.*,2019), and glucose reduction, energy inhibition elevates BACE1 levels and activity and is potentially amyloidogenic in APP transgenic mice and it could possibly be the early events in AD pathogenesis (Velliquette, *et al.*,2005), suggesting the capability of enhance glucose uptake of Gin could be the key for its role in modulating BACE1 level and activity. In addition, it is worth to note that with a higher concentration, Gin shows effect on the modulation of  $\gamma$ -secretase expression. However, this effect is not shown when cells are treated with T2, therefore it might not or only partially account for the A $\beta$  reduction in C99 transfected cell. This could be the effect of drug combination could be different from a single component's.(Table 1)

The two major isolated gradients of T2, Gin and Gen have been reported to have multiple clinical effects, including anti-oxidation, anti-tumor, Type 2 diabetes etc. (<http://en.wikipedia.org/wiki/Ginsenoside>; <http://en.wikipedia.org/wiki/Genipin>). Gin are class of steroid-like compounds, a main gradient of *Ginseng* or *Panax notoginseng* saponin (PNS), the latter of which is extracted from *Panax notoginseng*, the component of T2 and a valuable herb widely used in TCM for stroke. PNS has the effect of inhibiting platelet aggregation, increasing the heart and cerebral blood flow and promotes angiogenesis in DG areas of hippocampus and plays a protective role in cardiovascular and cerebrovascular diseases (Yang, *et al.*,2014;Xu, *et al.*,2015;Tan,2020). A bench of clinical trials has been administrated for the treatment of PNS in hypertensive intracerebral hemorrhage and ischemic stroke (ClinicalTrials.gov identifier: NCT02999048, NCT01636154, NCT02544087). Ginseng was shown to have beneficial effects against amnesia induced by  $\beta$ -amyloid peptides *in vivo* (Wang, *et al.*,2006), Gin also have been shown to promote learning and memory capability, reduced A $\beta$  level of AD mice (Chen, *et al.*,2006), as well as neuroprotective effect (Bao, *et al.*,2005), which is consistent to our study. GP is shown to inhibit microglia-mediated inflammatory responses, reduced the production of pro-inflammatory cytokines, decreased the cytotoxicity of A $\beta$  and induced neurotrophic effects as well (Li, *et al.*,2014;Li, *et al.*,2012).

What's more, it is well known that stroke results a hypoxic environment in the brain. Interestingly, Hypoxia was shown to facilitate AD pathogenesis by up-regulating BACE1 gene transcription; Hypoxia treatment markedly increased A $\beta$  deposition and neuritic plaque formation and potentiated the memory deficit in Swedish mutant APP transgenic mice (Sun, *et al.*,2006); Our recent study also showed that the death of brain cells caused by a stroke or head injury may cause generation of A $\beta$  by building up BACE1 protein level (Tesco, *et al.*,2007), raising the prospect that therapeutic intervention with such process could lower the risk of AD caused by stroke. What's more, since in pathological aspect, stroke and AD both are very

complicated and have multiple symptoms from blood vessel to neuron cell and multiple mechanism could be involved, the TCMs did not show effect on APP processing doesn't mean they do not alleviate other symptoms of AD, such as H4. As a whole, anti-stroke TCMs show different mechanisms on APP processing, and have the potential to be used in AD treatment; particularly, T2 with its relatively simple components may be a promising candidate for the treatment of AD (Fig. 7).

## 5. Conclusion

These studies suggest that TCMs used to treat stroke patients show different mechanisms on APP processing, and have the potential to be used in AD treatment; particularly, T2 with its relatively simple components may be a promising candidate for the treatment of AD.

## Declarations

### 6.1 Acknowledgements

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### 6.2 Authors' contributions

Yan Tan and Zihui Xu contributed to data organization and analysis and initial manuscript; all the authors partially contributed to the experiment operation. Qian Hua and Tonghua Liu conceptualized and designed the study, critically reviewed and revised the manuscript. Qian Hua and Yan Tan provided research funds.

