

# Geniposide, the component of the Chinese herbal formula *Tongluojiunao*, protects amyloid- $\beta$ peptide (1–42)-mediated death of hippocampal neurons *via* the non-classical estrogen signaling pathway

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## Abstract

*Tongluojiunao* (TLJN) is an herbal medicine consisting of two main components, geniposide and ginsenoside Rg1. TLJN has been shown to protect primary cultured hippocampal neurons. However, its mechanism of action remains unclear. In the present study, primary cultured hippocampal neurons treated with A $\beta_{1-42}$  (10  $\mu$ mol/L) significantly increased the release of lactate dehydrogenase, which was markedly reduced by TLJN (2  $\mu$ L/mL), specifically by the component geniposide (26  $\mu$ mol/L), but not ginsenoside Rg1 (2.5  $\mu$ mol/L). The estrogen receptor inhibitor, ICI182780 (1  $\mu$ mol/L), did not block TLJN- or geniposide-mediated decrease of lactate dehydrogenase under A $\beta_{1-42}$ -exposed conditions. However, the phosphatidylinositol 3-kinase or mitogen-activated protein kinase pathway inhibitor, LY294002 (50  $\mu$ mol/L) or U0126 (10  $\mu$ mol/L), respectively blocked the decrease of lactate dehydrogenase mediated by TLJN or geniposide. Therefore, these results suggest that the non-classical estrogen pathway (*i.e.*, phosphatidylinositol 3-kinase or mitogen-activated protein kinase) is involved in the neuroprotective effect of TLJN, specifically its component, geniposide, against A $\beta_{1-42}$ -mediated cell death in primary cultured hippocampal neurons.

**Key Words:** nerve regeneration; neurodegeneration; Alzheimer's disease; cell culture; hippocampus; neurons; A $\beta_{1-42}$ ; estrogen signaling pathway; phosphatidylinositol 3-kinase pathway; mitogen-activated protein kinase pathway; *Tongluojiunao* injection; geniposide; ginsenoside Rg1; NSFC grant; neural regeneration

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## Introduction

Alzheimer's disease is a common neurodegenerative disease in the aged population (Maurer et al., 1997; Dong and Chai, 2013). Amyloid- $\beta$  (A $\beta$ ) plaques and neurofibrillary tangles are the main pathological hallmarks of Alzheimer's disease, as well as the loss of neurons and synapses (Bard et al., 2000; Hardy and Selkoe, 2002; Taylor et al., 2002; Ross and Poirier, 2004). Women have a higher risk than men for developing Alzheimer's disease, mainly due to the abrupt decline in estrogen levels during menopause (Winkler and Fox, 2013). Studies have indicated that estrogen is one of the most important signals for maintaining neuronal function (Burek et al., 1995; Morale et al., 2006), and is protective against brain injury, neurodegeneration, and cognitive decline (Dubal et

al., 2001). Because of its anti-A $\beta$  action, estrogen was used as a potential drug to treat Alzheimer's disease. (Liang et al., 2010a). In addition to the important role of estrogen in the regulation of reproduction, the immune system, bone maintenance, and cardiovascular disease, recent reports have shown that estrogen exerts neuroprotective effects. However, large clinical trials in postmenopausal women indicated adverse side-effects of estrogens, such as increased incidence of breast cancer and endometrial carcinoma, thereby preventing clinical use of estrogen. Therefore, scientists have now turned to exploit phytoestrogens as a potential safer alternative for age-related cognitive decline (Lephart et al., 2002; Sumien et al., 2013).

