Journal of Integrative Neuroscience

Original Research

Geniposide protection against Aβ_{1-42} toxicity correlates with mTOR inhibition and enhancement of autophagy

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DOI:10.31083/j.jin.2021.01.242

Submitted: 13 August 2020 Revised: 13 January 2021 Accepted: 27 January 2021 Published: 30 March 2021

Overactivation of the PI3-K/Akt/mTOR signaling pathway and inhibition of autophagy in the brain are involved in Alzheimer's disease. The present paper's goal was to explore the potential mechanisms of geniposide to protect against Alzheimer's disease. We treated the human neuroblastoma SH-SY5Y cell line with Aβ_{1-42} as an Alzheimer's disease *in vitro* model to explore the potential mechanisms of geniposide to protect against Alzheimer's disease. Further, SH-SY5Y cells damaged by Aβ_{1-42} were treated with geniposide. Akt/mTOR-related proteins and autophagy-associated proteins were measured to reveal the molecular mechanisms by which geniposide protects against Aβ_{1-42}-induced toxicity. Results showed that Akt and mTOR's geniposide inhibited phosphorylation induced by Aβ_{1-42}, enhanced expression of the LC3II/LC3I ratio, and Atg7 and Beclin1 expression and inhibited expression of p62 induced by Aβ_{1-42}. Our results lead us to hypothesize that inhibition of the Akt/mTOR signaling pathway and autophagy enhancement are fundamental molecular mechanisms for geniposide to protect against Aβ toxicity.

Keywords

Geniposide; PI3K; Akt; mTOR; Autophagy; Aβ; Neurodegeneration; Alzheimer's disease

1. Introduction

Aging is a pivotal factor for numerous diseases, including neurodegeneration, obesity, diabetes, cardiovascular diseases, and metabolic disorders [1, 2]. Growth factors, nutrients, and energy metabolism are pivotal factors for cell growth, development, and proliferation. Activation of the mechanistic target of rapamycin (mTOR) promotes cell growth in response to favorable environmental cues and is viewed as a master regulator of this response [3]. Many studies have shown that mTOR signaling dysregulation is involved in age-related diseases, including neurodegenerative diseases, diabetes, metabolic disorders, and cancer [4]. mTOR signaling networks stimulate the synthesis of nucleotides, proteins, and lipids and block autophagic catabolic response at the post-translational and transcriptional levels [5]. The PI3-K/Akt/mTOR signaling pathway is widely regarded as a central signaling axis to regulate cell growth and proliferation, crucial metabolism processes, apoptosis, and secretion [6]. Protein kinase B (PKB, also known as Akt) performs its action as a central intersection between phosphoinositide 3-kinase (PI3-K) and mTOR by phosphorylating various substrates. Considering its crucial role in regulating vital cellular functions, dysregulation of PI3-K/Akt/mTOR is a critical molecular event in mental illnesses [7]. Specifically, abnormalities in PI3-K/Akt/mTOR signaling are involved in Alzheimer's disease (AD) [8]. Overactivation of PI3-K/Akt/mTOR signaling in the brain is regarded as an early pathogenic event in AD and an essential candidate for pathophysiological processes activated by β-amyloid [8]. Evidence gathered also indicates that insulin and IGF-1 can rescue and normalize the aberrant PI3-K/Akt/mTOR signaling and protect against AD's physiopathologic processes [9].

Recent studies focused on the regulators of longevity and health span showed that strategies to delay aging are therapeutic strategies for aging-related diseases such as AD [10, 11]. mTOR inhibition [12] and autophagy enhancement [13] are regarded as crucial regulators of longevity and health span, as well as the novel therapeutic strategies for aging-associated diseases. mTOR functions as a nutrient sensor by regulating "protective" autophagy programs [14]. Interestingly, activation of the mTOR signaling pathway is related to AD [15]. The inhibition of mTOR is being developed into a novel AD therapy [16].

Autophagy is a critical molecular mechanism in mediating the lifespan-extending effects of dietary restriction and mTOR inhibition [17]. Autophagy is a normal cellular process in which the lysosome degrades older cytosolic components due to nutrient deprivation [18]. Many studies have

shown that damage due to autophagy occurs at the early stages of the AD process. Studies also showed that autophagy performs a pivotal role in the production and metabolism of Aβ and AD progress [19]. As the self-degrading process, autophagy is key in maintaining cellular homeostasis. Defects in autophagy homeostasis are considered pivotal pathogenesis in shortening lifespan and promoting multifarious aged-related diseases, including obesity, insulin resistance, diabetes, dementia, atherosclerosis, and neoplasm. Preclinical evidence supports autophagy modulators' therapeutic promise to treat obesity and metabolic diseases [20]. Recent work has shown that glucagon-like peptide-1 (GLP-1)-based therapeutic approaches may positively affect autophagy in perivascular adipose tissue, thus improving obesity-related endothelial dysfunction [21]. To explore the effects of GLP-1 in GLP-1/insulin/insulin-like growth factor-1 (IGF-1) signaling pathway and the autophagic process, Candeias *et al.* [22] evaluated the effect of GLP-1 GLP-1 mimetics, exendin-4 (Ex-4) on insulin, and IGF-1, their downstream signaling and autophagic markers in brain of the T2D rats [22]. The results showed that Ex-4 protects T2D rats against hyperglycemia; insulin resistance enhances GLP-1 and IGF-1 levels in brain cortical and subsequent signaling pathways. Ex-4 also regulated autophagy markers (as mTOR, PI3K class III, LC3 II, Atg7, p62, LAMP-1, and Parkin).