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### 6.4 Competing interests

The authors declare that they have no competing interests.

### 6.5 Ethics approval and consent to participate

Not applicable.

### 6.6 Consent for publication

The manuscript is approved by all authors for publication.

## 6.7 Availability of data and materials

The datasets used in this study are available from the corresponding author upon reasonable request

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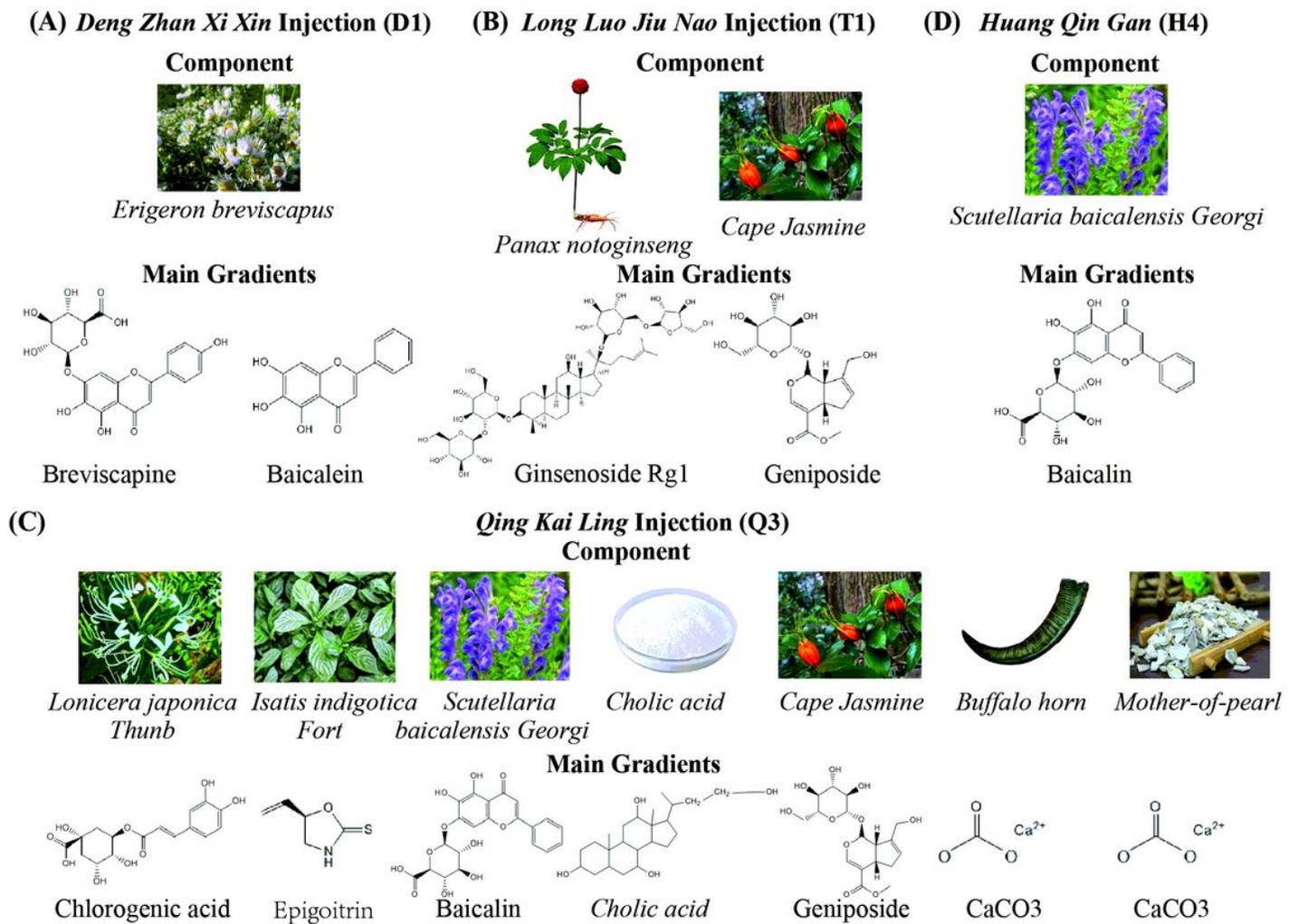
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## Tables