Two main estrogen signaling transduction pathways exist,

the classical and non-classical signaling pathways (Coleman and Smith, 2001; Hall et al., 2001). The classical signaling pathway is mediated by estrogen receptors, ER $\alpha$  and ER $\beta$  (Shughrue et al., 1997; Dubal et al., 2001), which are constitutively expressed in many brain regions, initiating gene transcription after specifically binding to estradiol. The non-classical signaling pathway acts mainly *via* phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) (Marin et al., 2005; Quesada et al., 2008). Studies of the classical estrogen pathway using the estrogen receptor steroidal inhibitor, ICI182780 (fulvestrant), have shown that this inhibitor prevents the translocation of the estrogen receptor into the nucleus where it is downregulated *via* homodimerization (Jakacka et al., 2001). This inhibitor also interferes with the binding site of the estrogen receptor in the nucleus and reduces the estrogen receptor and estrogen receptor response element, therefore completely blocking the estrogenic classical pathway (Dauvois et al., 1993; Chanda et al., 2000; Björnström and Sjöberg, 2005). In the non-classical estrogen pathway, estrogen requires the participation of intracellular signaling transduction molecules, mainly the PI3K and MAPK pathways. PI3K is a crucial signaling pathway that can be repressed by the inhibitor, LY294002 (Hui et al., 2005; Fan et al., 2006). MAPK is one of most important pathways in eukaryote signaling transmission and plays key roles in regulating gene expression and the functional activity of the cytoplasm. U0126 is an inhibitor of the MAPK pathway (MacDonald et al., 2001; Martin et al., 2003).

Senile plaques are one of the characteristic hallmarks of Alzheimer's disease and are formed predominantly of A $\beta$ , which is a 42-aminoacid peptide and a potential target in the treatment of this disease (Gilbert, 2013; Honig and Boyd, 2013). Accumulating evidence has suggested that A $\beta$ <sub>1-42</sub> is neurotoxic and exhibits apoptotic effects in cultured hippocampal neurons (Loo et al., 1993). Therefore, in the present study, A $\beta$ -mediated injury of primary cultured neurons was used as an *in vitro* model of Alzheimer's disease. *Tongluojunao* (TLJN) injection is a traditional Chinese medicine preparation, clinically efficacious in the treatment of ischemic cerebral stroke and dementia (Hui et al., 2005; Hua et al., 2010). The main ingredients of TLJN injection are ginsenoside and ginsenoside Rg1 (Hua et al., 2010). Ginsenoside Rg1 is one of main components of TLJN, which has been reported to be neuroprotective and reduce toxicity (Gong and Zhang, 1999; Chen et al., 2003; Leung et al., 2007; Gao et al., 2009). Ginsenoside Rg1 is neuroprotective against glutamate-exposed mesencephalic dopaminergic cells (Radad et al., 2004). Geniposide is another main component of *Gardenia jasminoides*, which is widely used in Chinese traditional medicine, and has been shown to be protective against A $\beta$ -, CoCl<sub>2</sub>-, and H<sub>2</sub>O<sub>2</sub>-mediated neuronal apoptosis (Guo et al., 2009; Liu et al., 2009; Liang et al., 2010b). Geniposide protects PC12 cells from hydrogen peroxide-induced cell death *via* the activation of glucagon-like peptide 1 receptor (Liu et al., 2009). Furthermore, geniposide regulates insulin secretion by activating the glucagon-like peptide 1 receptor (Radad et al., 2004). TLJN has been reported to reduce brain

ischemic damage and increase the expression of brain derived neurotrophic factor (Alesheikh et al., 2011). Moreover, previous reports have shown that TLJN is neuroprotective against A $\beta$ -induced injuries (Hua et al., 2010; Liu et al., 2011; Li et al., 2012).

We have previously reported that TLJN improves cognitive performance, primarily *via* the up-regulation of insulin-degrading enzyme and neprilysin, which promote the degradation of A $\beta$  and clear amyloid plaque from the brain of Alzheimer's disease rats (Liu et al., 2011). Previous unpublished data have shown that improvement of learning and memory by TLJN in Alzheimer's disease rats is gender dependent, in which male Alzheimer's disease rats perform better than female counterparts after drug treatment.

In the present study, we compared the effects of TLJN injection and estrogen on A $\beta$ -exposed primary cultured hippocampal neurons as Alzheimer's disease-like cell models.