Geniposide is a traditional Chinese medicine monomer isolated from the herb Gardenia jasminoides. Its extensive pharmacological effects, including anti-diabetes, anti-inflammation, antioxidation, neuroprotection, and anti-asthma, have been noted [23]. The protective effects of geniposide in neurodegenerative diseases have been of keen interest. A glucagon-like peptide-1 receptor (GLP-1R)-the dependent mechanism-protected geniposide [24, 25]. Further, activation of PI3K/AKT signaling may also involve a geniposide-induced protective effect [26]. Li *et al.* [27] showed that although geniposide was a useful bioactive substance in treating AD, its toxicity was apparent at a dose higher than 50 mg/kg/d. Dinda *et al.* [28] reviewed the therapeutic potential of plant iridoids, including geniposide, in AD and Parkinson's disease. Plant iridoids exhibit the property of retarding the process of neurodegeneration in AD and Parkinson's disease. Geniposide performed its protective effects after passing the blood-brain barrier [29]. Plant iridoids, including geniposide, can ameliorate AD by increasing the expression of PPAR-γ, and α-secretase, insulin-degrading enzyme, neprilysin, and decreasing the levels of Aβ oligomers (Aβ_O) deposited in brain neurons. The molecular mechanism has been extensively explored. It is suggested that plant iridoids, including geniposide, may: 1. Decrease expression of GSK-3β and its receptor gene; 2. Improve the lysosomal autophagy process by increasing the expression of LC3II, Beclin-1, and cathepsin B genes for the clearance of Aβ and neurofibrillary tangles (NFT); 3. Enhanced expression of transporter proteins, such as P-glycoprotein and low-density lipoprotein receptor-related protein-1, for the clearance of Aβ load from brain across the blood-brain barrier; 4. Enhanced expression of PPAR-γ and ApoE proteins for clearance of Aβ in ApoE mediated pathway from the brain. Further, plant iridoids may decrease cognitive impairment by enhancing the expression of synaptic proteins, such as SNAP-25, BDNF, PSD-95, GAP-43 and SYP, to improve learning memory ability in AD. Some of those plant iridoids, including geniposide, may improve the expression of TH-positive neurons, GDNF, and Bcl-2 proteins by increasing the levels of antioxidant enzymes, such as GSH-P$_X$ and SOD, and down-regulate insulin/IGF signaling by activating MEK. Furthermore, geniposide may enhance the expression of autophagy-related LAMP-2A-protein for clearance of LB from dopaminergic neurons in the PD brain via improving the lysosomal autophagy process.

Song *et al.* [30] pretreated differentiated SH-SY5Y cells or primary hippocampal neurons with Schizandrol A and subsequently subjected the cells to β-amyloid peptides of 1-42 amino acids (Aβ_{1-42}) and estimated the effect of Schizandrol A by testing its effects on cell viability, apoptosis, oxidative stress, and autophagy. Further, these investigators explored the molecular mechanism underlying this effect by treating cells with an mTOR inhibitor (rapamycin) and a PI3K inhibitor (LY294002) to analyze the role of the PI3K/AKT/mTOR pathway. Their results showed that Schizandrol A effectively inhibited Aβ_{1-42}-triggered increases in apoptotic cell number and pro-apoptotic protein expression, reduction of viable cells, as well as alterations in markers of oxidative stress. Also, Schizandrol A enhanced LC3-II/LC3-I and Beclin-1 and reduced the expression of p62. At the molecular level, they showed Schizandrol A rescued the PI3K/AKT/mTOR-autophagy pathway dysregulation resulting from Aβ_{1-42} exposure.

Based on the overlapping functions between GLP-1 and mTOR inhibition, including energy balance, AD protection and diabetes treatment, we hypothesized in an earlier study that mTOR inhibition may mediate the protective effect of GLP-1 in AD [31]. Similarly, Jiang *et al.* [32] explored molecular mechanisms underlying the effect of GLP-1 to improve insulin signaling in ER-stressed adipocytes. These investigators showed GLP-1 directly modulated ER stress response, in part, by inhibiting the mTOR signaling pathway. Further, a study from our group showed that the downregulation of mTOR signaling and enhancement of autophagy in APP/PS1 mice mediated the effect of geniposide to protect against amyloid deposition and behavioral impairment [33].

In this paper, we test the hypothesis that mTOR inhibition and autophagic activity are key molecular events that control the protective effects of geniposide against Aβ *in vitro*.

2. Materials and methods

2.1 Chemicals and reagents

The SH-SY5Y cell line's human neuroblastoma was obtained from the Stem Cell Bank, Chinese Academy of Sciences. Geniposide (purity $\geq$ 98%) was purchased from

Aladdin Bio-Chem Technology Company, LTD, Shanghai, PR China. Aβ_{1-42} (CAT: 1932-2-15, Peptide Sequence: Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val-Ile-Ala) was purchased from Qiangyao Biotechnology Company. Anti-LC3II antibody (CAT: L8918) was purchased from Sigma, USA. Anti-mTOR antibody (CAT: ab134903), anti-p-mTOR (Ser2448) antibody (CAT: ab109268), anti-Akt (Ser473) antibody (CAT: ab81283), anti-Akt antibody (CAT: ab238477), anti-Atg7 antibody (CAT: ab133528), anti-Beclin1 antibody (CAT: ab210498), and anti-P62 antibody (CAT: ab210498) were purchased from Abcam, UK. Anti-Bcl2 antibody (CAT: BS70205), anti-Bax antibody (CAT: BS6420), β-action antibody, and HRP-labeled Goat anti-Rabbit IgG were purchased from Bioworld Technology Company, Shanghai, PR China. Fetal bovine serum was purchased from Cellmax technology Company. Beijing, PR China.

2.2 Cell culture

SH-SY5Y cells (ATCC CRL-2266, Shanghai, PR China) were cultured in DMEM/F-12 medium containing streptomycin (100 μg/mL), penicillin (100 U/mL), and 10% heat-inactivated fetal bovine serum at 37 °C in a humidified incubator based on 5% CO_2 and 95% air. Aβ_{1-42} was dissolved in 100% 1, 1, 1, 3, 3, 3-hexafluoro-2-propanol (HFIP) to a concentration of 1 mg/mL. This solution was incubated at room temperature (RT) for 1 h and, after that, sonicated for 10 min. The HFIP/Aβ_{1-42} solution was subsequently dried down in a gentle stream of nitrogen, and the dried Aβ_{1-42} was resuspended in 1 mM DMSO. The preparation was incubated for 12 min at RT and then pipetted and stored at -80 °C. Before use, the preparation was rapidly thawed, utilizing 0.1 M PBS, and a final Aβ_{1-42} concentration of 20 μM was prepared. Neurons were grouped into control; Aβ_{1-42} treatment, the only treatment of geniposide, and Aβ_{1-42} + geniposide treatment.

2.3 Cell viability (MTT) assay

The viability of SH-SY5Y cells was measured utilizing a 3, (4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide (MTT) assay. Before analysis, SH-SY5Y cells were seeded into 96-well density, and the cell density was adjusted to 5,000 cells/well and incubated for 24 h before treatment. For selecting an appropriate concentration of Aβ_{1-42}, the cells were treated with different concentrations of Aβ_{1-42} (0, 5, 10, 20, 40 μM). Apparentcytotoxicity was seen in cells treated by 20, 40 μM Aβ_{1-42} and the concentration of 20 μM Aβ_{1-42} was selected to conduct our study. Where indicated, cells treated with 20 μM Aβ_{1-42} were also treated with different concentrations of geniposide (0, 5, 10, 20, 40 μM).