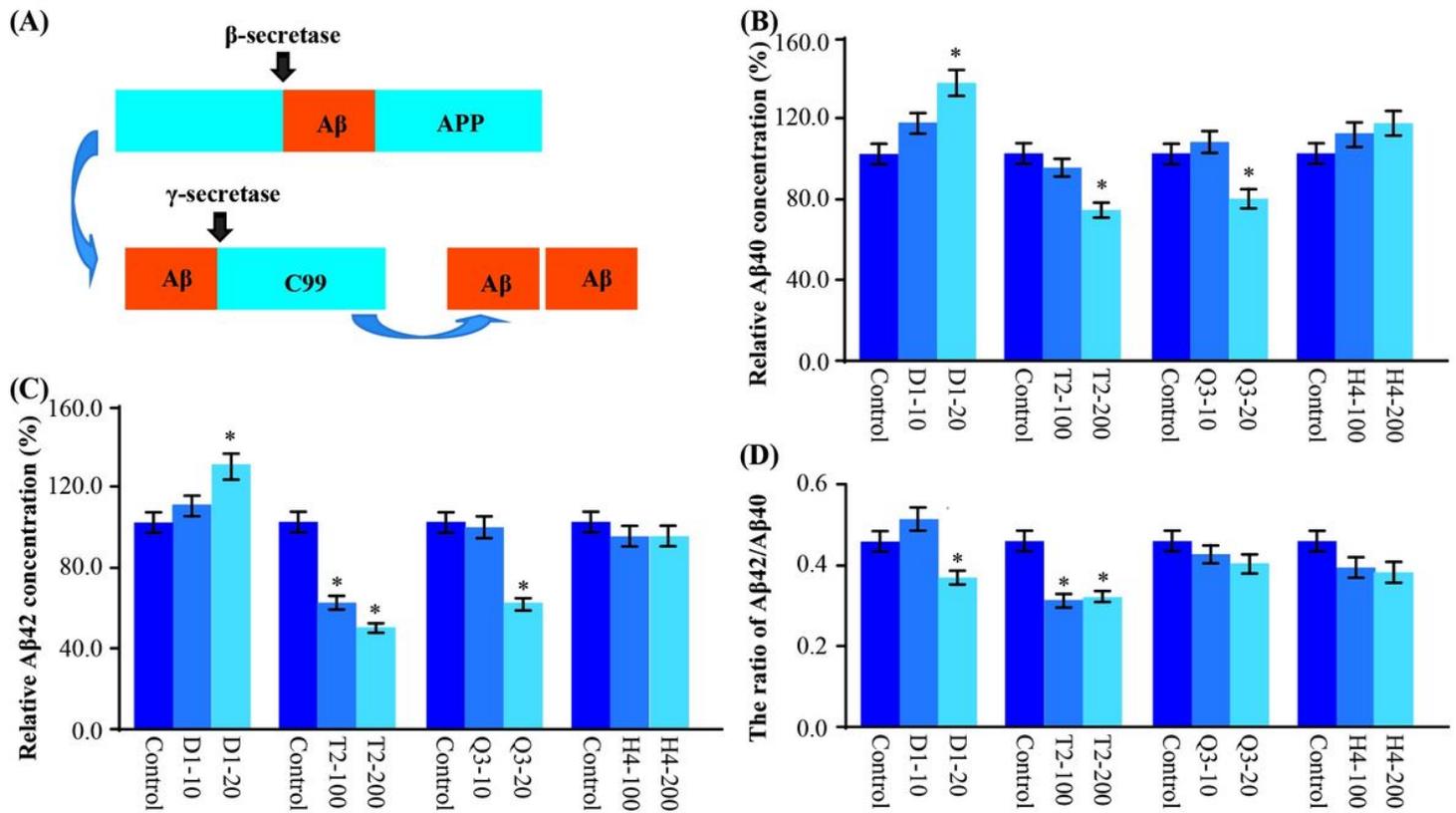
Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