## Materials and Methods

### Animals

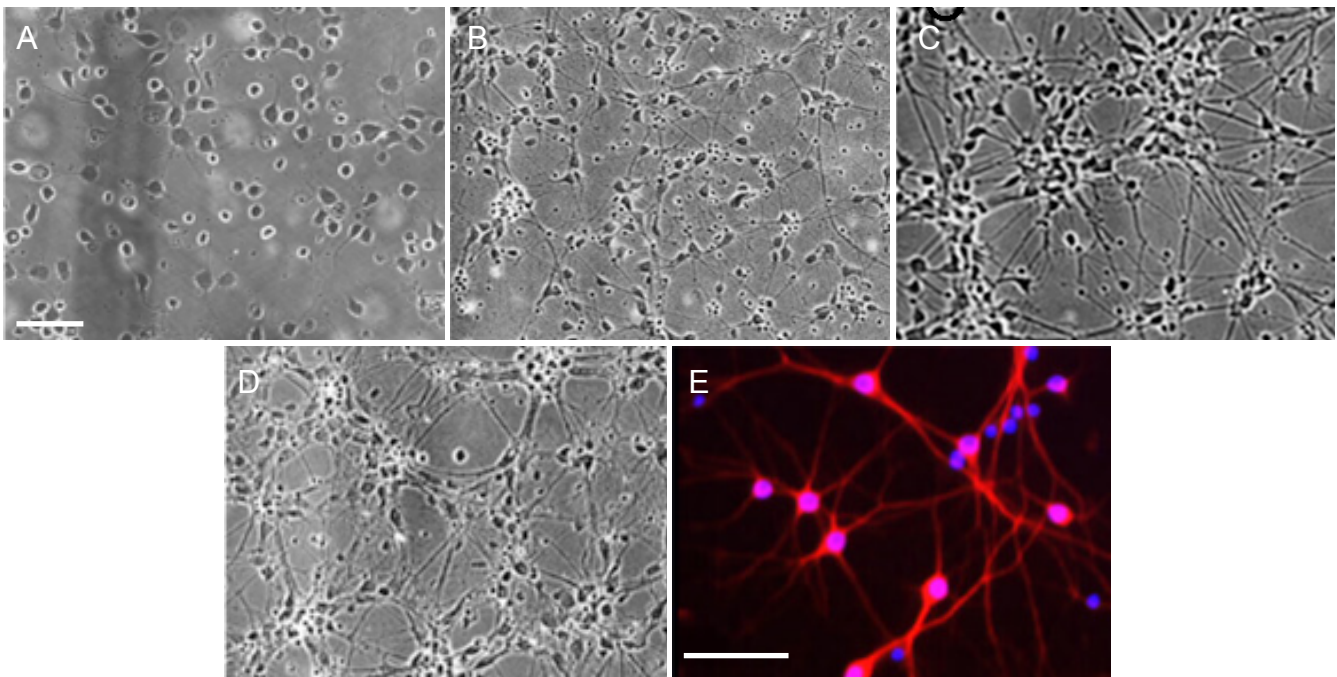
Sprague-Dawley rats (gestational age 16–18 days) were obtained from the Institute of Biophysics, Chinese Academy of Science, China (license No. SYXK (SPF) 2007-14). The animals were raised in a specific pathogen-free facility under a 12-hour light/dark cycle with *ad libitum* access to food and water. All experimental procedures were performed in accordance with the animal research regulation of the Chinese National Natural Science Foundation and the animal care guidelines of the National Institutes of Health.

### Drugs

TLJN injection was provided by the Pharmaceutical Factory of Beijing University of Chinese Medicine (Beijing, China; Chinese SFDA: 2004 L01620). The concentrations of geniposide (4.95 mg/mL) and ginsenoside Rg1 (1.02 mg/mL) in TLJN were determined by high performance liquid chromatography (Hua et al., 2010). Purified geniposide (lot: 110749-200714) and ginsenoside Rg1 (lot: 110703-200726) were purchased from the National Institutes for Food and Drug (Beijing, China).