SH-SY5Y cells in various treatment groups (Aβ_{1-42} only, geniposide only, and Aβ_{1-42} and geniposide) were treated 24 h. After this, MTT was added to the culture media (0.5 mg/mL final concentration) and incubated for 4 h at 37 °C in a CO_2 incubator. The culture medium was mixed with extraction buffer, and then absorbance was measured at 490 nm after an overnight incubation utilizing a microplate absorbance reader (Bio-Rad Instruments). Untreated cells were used as controls, and cell viability was calculated using the formula:

Cell viability = **A** of a sample (treated by Aβ_{1-42}, sole geniposide and Aβ_{1-42} + geniposide separately) / **A** of the control sample where **A** = absorbance.

2.4 Western blot

SH-SY5Y cells were lysed with RIPA protein lysis buffer containing 1 mM PMSF (Beyotime Biotechnology, Shanghai, PR China) for 30 minutes after washing with cold PBS. Total proteins in the supernatant were quantified using a BCA protein assay (Beyotime Biotechnology, Shanghai, PR China) after centrifugation of the cell lysate at 12000 r/min for 20 min at 4 °C. Proteins were subsequently resolved in 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels (Beyotime Biotechnology, Shanghai, PR China) and transferred to polyvinylidene difluoride membranes (Beyotime Biotechnology, Shanghai, PR China). Membranes were incubated in 5% BSA (TBST) for 2 h at room temperature and after that were incubated with primary antibodies against Anti-LC3II, mTOR, p-mTOR, Akt, p-Akt, Atg7, Beclin1, and P62 overnight at 4 °C. Membranes were subsequently washed and treated with horseradish peroxidase-conjugated secondary antibody (1 : 5000) for 2 h at room temperature. Proteins were visualized utilizing an enhanced chemiluminescence method, and β-actin was used as a loading control.

The protein bands were visualized using the Chemi-Doc XRS + imaging system (Bio-Rad). The Western blots were subjected to quantification of the protein band density using the Image Pro.

2.5 Statistical analyses

The results were expressed as mean $\pm$ SD. A one-way ANOVA analysis was used to determine statistical significance. The contrast between multiple groups was performed by one-way ANOVA based on SPSS 19.0 software, and the differences observed were further analyzed by the least significant difference (LSD)-*t*-test. A *P*-value of less than 0.05 was considered statistically significant.

3. Results

3.1 Geniposide reverses loss of cell viability induced by Aβ_{1-42} in SH-SY5Y cells

To investigate the effect of Aβ_{1-42} on SH-SY5Y cells, an MTT assay was conducted to quantify cell viability. Results indicated a concentration-dependent effect of Aβ_{1-42} on cell viability (Fig. 1A). Lower doses of Aβ_{1-42} (5 and 10 μM) did not affect cell viability, whereas higher concentrations of Aβ_{1-42} (20 and 40 μM) had measurable effects on cell viability. Aβ_{1-42} (20 μM) treatment significantly decreased the cell viability to 61.8 $\pm$ 4.1% versus control (100%).

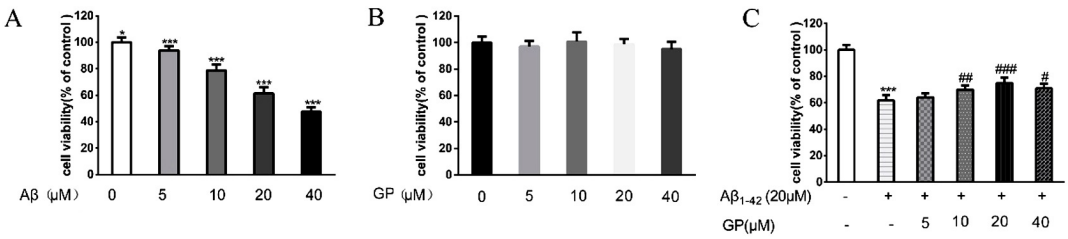

Fig. 1. Geniposide reverses cellular toxicity induced by Aβ in SH-SY5Y cells. There was no significant difference in cell viability between the cells treated by different concentrations of geniposide and control. Cells treated with 20 μM Aβ_{1-42} for 24 hours. Cell viability was measured utilizing an MTT assay. Values were denoted as mean $\pm$ SD. $^{***}P < 0.001$, $^{*}P < 0.05$ vs. control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. Aβ_{1-42} treatment.

Based on these findings, 20 μM concentrations of Aβ_{1-42} were selected for further study. Treatment of SH-SY5Y cells with various concentrations of geniposide did not affect the cells' viability versus untreated controls (Fig. 1B). However, a concentration-dependent relationship of geniposide to protect against lost cell viability following Aβ_{1-42} exposure was observed (Fig. 1C). Specifically, cell viability was restored from 61.8 $\pm$ 4.1% in cells treated with 20 μM Aβ_{1-42} to 64.1 $\pm$ 3.2%, 70.0 $\pm$ 3.2%, 74.8 $\pm$ 4.3%, and 69.0 $\pm$ 3.517% after different concentrations (5, 10, 20, and 40 μM, respectively) of geniposide. 20 μM geniposide was selected for further studies based on the maximum effect to improve viability induced by 20 μM Aβ_{1-42} treatment.

3.2 Geniposide protects against Aβ_{1-42} by downregulating mTOR signaling

mTOR signaling was upregulated in the SH-SY5Y cells treated by Aβ_{1-42}. phosho-AKT (Ser473)/AKT ratio increased from 0.370 $\pm$ 0.087 in control to 0.748 $\pm$ 0.131 in SH-SY5Y cells treated by Aβ_{1-42} (Fig. 2A), and the phospho-mTOR (Ser2448)/mTOR ratio increased from 0.476 $\pm$ 0.076 in control to 0.907 $\pm$ 0.160 in SH-SY5Y cells treated with Aβ_{1-42} (Fig. 2B).