# Figures



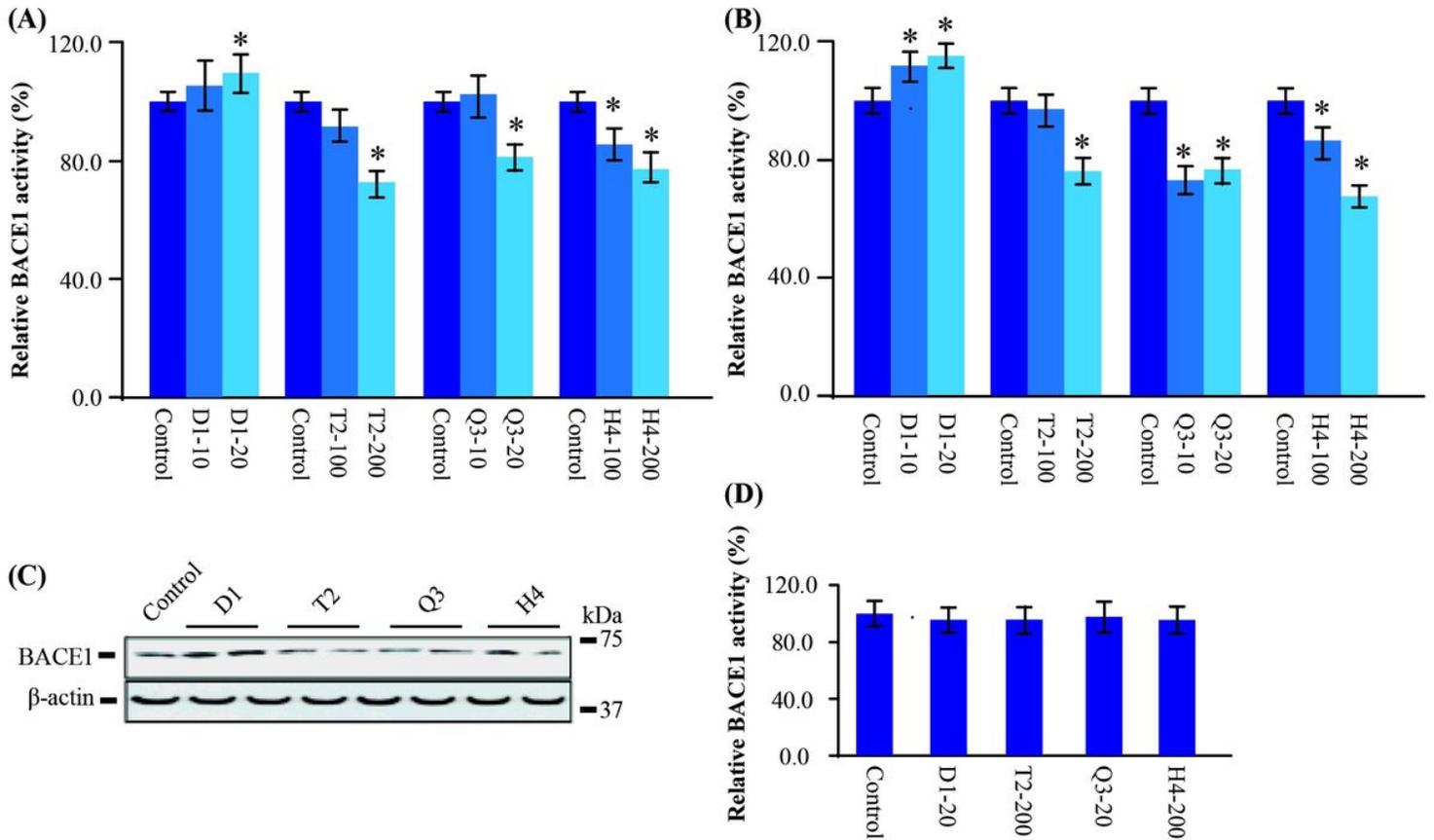
**Figure 1**

The composition of these four anti-stroke TCM drugs and their main ingredients. A) The component and main gradients of DengZhanXiXin injection (D1). B) The components and main gradients of TongLuoJiuNao injection (T2). C) The components and main gradients of QingKaiLing injection (Q3). D) The component and main gradients of HuangQinGan (H4).