### Primary hippocampal neuronal cultures

Primary hippocampal neurons were prepared from Sprague-Dawley rats at embryonic days 16–18. Pregnant rats were anesthetized with 10% chloral hydrate (400 mg/kg) and disinfected with 75% alcohol, and fetuses were removed. After fetuses were sacrificed with an over-dose of chloral hydrate, the skull was opened, the cerebral cortex removed (by ophthalmological forceps), and the separated to reveal the two hippocampi (Joseph, 2008). The hippocampi were dissected, rinsed in Hank's Balanced Salt solution, and then cut into pieces before tissue digestion with 0.25% trypsin for 15 minutes at 37°C. Digestion was stopped by the isometric trypsin inhibitor. The tissue suspension was gently triturated with a pipette and filtered through a 200 mesh screen. To remove the supernatant, the filtrate was centrifuged at 1,580  $\times$  g for 5 minutes at 4°C. Cells were plated at a density of 1  $\times$



**Figure 1 Morphology of hippocampal neurons.**

(A–D) Hippocampal neurons were cultured for 1 (A), 5 (B), 7 (C), and 9 days (D). (E) Immunofluorescence for microtubule-associated protein 2 (red) on 7-day-old cultures. Nuclei were stained with Hoechst (blue fluorescence). Scale bars: 100  $\mu\text{m}$  (A–D), 50  $\mu\text{m}$  (E).

$10^6$  cells/mL and grown in Neurobasal Medium (Invitrogen, Carlsbad, CA, USA) containing B27 supplement (Invitrogen), without fetal bovine serum, and maintained in 5%  $\text{CO}_2$ , 90% humidity at  $37^\circ\text{C}$  for 4 days (Wu et al., 2012; Quan et al., 2013).

### Treatments

Hippocampal cultures were exposed to  $10 \mu\text{mol/L}$   $\text{A}\beta_{1-42}$  (American Peptide, Sunnyvale, CA, USA) for 72 hours (Kashiwaya et al., 2000). The cell medium was then freshly replaced and cells were incubated with  $1 \mu\text{mol/L}$  ICI182780 (Tocris Bioscience, Ellisville, MO, USA) for 30 minutes,  $50 \mu\text{mol/L}$  LY294002 (Cell Signaling, Boston, MA, USA) for 1 hour, or  $10 \mu\text{mol/L}$  U0126 (Cell Signaling) for 2 hours (Chaulet et al., 2001; Lee et al., 2006; Zhang et al., 2010). The inhibitors were removed by replacing the medium with neurobasal medium (Invitrogen, Carlsbad, CA, USA) containing B27 supplement, without fetal bovine serum, and the cells were then treated with  $2 \mu\text{L/mL}$  TLJN,  $26 \mu\text{mol/L}$  geniposide,  $2.5 \mu\text{mol/L}$  ginsenoside Rg1, or  $1 \text{ nmol/L}$  estradiol ( $\text{E}_2$ ) for 72 hours before analyses (Hua et al., 2010; Sun et al., 2013).

### Morphology of hippocampal neurons

The morphology of hippocampal neurons was observed in ten randomly selected visual fields under an inverted microscope (NIKON TE2000-S, Tokyo, Japan) 1, 5, 7 and 9 days after treatment.

### Immunofluorescence for neurons

Microtubule-associated protein 2 (MAP-2) was used to identify neurons, and Hoechst 33342 was used to stain the nucleus (Gong et al., 2012). Neurons were cultured for 7 days

at a density of  $1 \times 10^6$  cell/mL on 12-mm cover slips coated with poly-L-lysine. Cells were fixed with cold 4% paraformaldehyde for 15 minutes, followed by 10 minute permeabilization in 0.1% Triton X-100 at room temperature, then rinsed three times for 5 minutes with PBS, and blocked with 0.5% bovine serum albumin solution for 30 minutes. Cells were then incubated with rabbit MAP-2 polyclonal antibody (1:500; Chemicon, Billerica, MA, USA) overnight at  $4^\circ\text{C}$  followed by three washes with PBS. Cells were incubated with fluorescein isothiocyanate goat anti-rabbit IgG (1:50; Cwbio, Beijing, China) for 1 hour at room temperature, then washed with PBS and double stained with Hoechst 33342 ( $10 \mu\text{g/mL}$ ) (Beyotime, Haimen, Jiangsu Province, China) for 5 minutes at room temperature. Staining was observed under a confocal laser scanning microscope (Zeiss, LSM 510, Oberkochen, Baden-Württemberg, Germany). Cell and nuclei counts were performed from ten randomly selected visual fields. The purity of hippocampal neurons was calculated as the number of hippocampal neurons/the number of cell nuclei  $\times 100\%$ .