Treatment of SH-SY5Y cells with geniposide only did not influence mTOR signaling as the phospho-AKT (Ser473)/AKT ratio (0.400 $\pm$ 0.050) as the phospho-mTOR (Ser2448)/mTOR ratio (0.498 $\pm$ 0.085) in the SH-SY5Y cells treated with geniposide only were not statistically different from control cells. Geniposide reversed mTOR signaling upregulation induced by Aβ_{1-42} as the phospho-AKT (Ser473)/AKT, and phospho-mTOR (Ser2448)/mTOR ratios were upregulated in the SH-SY5Y cells treated by Aβ_{1-42}. Specifically, we measured a 0.415 $\pm$ 0.052 in phospho-AKT (Ser473)/AKT ratio (Fig. 2A) and a 0.570 $\pm$ 0.0239 in phospho-mTOR (Ser2448)/mTOR ratio (Fig. 2B) after geniposide treatment.

3.3 Geniposide protects against Aβ_{1-42} toxicity by enhancing autophagy

Autophagy was inhibited in SH-SY5Y cells treated by Aβ_{1-42} (Fig. 3). Specifically, the LC3-II/LC3-I ratio decreased from 0.330 $\pm$ 0.080 in control to 0.204 $\pm$ 0.034 in SH-SY5Y cells treated with Aβ_{1-42} (Fig. 3A). Beclin1 decreased from 0.358 $\pm$ 0.102 in control to 0.131 $\pm$ 0.044 in SH-SY5Y cells treated with Aβ_{1-42} (Fig. 3B), and Atg7 decreased from 0.806 $\pm$ 0.241 in control to 0.317 $\pm$ 0.142 in SH-SY5Y cells treated with Aβ_{1-42} (Fig. 3C). Finally, expression of p62 increased from 0.306 $\pm$ 0.137 to 0.728 $\pm$ 0.170 in SH-SY5Y cells treated with Aβ_{1-42} (Fig. 3D).

The LC3-II/LC3-I ratio (0.323 $\pm$ 0.038), and expression of Beclin1 (0.332 $\pm$ 0.119), Atg7 (0.723 $\pm$ 0.270), and p62 (0.383 $\pm$ 0.108) in the SH-SY5Y cells treated by only treatment of geniposide were not statistically different from those measured in control cells, indicating that treatment of SH-SY5Y cells with geniposide only did not influence autophagy-related signaling. Geniposide did reverse the inhibition of autophagy induced by Aβ_{1-42}. Specifically, geniposide treatment increased the level of LC3-II/LC3-I ratio to 0.317 $\pm$ 0.066 (Fig. 3A), Beclin1 expression to 0.310 $\pm$ 0.075 (Fig. 3B), and Atg7 to 0.705 $\pm$ 0.247 (Fig. 3D). Similarly, geniposide treatment decreased the expression of p62 to 0.506 $\pm$ 0.155 (Fig. 3C).

3.4 Geniposide protects against Aβ_{1-42} by inhibiting Apoptosis

Apoptosis was activated in the SH-SY5Y cells following treatment with Aβ_{1-42}. The Bax/Bcl-2 ratio was increased after a 24 hours treatment with Aβ_{1-42} (1.864 $\pm$ 0.333) versus control (0.391 $\pm$ 0.194) (Fig. 4). However, geniposide alone did not influence the Bax/Bcl-2 ratio in SH-SY5Y (0.421 $\pm$ 0 .140) cells treated with only geniposide. In contrast, geniposide blunted apoptosis activation induced by Aβ_{1-42} as the Bax/Bcl-2 ratio fell dramatically to 0.499 $\pm$ 0.185 in SH-SY5Y cells treated with geniposide and Aβ_{1-42} (Fig. 4).

In sum, data gathered during this study provides evidence that geniposide can protect against the toxic effects of Aβ_{1-42} by inhibiting mTOR (Fig. 5). Evidence supporting this conclusion comes from the observations that phospho-AKT (Ser473)/AKT and phospho-mTOR (Ser 2248)/mTOR ratios were restored to near control levels with geniposide, and geniposide enhanced autophagy by increasing the LC3-II/LC3-I ratio, increasing expression of Beclin 1, Atg7, and inhibiting expression of p62. Finally, we observed that geniposide blunted the apoptotic response to Aβ_{1-42}, as evidenced by measuring the Bax/Bcl-2 ratio.

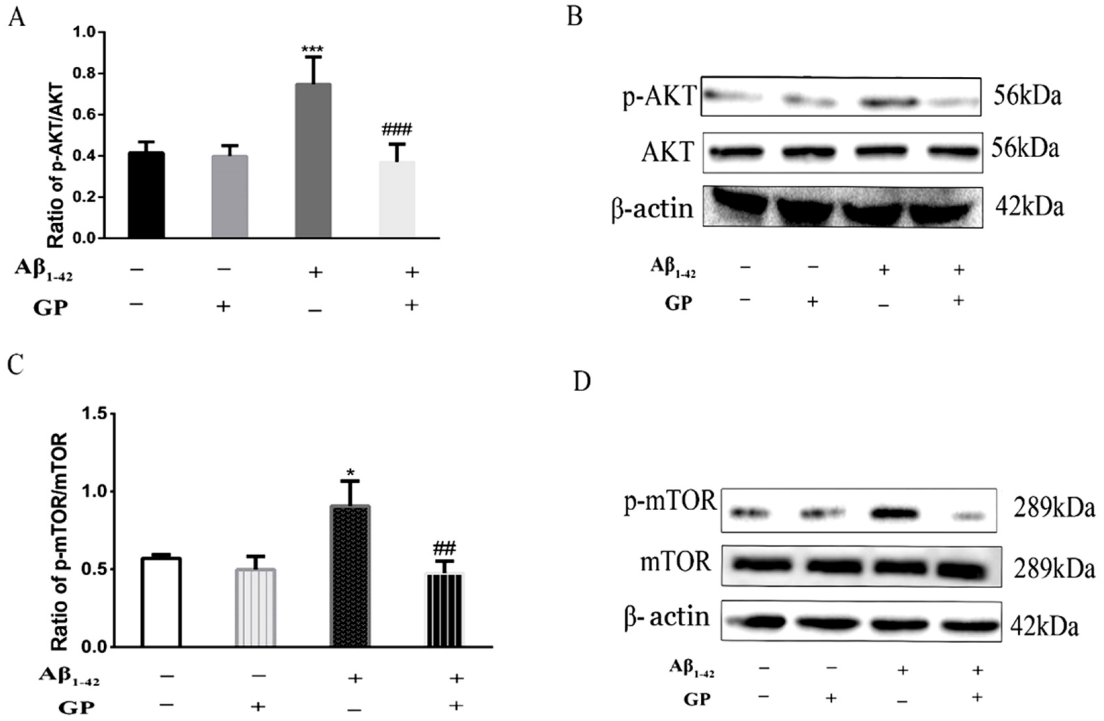

Fig. 2. Changes in mTOR signaling in SH-SY5Y cells treated with Aβ_{1-42} and geniposide. Western blot analysis was conducted to measure the phospho-AKT (Ser473)/AKT ratio, phospho-mTOR (Ser2448)/mTOR ratios in treated SH-SY5Y cells. Geniposide inhibited increases in phospho-AKT (Ser473)/AKT ratio and phospho-mTOR (Ser2448)/mTOR ratios induced by Aβ_{1-42}. β-actin was used as an internal control. All results are presented as the mean $\pm$ SD (n = 6). $^{*}P < 0.05$, $^{**}P < 0.001$ vs. control. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. Aβ_{1-42} treatment.