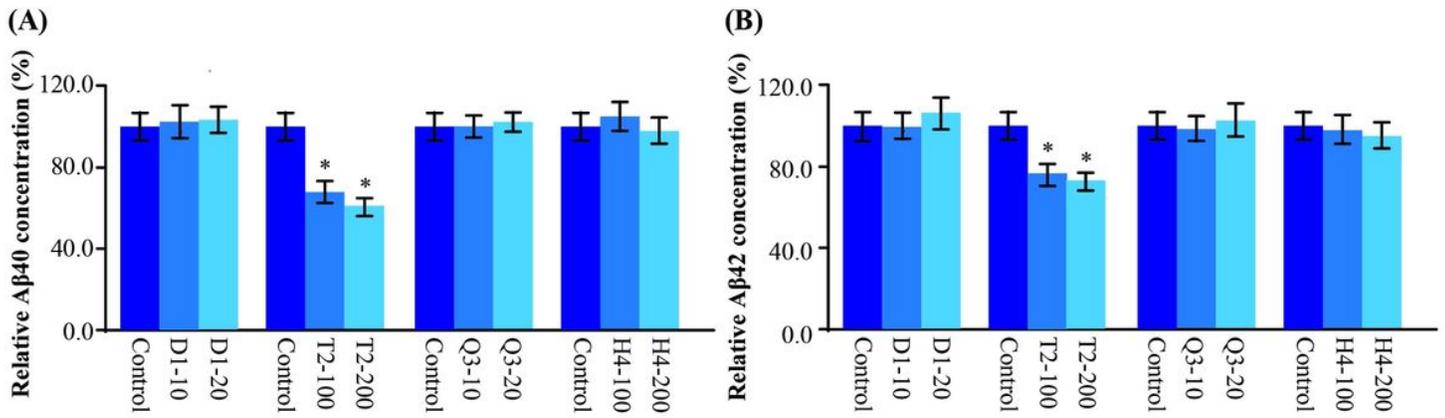
**Figure 2**

Relative Aβ40, Aβ42 levels in APP stable transfected cells. A) The sketch of APP processing. B) The level of Aβ40 is significantly decreased under T2 and Q3 administration, but increased in D1 group with a clear dose-dependent manner. B) The level of Aβ42 is significantly decreased under T2 and Q3 administration, but increased under D1 with a dose-dependent manner. C) The ration of Aβ42/Aβ40 is significantly reduced in D1 and T2 groups. Data are represented as mean ± SEM. \*p < 0.05.