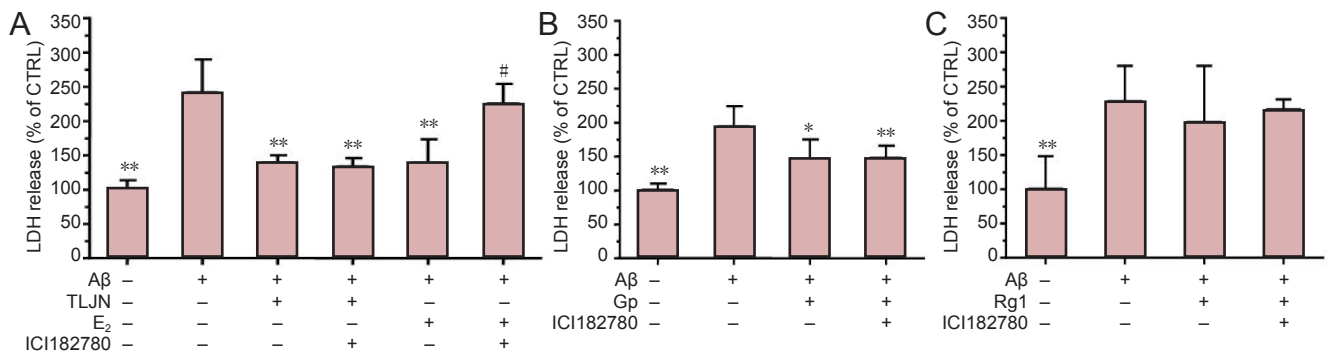
### Cell viability analysis

Cell viability was analyzed at day 11 by measuring the release of lactate dehydrogenase (LDH) (Decker and Lohmann-Matthes, 1988) using the LDH kit (Roche, Basel, Switzerland).

### Statistical analysis

All data are expressed as mean  $\pm$  SD (with each experiment in at least triplicate) and were analyzed either by the one-way analysis of variance or the independent samples *t*-test using GraphPad Prism software (version 6.00; GraphPad Software Inc., CA, USA). A value of  $P < 0.05$  was considered statistically significant.





**Figure 2** *Tongluojiunao* (TLJN), including its component, geniposide, reduces amyloid-peptide (1–42) ( $A\beta_{1-42}$ )-induced increase of lactate dehydrogenase (LDH) release independent of the classical estrogen pathway.

(A) The classical estrogen pathway inhibitor, ICI182780, does not block (A) TLJN- or (B) geniposide-mediated reduction of  $A\beta_{1-42}$ -induced LDH release. (C) Ginsenoside Rg1 does not affect LDH release under  $A\beta_{1-42}$ -exposed conditions. Results are presented as a ratio of control cells (CTRL). All data are expressed as mean  $\pm$  SD (with each experiment in at least triplicate) and were analyzed either by the one-way analysis of variance or the independent samples *t*-test. \* $P < 0.05$ , \*\* $P < 0.01$ , vs.  $A\beta$  group; # $P < 0.01$ , vs.  $A\beta + E_2$  group. Gp: Geniposide;  $E_2$ : 17- $\beta$  estradiol.

## Results

### Morphological observation and identification of hippocampal neurons

Cells began to adhere 2 hours after seeding and were firmly attached within 6–12 hours. The cells were evenly distributed on the bottom of plate 24 hours later, with one or two neurites found in few cells (Figure 1A). On day 5, many neurites extended from the somas and neuronal length increased (Figure 1B). On day 7, the somas were round or oval and the neurites were interweaved into a network. Haloes of the neurons were evident (Figure 1C). On day 9, the neurons matured and aggregated into clumps (Figure 1D). MAP-2 immunofluorescence staining on day 7 (Figure 1E) confirmed that the culture was predominantly (approximately 90%) neuronal (data not shown).