4. Discussion

A prior study showed that geniposide-mediated protection against pathological hallmarks of AD and behavioral impairment correlates with downregulation of mTOR signaling and enhanced autophagy in APP/PS1 double transgenic mice [33, 34]. In the present study, we sought to determine the mechanism by which geniposide prevents Aβ-associated toxicity. Considering that geniposide can activate the glucagon-like-1 receptor (GLP-1R) and adenylyl cyclase (AC)/cAMP signaling pathways and promotes insulin secretion and inhibition of protein kinase A (PKA) [35], we hypothesized that geniposide prevents Aβ toxicity by inhibiting the PI3-K/Akt/mTOR signaling pathway, and enhances the autography as an agonist of the GLP-1 receptor. Our results showed that geniposide protected SH-SY5Y cells against lost cell viability induced by Aβ_{1-42}. Further, we showed mTOR signaling was upregulated in the SH-SY5Y cells treated by Aβ_{1-42}. phosho-AKT (Ser473)/AKT ratio and the phospho-mTOR (Ser2448)/mTOR ratio increased in SH-SY5Y cells treated with Aβ_{1-42}. Geniposide reversed mTOR signaling upregulation induced by Aβ_{1-42}. The phospho-AKT (Ser473)/AKT and phospho-mTOR (Ser2448)/mTOR ratios were reversed after geniposide treatment. This finding implies that inhibition of the PI3-K/Akt/mTOR pathway may be a pivotal molecular event controlling geniposide's ability to prevent the toxic effects of Aβ.

Autophagy is a primary physiologic function for clearing abnormal proteins within mammalian cells and contributes to protein homeostasis and neuronal health. An autophagy deficit is found in early AD pathogenesis, and autophagy plays a critical role in the formation and metabolism of Aβ [31]. In the present study, we assessed autophagy by measuring the LC3-II/LC3-1 ratio, as well as Atg7, p62, and Beclin1 expression utilizing western blotting in SH-SY5Y cell lines treated with Aβ_{1-42}. Our results showed that geniposide protected against the cellular damage induced by Aβ_{1-42} in SH-SY5Y cells. Further, we showed that geniposide reversed the LC3-II/LC3-I ratio and repression of Atg7 and Beclin1 induced by Aβ_{1-42} and reversed the expression of p62 enhanced by Aβ_{1-42} in SH-SY5Y cells. The cytosolic form of LC3-I is converted to the phosphatidylethanolamine-conjugated form (LC3-II) and binds to autophagosomes' membranes [36]. Thus, the LC3-II/LC3-I ratio is an often-used marker for autophagy in various tissues, including the brain [37]. We observed a decrease of the LC3-II/LC3-I ratio after treatment of SH-SY5Y cells with Aβ_{1-42}, which suggests that Aβ damages the brain by, in part, inhibiting autophagy. The ratio was reversed after the treatment by geniposide, indicating that geniposide protects against AD by enhancing autophagy. Atg7 is an E1-like activating enzyme that is down-regulated during aging [38] and is needed for the autophagic conjugation system and formation of autophagosomes [39]. Sim-

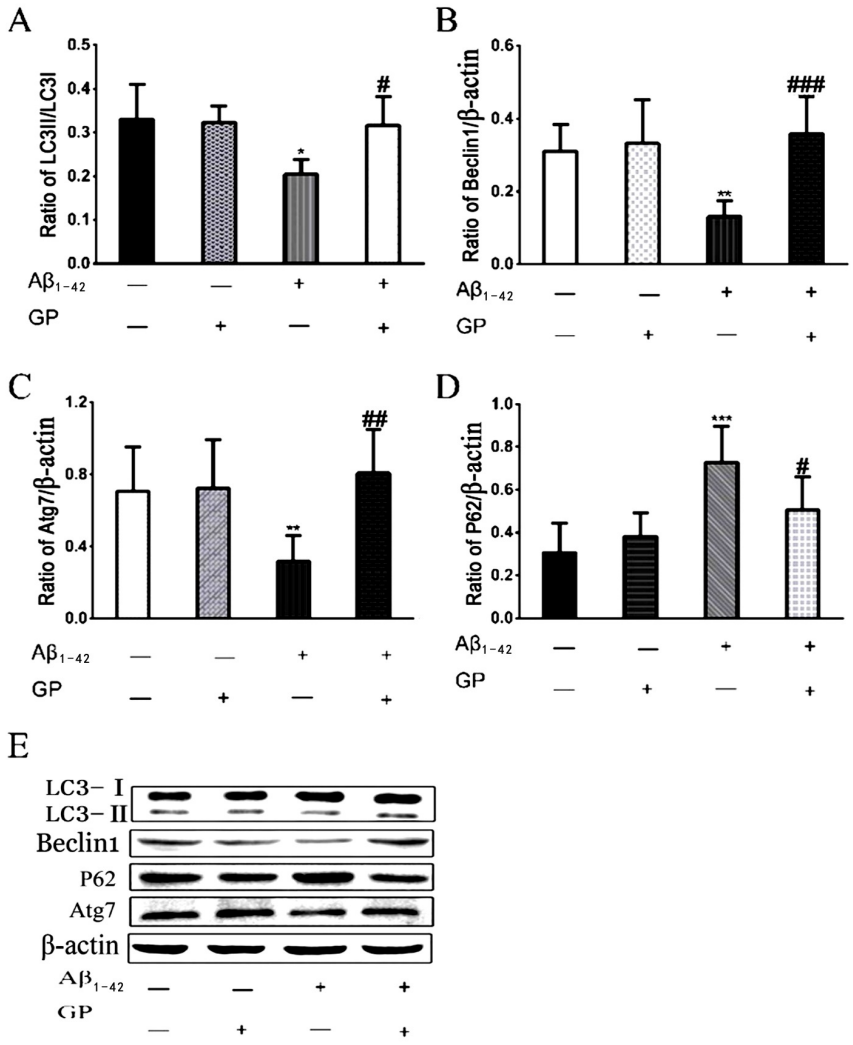

Fig. 3. Changes in autophagy-related proteins in SH-SY5Y cells. Western blot analysis of the LC3-II/LC3-I ratio, Beclin1, Atg7, and p62 expression was quantified by western blot. Geniposide increased the LC3-II/LC3-I ratio and Beclin1 and Atg7 expression and decreased the expression of p62 induced by Aβ_{1-42}. β-actin was used as an internal control. All results are presented as the mean ± SD (n = 6). * $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$ vs. control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. Aβ_{1-42} treatment.