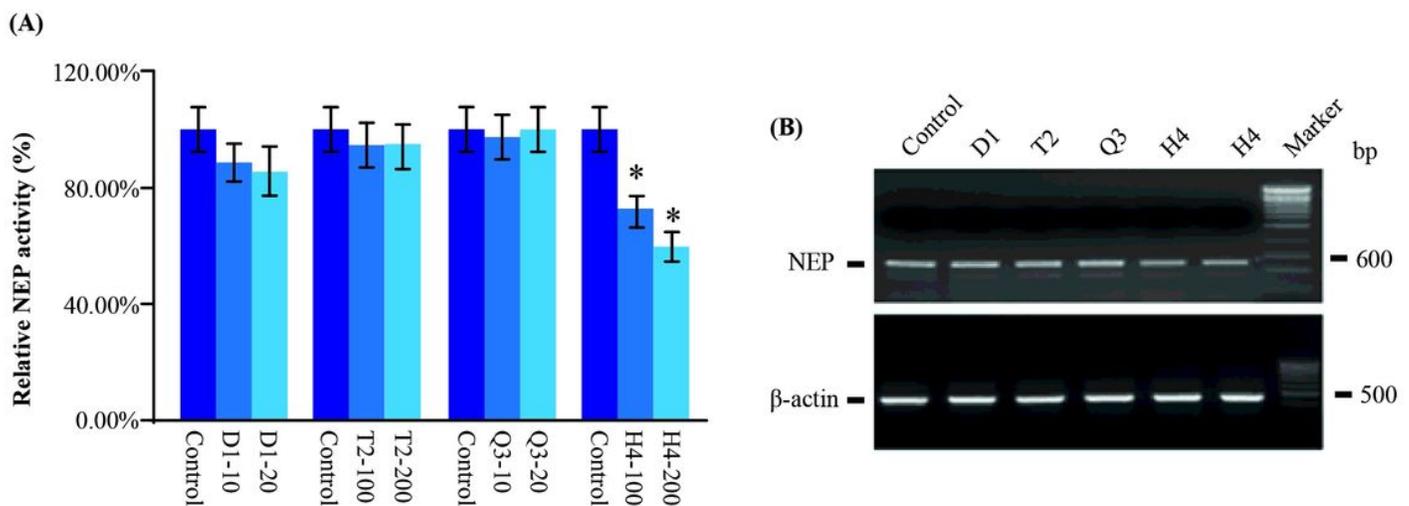
**Figure 3**

TCMs affect BACE1 expression and activity. A) In the APP stable transfected cells, the BACE1 activity is significantly increased under D1 administration, but decreased under T2, Q3 and H4 intervention. B) In the BACE1 stable transfected cells, the BACE1 activity is significantly increased under D1, while decreased under T2, Q3 and H4. C) In the BACE1 stable cells, the protein level of BACE1 has a higher expression under D1 administration, but with a lower tendency under T2, Q3 and H4. D) Direct effect of TCMs on BACE1 activity, BACE1 activity assay was carried out in the presence of TCMs in BACE1 stable transfected cells. There is no significant difference among all groups. Data are represented as mean  $\pm$  SEM. \* $p < 0.05$ .