### TLJN decreased $A\beta_{1-42}$ -induced LDH release independent of the estrogen receptor pathway

Results showed that the release of LDH was significantly ( $P < 0.01$ ) increased in cultures exposed to  $A\beta_{1-42}$  compared with the control (Figure 2A), indicating that the *in vitro* Alzheimer's disease model was reliable. Compared with the  $A\beta_{1-42}$  alone group, these levels were significantly ( $P < 0.01$ ) reduced in  $A\beta_{1-42}$ -exposed cultures with TLJN and  $E_2$  (Figure 2A). Furthermore, of the two components of TLJN, geniposide significantly ( $P < 0.05$ ) reduced LDH in  $A\beta_{1-42}$ -exposed cultures (Figure 2B) and ginsenoside Rg1 had no effect (Figure 2C).

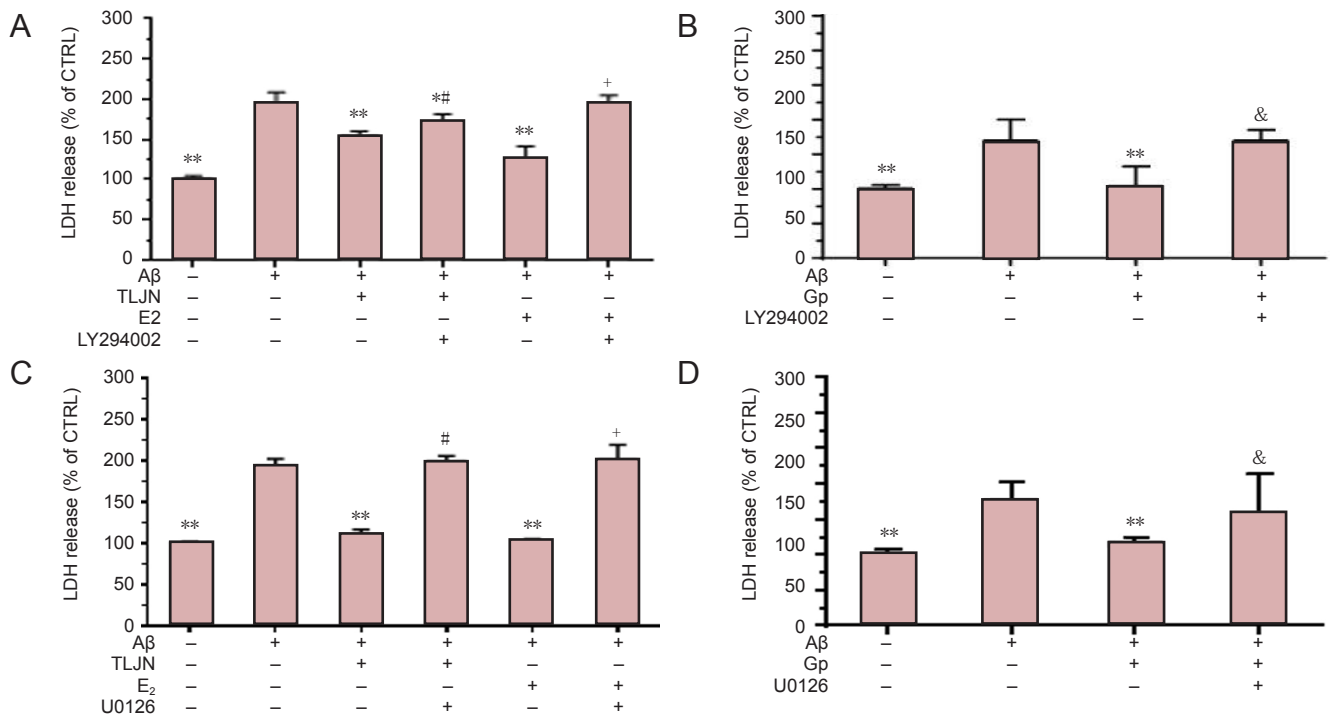
Similarly to TLJN, 17- $\beta$  estradiol markedly ( $P < 0.01$ ) attenuated the  $A\beta$ -induced release of LDH (Figure 2A). Blocking the classical estrogen receptor pathway with ICI182780 significantly ( $P < 0.01$ ) attenuated the protective effect of 17- $\beta$  estradiol under  $A\beta$  conditions (Figure 2A). However, this effect was significantly ( $P < 0.01$ ) reversed by the subsequent addition of TLJN (Figure 2A) or geniposide (Figure 2B). These results indicated that the classical estrogen signaling pathway did not participate in TLJN's protective function.