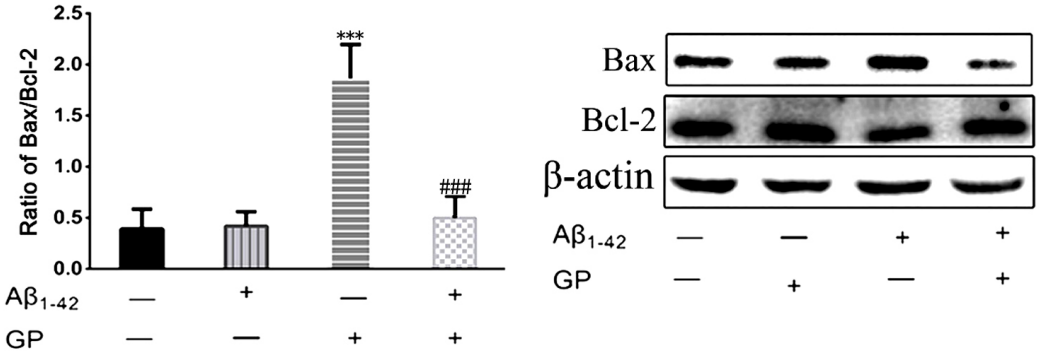

Fig. 4. Changes in apoptosis-associated proteins in SH-SY5Y cells. Quantitative western blot analyses of Bax and Bcl-2 expression were conducted. β-actin was used as an internal control. All results are presented as the mean ± SD (n = 6). ***$P < 0.001$ vs. control. $^{\#\#\#}P < 0.001$ vs Aβ_{1-42} treatment.

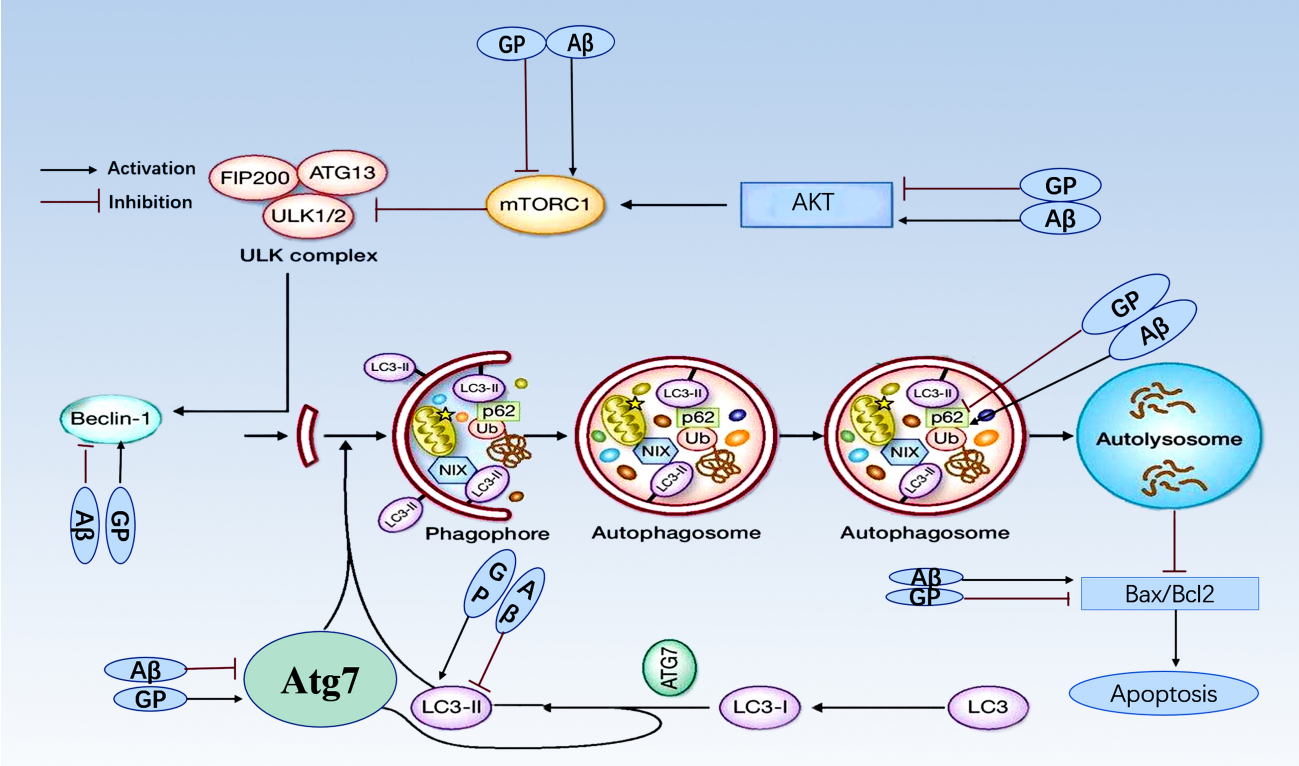

Fig. 5. Molecular mechanism for geniposide protection. Geniposide performs its protection against Aβ_{1-42} toxicity by inhibiting mTOR and enhancing autophagy. Geniposide reverses increase of AKT and mTOR induced by to Aβ_{1-42}. Geniposide reverses a decrease in the LC3-II/LC3-I ratio, decreases expression of Beclin 1 and Atg, and increases expression of p62 induced by Aβ_{1-42}. Geniposide also blunts the Aβ_{1-42}-induced apoptotic response by reducing the Bax/Bcl-2 ratio.

ilarly, the expression of Beclin-1, an autophagy-associated gene, is also widely used to reliably quantify autophagosome formation. There is a close relationship between AD and Beclin1, as Pickford *et al.* [40] showed a decrease of Becline 1 in the brain of patients with AD. Our results indicate that the enhancement of autophagy-related proteins, including the LC3-II/LC3-I ratio, Atg7, and Beclin-1, maybe a critical molecular event in the protective effects of geniposide during the toxic response to Aβ.