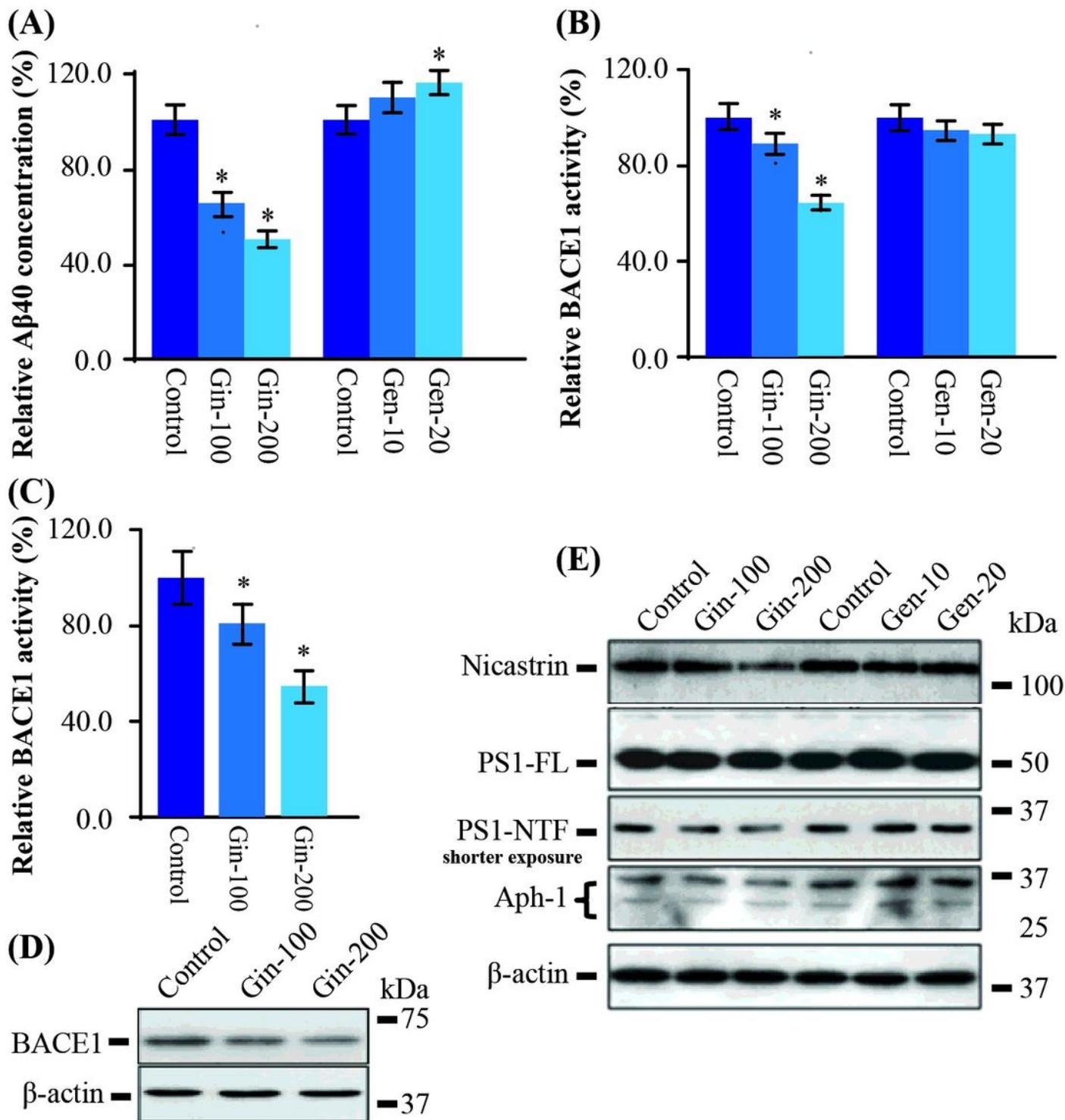
**Figure 4**

TCMs affect  $\gamma$ -secretase activity. A) In the C99 transfected cells, the production of A $\beta$ 40 is significantly decreased under T2 administration. B) In the C99 transfected cells, the production of A $\beta$ 42 is significantly decreased under T2 administration. Data are represented as mean  $\pm$  SEM. \*p < 0.05.



**Figure 5**

TCMs affect NEP activity and its mRNA level. A) In the APP stable transfected cells, only H4 significantly reduced the NEP activity. B) By the analysis of RT-PCR, again, the mRNA level of NEP was reduced under H4 administration. Data are represented as mean  $\pm$  SEM. \*p < 0.05.



**Figure 6**

The major gradient of T2-ginsenoside mainly contributes the activity and expression of BACE1. A) In the APP stable transfected cells, Ginsenoside (Gin) significantly reduced the production of A $\beta$ 40 in a dosage manner; Geniposide (Gen) significantly increased A $\beta$ 40 production in a dosage manner. B) The BACE1 activity was significantly reduced under Gin administration with a dosage manner, while there was no significant change under Gin treatment. C) In the BACE1 stable transfected cells, again, Gin significantly

reduced the BACE1 activity in a dosage manner. D) The protein level of BACE1 was significantly reduced with a dosage manner under Gin. E) In the C99 stable cells, there is no significant change in Western blot of  $\gamma$ -secretase subunits. But Gin slightly reduced the level of Nicastrin, PS1-NTF and Aph-1 in a dose-dependent manner. Data are represented as mean  $\pm$  SEM. \* $p < 0.05$ .