### The neuroprotective effect of TLJN was partially attributed to the non-classical estrogen signaling pathway

Because the neuroprotective effect of TLJN was found to be

independent of the estrogen receptor classical pathway, we proposed that other signaling cascades could be involved. Therefore, we investigated the effect of the PI3K inhibitor, LY294002. LY294002 plus 17- $\beta$  estradiol markedly ( $P < 0.01$ ) attenuated the  $A\beta_{1-42}$ -induced release of LDH compared with 17- $\beta$  estradiol alone under  $A\beta_{1-42}$ -exposed conditions (Figure 3A), suggesting that this PI3K inhibitor effectively blocked the neuroprotection mediated through the non-classical estrogen signaling pathway. However, this effect was significantly ( $P < 0.01$ ) reversed by the subsequent addition of TLJN compared with TLJN alone under  $A\beta_{1-42}$ -exposed conditions (Figure 3A), indicating that the PI3K pathway plays a role in the neuroprotective effect of TLJN. However, the protective effect of TLJN protection on cells was not completely blocked by LY294002 because the LDH level was significantly ( $P < 0.05$ ) lower than that of cells exposed to  $A\beta_{1-42}$  alone (Figure 3A). The effect of TLJN was found to be mainly attributed to geniposide ( $P < 0.01$ ; Figure 3B). These results suggested that PI3K signaling pathway partially contributed to the neuroprotective effect of TLJN.

Because the non-classical estrogen pathway also includes the MAPK signaling pathway, we next explored if this pathway participated in the protective effect of TLJN under  $A\beta_{1-42}$ -exposed conditions using the MAPK inhibitor, U0126 (Duncia et al., 1998). U0126 plus 17- $\beta$  estradiol significantly ( $P < 0.01$ ) increased  $A\beta_{1-42}$ -induced release of LDH compared with 17- $\beta$  estradiol alone under  $A\beta$ -exposed conditions (Figure 3C), indicating the inhibition of neuroprotection *via* a non-classical estrogen pathway. LDH release was markedly ( $P < 0.05$ ) higher with the subsequent addition of TLJN compared with TLJN alone under  $A\beta_{1-42}$ -exposed conditions (Figure 3C), indicating that the MAPK pathway participated in the neuroprotective effect of TLJN. Moreover, the protective effect of TLJN was almost completely blocked by U0126 because LDH release was much higher (close to that of  $A\beta_{1-42}$  alone) than that of TLJN treatment under  $A\beta_{1-42}$ -exposed conditions. The subsequent addition of geniposide markedly ( $P < 0.01$ ) attenuated the neuroprotective effect of geniposide (Figure 3D). These findings demonstrated that the MAPK signaling pathway was required for the neuroprotective effect of TLJN.



**Figure 3** The phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling pathways are partially attributed to the neuroprotective effect of *Tongluojiunao* (TLJN), including geniposide.

(A) The reduced release of LDH by TLJN is partially blocked by the PI3K inhibitor, LY294002, under Aβ<sub>1-42</sub>-exposed conditions. (B) The reduced release of LDH by geniposide (Gp) is completely inhibited by LY294002. (C) The neuroprotective effect of TLJN is dependent on the MAPK signaling pathway. (D) Gp protects cells *via* the MAPK estrogen signaling pathway. Results are presented as a ratio of control cells (CTRL). All data are expressed as mean ± SD (with each experiment in at least triplicate) and were analyzed either by the one-way analysis of variance or the independent samples *t*-test. \**P* < 0.05, \*\**P* < 0.01, vs. Aβ<sub>1-42</sub> group; #*P* < 0.05, ##*P* < 0.01, vs. Aβ<sub>1-42</sub> + TLJN group; +*P* < 0.01, vs. Aβ<sub>1-42</sub> + E<sub>2</sub> group; &*P* < 0.01, vs. Aβ<sub>1-42</sub> + Gp group. E<sub>2</sub>: 17-β estradiol; U0126: MAPK inhibitor; LDH: lactate dehydrogenase.