LC3B-II is a trustworthy indicator for the formation of autophagic vacuoles, just as the lipidized form LC3B-I and p62 are markers for autophagic flux as an adapter for selective autophagy [41]. The degradation of p62 is widely utilized as a marker to monitor the autophagic activity because p62 can directly bind to LC3 and is selectively degraded during autophagy [42]. To estimate the effect of IL-4 on the formation of autophagic vacuoles and promote autophagic flux in microglia, Tang *et al.* [43] measured LC3 B-II and p62 in microglia and found an enhancement of LC3 B-II and an attenuation of p62 in microglia treated with IL-4. Song *et al.* [44] showed that the treatment of selenium-enriched yeast (Se-yeast) also significantly attenuated the levels of p62 accompanying an increase of turnover of Aβ and APP in AD mice. Similarly, by these studies, we showed that geniposide lowered the expression of p62, which was increased in SH-SY5Y cells treated by Aβ_{1-42}.

In summary, we speculate that mTOR inhibition and enhancement of autophagy induced by mTOR inhibition may be a critical molecular event in geniposide mitigating Aβ-induced toxicity.

Abbreviations

Aβ, β-amyloid; Aβ_O, Aβ oligomers; AC, adenylyl cyclase; AD, Alzheimer's disease; Atg7, autophagy-related gene 7; Ex-4, exendin-4; GLP-1, glucagon-like peptide-1; GLP-1R, glucogen-like peptide-1 receptor; HFIP, 1, 1, 1, 3, 3, 3-hexafluoro-2-propanol; IGF-1, insulin-like growth factor-1; mTOR, mechanistic target of rapamycin; NFT, neurofibrillary tangles; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKB (also known as Akt), Protein kinase B; RT, room temperature.

Author contributions

Dong-Xing Liu, Yan-fang Chang were involved in the design and execution of the experimental job and the statistical analysis of the data. Di Zhang and Wei-min Hu contributed to the statistical analysis of the data and manuscript writing. Xiao-hui Wang and Lin Li were involved in the design and execution of the study. All authors contributed to the development of the manuscript and reviewed and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Acknowledgment

We thank three anonymous reviewers for excellent criticism of the article.

Funding

Research project was supported by Shanxi Scholarship Council of China (2017- important 4), and by the Fund for Shanxi "1331 Project" Key Subjects Construction.

Conflict of interest

The authors declare no conflict of interest.

Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

References

[1] Longo VD, Antebi A, Bartke A, Barzilai N, Brown-Borg HM, Caruso C, *et al.* Interventions to slow aging in humans: are we ready? Aging Cell. 2015; 14: 497-510.

[2] Niccoli T, Partridge L. Ageing as a risk factor for disease. Current Biology. 2012; 22: R741-R752.

[3] Kim YC, Guan K. MTOR: a pharmacologic target for autophagy regulation. The Journal of Clinical Investigation. 2015; 125: 25-32.

[4] Laplante M, Sabatini DM. MTOR signaling in growth control and disease. Cell. 2012; 149: 274-293.

[5] Shimobayashi M, Hall MN. Making new contacts: the mTOR network in metabolism and signalling crosstalk. Nature Reviews Molecular Cell Biology. 2014; 15: 155-162.

[6] Cai Z, Chen G, He W, Xiao M, Yan L. Activation of mTOR: a culprit of Alzheimer's disease? Neuropsychiatric Disease and Treatment. 2015; 11: 1015-1030.

[7] Kitagishi Y, Kobayashi M, Kikuta K, Matsuda S. Roles of PI3K/AKT/GSK3/mTOR pathway in cell signaling of mental illnesses. Depression Research and Treatment. 2012; 2012: 752563.

[8] O' Neill C. PI3-kinase/Akt/mTOR signaling: impaired on/off switches in aging, cognitive decline and Alzheimer's disease. Experimental Gerontology. 2013; 48: 647-653.

[9] Solano DC, Sironi M, Bonfini C, Solerte SB, Govoni S, Racchi M. Insulin regulates soluble amyloid precursor protein release via phosphatidyl inositol 3 kinase-dependent pathway. The FASEB Journal. 2000; 14: 1015-1022.

[10] Fontana L, Partridge L, Longo VD. Extending healthy life span-from yeast to humans. Science. 2010; 328: 321-326.

[11] Timmons JA, Volmar C-H, Crossland H, Phillips BE, Sood S, Janczura KJ, *et al.* Longevity-related molecular pathways are subject to midlife "switch" in humans. Aging Cell. 2019; 18: e12970.

[12] Weichhart T. MTOR as regulator of lifespan, aging, and cellular senescence: a mini-review. Gerontology. 2018; 64: 127-134.

[13] Nakamura S, Yoshimori T. Autophagy and longevity. Molecules and Cells. 2018; 41: 65-72.

[14] Yang F, Chu X, Yin M, Liu X, Yuan H, Niu Y, *et al.* MTOR and autophagy in normal brain aging and caloric restriction ameliorating age-related cognition deficits. Behavioural Brain Research. 2014; 264: 82-90.

[15] Tramutola A, Triplett JC, Di Domenico F, Niedowicz DM, Murphy MP, Coccia R, *et al.* Alteration of mTOR signaling occurs early in the progression of Alzheimer disease (AD): analysis of brain from subjects with pre-clinical AD, amnestic mild cognitive impairment and late-stage AD. Journal of Neurochemistry. 2015; 133: 739-749.

[16] Van Skike CE, Jahrling JB, Olson AB, Sayre NL, Hussong SA, Ungvari Z, *et al.* Inhibition of mTOR protects the blood-brain barrier in models of Alzheimer's disease and vascular cognitive impairment. American Journal of Physiology Heart and Circulatory Physiology. 2018; 314: H693-H703.

[17] Kapahi P, Kaeberlein M, Hansen M. Dietary restriction and lifespan: lessons from invertebrate models. Ageing Research Reviews. 2017; 39: 3-14.

[18] Feng Y, He D, Yao Z, Klionsky DJ. The machinery of macroautophagy. Cell Research. 2014; 24: 24-41.

[19] Li Q, Liu Y, Sun M. Autophagy and Alzheimer's disease. Cellular and Molecular Neurobiology. 2017; 37: 377-388.