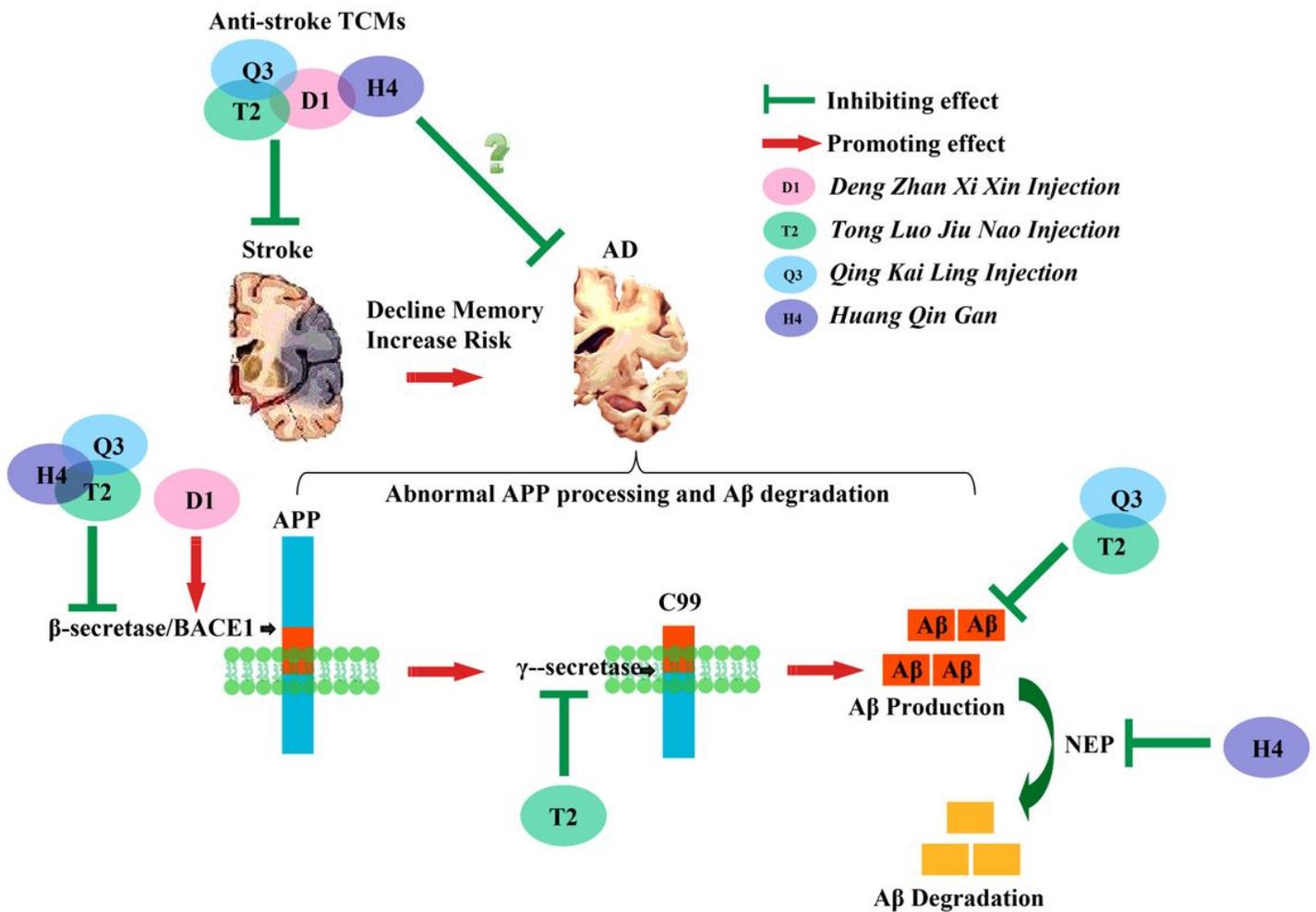


Figure 7

TCMs for stroke show a potential role in AD treatment. Stroke increased the risk of AD by up-regulating BACE1 activity. Based on this research, among these four anti-stroke TCMs, D1, T2, Q3 and H4, T2 shows the most evident effect on A $\beta$  production by targeted on both  $\beta$ -secretase and  $\gamma$ -secretase.

## Supplementary Files

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