## Discussion

Previous reports have shown that ginsenoside Rg1 alone is neuroprotective (Gong and Zhang, 1999; Chen et al., 2003; Leung et al., 2007; Gao et al., 2009). However, in our study, ginsenoside Rg1 was not protective against the neuronal cells in our model. This discrepancy may be due to its regulated effects in a dose-dependent manner, as well as the differences between primary cultured rat hippocampal neurons and PC12 cells (Chen et al., 2003). Reports have illustrated that the neuroprotective effect of Rg1 through the estrogen classical pathway occurs at a concentration as low as  $3 \times 10^{-7}$  μmol/L (Chen et al., 2003). Furthermore, Rg1 has also been shown to be neuroprotective between 5 μmol/L and 20 μmol/L (Chen et al., 2003). However in this study, ginsenoside Rg1 was used at a lower concentration (2.5 μmol/L) than the other studies. The protective effect of ginsenoside Rg1 has been shown to be mediated by the upregulated and downregulated activity of nuclear factor-kappa B in neurons and astrocytes, respectively (Quan et al., 2013), suggesting that ginsenoside Rg1 decreases Aβ<sub>1-42</sub> levels by upregulating the expression of peroxisome proliferator-activated receptor γ and insulin-degrading enzyme. Wu et al. (2012) have also demonstrated that ginsenoside Rg1 protects Aβ<sub>25-35</sub>-induced injury of primary cultures of rat cortical neurons through the mitochondrial-mediated antiapoptotic pathway. These findings suggest that ginsenoside Rg1 induces a neuroprotective

effect through a pathway different from that of estrogen. Future studies should aim to determine a concentration-response effect of ginsenoside Rg1 for its neuroprotective action.

In the present study, TLJN, geniposide, and 17-β estradiol exerted different effects when pre-treated with the different kinds of inhibitors. The integrity of cell membranes was preserved and therefore LDH release was decreased in the treatment of ICI182780 plus 17-β estradiol. However, the other two inhibitors had no effect. These findings indicate that 17-β estradiol exerts its effects predominantly through the classical pathway. Interestingly however, the neuroprotective effect of TLJN and geniposide was inhibited by LY294002 and U0126, and not ICI182780. LY294002 only partially blocked the neuroprotective effect of TLJN/geniposide and U0126 completely reversed this response. These results indicate that the neuroprotective effect of TLJN and geniposide against Aβ<sub>1-42</sub> occurs through the non-classical (*i.e.*, PI3K and MAPK) signaling pathways instead of the classical (estrogen receptor) signaling pathway.

Although the protective mechanism of TLJN remains unclear, we hypothesized that it exhibits phytoestrogen-like actions and therefore has a neuroprotective effect. Furthermore, elucidating whether the classical or non-classical signaling pathway is involved remains to be determined. The present study explored the effects of TLJN and its com-

ponents, geniposide and ginsenoside Rg1, on A $\beta$ -mediated neuronal injury and their possible signaling pathways as a mechanism of action.

In conclusion, TLJN, and its active ingredient, geniposide, protected hippocampal neurons *via* a non-classical estrogen signaling pathway, suggesting that geniposide exhibits a phytoestrogen-like function. Therefore, TLJN/geniposide requires further investigation as a possible alternative treatment for neurodegenerative diseases.

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**Author contributions:** Li J and Chen JY performed statistical analysis and wrote the manuscript. Wang F, Ding HM, Zhao YN, Tan Y, Zhang Q and Wang X were involved in the manuscript preparation. Jin CY and Li XJ were responsible for the study design and data collection. Chen WJ, Sun P and Fan AR performed some of the experiments. Hua Q was responsible for the study design and research funding. All authors approved the final version of the manuscript.

**Conflicts of interest:** None declared.

**Peer review:** The present study showed that, TLJN injection and its main ingredient, geniposide, exerted similar protective effects to the estrogen against A $\beta_{1-42}$ -induced damage. The underlying mechanism is not mediated by classical estrogen receptor pathway, but by non-classical PI3K and MAPK signaling pathways. Our findings can not only provide important evidence to reveal neuroprotective mechanism of TLJN injection, but also suggests that TLJN is a potential therapy with estrogenic effect.

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