[20] Cai X, Li J, Wang M, She M, Tang Y, Li J, *et al.* GLP-1 treatment improves diabetic retinopathy by alleviating autophagy through GLP-1R-ERK1/2-HDAC6 signaling pathway. International Journal of Medical Sciences. 2017; 14: 1203-1212.

[21] Cai X, She M, Xu M, Chen H, Li J, Chen X, *et al.* GLP-1 treatment protects endothelial cells from oxidative stress-induced autophagy and endothelial dysfunction. International Journal of Biological Sciences. 2018; 14: 1696-1708.

[22] Candeias E, Sebastião I, Cardoso S, Carvalho C, Santos MS, Oliveira CR, *et al.* Brain GLP-1/IGF-1 signaling and autophagy mediate exendin-4 protection against apoptosis in type 2 diabetic rats. Molecular Neurobiology. 2018; 55: 4030-4050.

[23] Huang B, Chen P, Huang L, Li S, Zhu R, Sheng T, *et al.* Geniposide attenuates post-ischaemic neurovascular damage via GluN2a/AKT/ERK-dependent mechanism. Cellular Physiology and Biochemistry. 2017; 43: 705-716.

[24] Gong N, Fan H, Ma A, Xiao Q, Wang Y. Geniposide and its iridoid analogs exhibit antinociception by acting at the spinal GLP-1 receptors. Neuropharmacology. 2014; 84: 31-45.

[25] Guo L, Xia Z, Gao X, Yin F, Liu J. Glucagon-like peptide 1 receptor plays a critical role in geniposide-regulated insulin secretion in INS-1 cells. Acta Pharmacologica Sinica. 2012; 33: 237-241.

[26] Yin F, Liu J, Zheng X, Guo L, Xiao H. Geniposide induces the expression of heme oxygenase-1 via PI3K/Nrf2-signaling to enhance the antioxidant capacity in primary hippocampal neurons. Biological & Pharmaceutical Bulletin. 2010; 33: 1841-1846.

[27] Li Y, Kamo S, Metori K, Koike K, Che Q, Takahashi S. The promoting effect of eucommiol from eucommiae cortex on collagen synthesis. Biological & Pharmaceutical Bulletin. 2000; 23: 54-59.

[28] Dinda B, Dinda M, Kulsi G, Chakraborty A, Dinda S. Therapeutic potentials of plant iridoids in Alzheimer's and Parkinson's diseases: a review. European Journal of Medicinal Chemistry. 2019; 169: 185-199.

[29] Chen Z, Lu Y, Du S, Shang K, Cai C. Influence of borneol and muscone on geniposide transport through MDCK and MDCK-MDR1 cells as blood-brain barrier *in vitro* model. International Journal of Pharmaceutics. 2013; 456: 73-79.

[30] Song L, Yao L, Zhang L, Piao Z, Lu Y. Schizandrol a protects against $A\beta_{1-42}$-induced autophagy via activation of PI3K/AKT/mTOR pathway in SH-SY5Y cells and primary hippocampal neurons. Naunyn-Schmiedeberg's Archives of Pharmacology. 2020; 393: 1739-1752.

[31] Li L. The molecular mechanism of glucagon-like peptide-1 therapy in Alzheimer's disease, based on a mechanistic target of rapamycin pathway. CNS Drugs. 2017; 31: 535-549.

[32] Jiang Y, Wang Z, Ma B, Fan L, Yi N, Lu B, *et al.* GLP-1 improves adipocyte insulin sensitivity following induction of endoplasmic reticulum stress. Frontiers in Pharmacology. 2018; 9: 1168.

[33] Zhang Z, Wang X, Zhang D, Liu Y, Li L. Geniposide-mediated protection against amyloid deposition and behavioral impairment correlates with downregulation of mTOR signaling and enhanced autophagy in a mouse model of Alzheimer's disease. Aging. 2019; 11: 536-548.

[34] Zhang Z, Gao W, Wang X, Zhang D, Liu Y, Li L. Geniposide effectively reverses cognitive impairment and inhibits pathological cerebral damage by regulating the mTOR Signal pathway in APP/PS1 mice. Neuroscience Letters. 2020; 720: 134749.

[35] Zhang Y, Ding Y, Zhong X, Guo Q, Wang H, Gao J, *et al.* Geniposide acutely stimulates insulin secretion in pancreatic β-cells by regulating GLP-1 receptor/cAMP signaling and ion channels. Molecular and Cellular Endocrinology. 2016; 430: 89-96.

[36] Otomo C, Metlagel Z, Takaesu G, Otomo T. Structure of the human ATG12∼ATG5 conjugate required for LC3 lipidation in autophagy. Nature Structural & Molecular Biology. 2013; 20: 59.

[37] Fritzen AM, Frøsig C, Jeppesen J, Jensen TE, Lundsgaard A, Serup AK, *et al.* Role of AMPK in regulation of LC3 lipidation as a marker of autophagy in skeletal muscle. Cellular Signalling. 2016; 28: 663-674.

[38] Lipinski MM, Zheng B, Lu T, Yan Z, Py BF, Ng A, *et al.* Genome-wide analysis reveals mechanisms modulating autophagy in normal brain aging and in Alzheimer's disease. Proceedings of the National Academy of Sciences. 2010; 107: 14164-14169.

[39] Zhang J, Mao S, Wang L, Zhang W, Zhang Z, Guo Y, *et al.* MicroRNA-154 functions as a tumor suppressor in bladder cancer by directly targeting ATG7. Oncology Reports. 2019; 41: 819-828.

[40] Pickford F, Masliah E, Britschgi M, Lucin K, Narasimhan R, Jaeger PA, *et al.* The autophagy-related protein beclin 1 shows reduced expression in early Alzheimer disease and regulates amyloid beta accumulation in mice. Journal of Clinical Investigation. 2008; 118: 2190-2199.

[41] Yoshii SR, Mizushima N. Monitoring and measuring autophagy. International Journal of Molecular Sciences. 2017; 18: 1865.

[42] Bjørkøy G, Lamark T, Brech A, Outzen H, Perander M, Øvervatn A, *et al.* P62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. Journal of Cell Biology. 2005; 171: 603-614.

[43] Tang R, Qi R, Liu H. Interleukin-4 affects microglial autophagic flux. Neural Regeneration Research. 2019; 14: 1594-1602.

[44] Song G, Chen C, Wu Q, Zhang Z, Zheng R, Chen Y, *et al.* Selenium-enriched yeast inhibited β-amyloid production and modulated autophagy in a triple transgenic mouse model of Alzheimer's disease. Metallomics. 2018; 10: 1107-1115.