

β -asarone modulates Beclin-1, LC3 and p62 expression to attenuate $A\beta_{40}$ and $A\beta_{42}$ levels in APP/PS1 transgenic mice with Alzheimer's disease

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Abstract. Alzheimer's disease (AD) is a common neurodegenerative disease in the elderly population. Autophagy is a well-known regulator of neurodegenerative diseases and β -asarone has been discovered to have certain neuropharmacological effects. Thus, the present study aimed to analyze the potential effects of β -asarone in AD and its possible mechanism of action in relation to autophagy. The present study investigated the effects of β -asarone on the number of senile plaques and amyloid $\beta(A\beta)_{40}$, $A\beta_{42}$, amyloid precursor protein (APP) and Beclin-1 mRNA levels in the hippocampus of APP/presenilin-1 (PS1) transgenic mice. The possible mechanism of β -asarone on autophagy-related proteins, including Beclin-1, light chain (LC)3A, LC3B and p62 levels, and the number of autophagosomes was also investigated. Mice were divided into a normal control group, a model group, a β -asarone-treated group, a 3-MA-treated group and a rapamycin-treated group. Treatments were continuously administered to all mice for 30 days by intragastric administration. The mice, including those in the normal and model control groups, were given equal volumes of saline. It was demonstrated that β -asarone treatment reduced the number of senile plaques and autophagosomes, and decreased $A\beta_{40}$, $A\beta_{42}$, APP and Beclin-1 expression in the hippocampus of model mice compared with untreated model mice. β -asarone also inhibited LC3A/B expression levels, but increased p62 expression. It was deduced that the neuroprotective effects of β -asarone in APP/PS1 transgenic mice resulted from its

inhibition of autophagy. In conclusion, the data suggested that β -asarone should be explored further as a potential therapeutic agent in AD.

Introduction

Alzheimer's disease (AD) is a progressive, age-related neurodegenerative disease that presents with progressive intellectual deterioration involving memory, language and judgement, ultimately leading to a total dependence on nursing care for affected patients (1). One of the major histopathological hallmarks of AD is cerebral deposits of extracellular amyloid $\beta(A\beta)$ peptides. Senile plaques, composed of $A\beta$, are an important criterion for the verification of AD (2). $A\beta_{40}$ and $A\beta_{42}$, which are derived from amyloid precursor protein (APP), affect the pathogenesis of AD because of their strong aggregative ability and neurotoxicity (3). However, there is no effective therapy for AD that can reverse or slow its progression.

Autophagy is one of the main mechanisms of maintaining cellular homeostasis; it degrades and recycles old proteins and organelles using cellular machinery (4). However, excessive autophagy may also lead to autophagic neuron death and apoptosis (5). Previous studies have reported that autophagy may be involved in AD pathogenesis (6). In addition, autophagy serves an important role in clearing $A\beta$ aggregation and preserving neuronal function in AD (7). Another recent study also indicated that $A\beta_{1-42}$ can induce autophagic cell death and autophagic vacuoles (8). Autophagosomes can be observed during $A\beta_{1-42}$ -induced cytotoxicity in PC12 cells (8,9), and it has been reported that autophagy exhibits protective effects by reducing the deposition of $A\beta$ and attenuating damage related to memory and cognitive dysfunction in AD animal models (10). β -asarone, a chief constituent of *Acorus tatarinowii* Schott, can easily pass through the blood brain barrier and exhibits various neuroprotective effects against neurodegenerative disease *in vivo* and *in vitro* models (11-14). A study using SH-SY5Y cells demonstrated that β -asarone prevents $A\beta_{25-35}$ -induced inflammatory responses and autophagy through the downregulation of Beclin-1 and light chain (LC)3B and the upregulation of Bcl-2 (15). In addition, it has

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been identified that the upregulation of Beclin-1-dependent autophagy protects against β -amyloid-induced cell injury in PC12 cells (16). β -asarone also has cerebrovascular protective effects in AD rats (17). However, its effect on AD remains to be elucidated.

The present study evaluated the neuroprotective effect of β -asarone in an APP/presenilin-1 (PS1) transgenic mouse model of AD and identified its underlying mechanism. APP/PS1 mutant transgenic mouse models are used to assess the pharmacodynamics of potential amyloidosis-lowering and pro-cognitive compounds (18). Control groups were treated with the autophagy inhibitor 3-methyladenine (3-MA) or the autophagy activator rapamycin. By monitoring autophagy, the present study aimed to explore if β -asarone may be a potential therapeutic agent for the prevention and treatment of AD by affecting autophagy.

Materials and methods

Animals. A total of 60 APP/PS1 double transgenic mice (30 males and 30 females; weight, 20–25 g; age, 3 months), that is, C57BL/6 mice co-expressing mutant human APP^{swE} and mutant human PS1 gene lacking exon 9, PS1- Δ E9; and 10 wild-type littermates (5 males and 5 females; weight, 20–25 g; age, 3 months) were purchased from Nanjing Biomedical Research Institute of Nanjing University. The animals were housed under standard conditions, at a temperature of 20–22°C and 40–60% humidity, with a 12-h light/dark cycle and free access to water and food. All procedures were approved by the Guangzhou University of Chinese Medicine Institutional Animal Care and Use Committee (Guangzhou, China) (ethics no. 2014013).

Preparation of β -asarone. β -asarone was extracted from *Acorus tatarinowii* Schott and then purified by freezing crystallization, as reported previously (19). The obtained β -asarone was $\leq 99.55\%$ pure (17), as confirmed by the China National Analytical Center using gas chromatography-mass spectrometry, infrared spectroscopy and nuclear magnetic resonance spectroscopy.

Experimental design. The normal control group comprised 10 wild-type C57BL/6 mice. The APP/PS1 transgenic mice were randomly divided into 6 groups (n=10 each): i) model group; ii) low-, iii) medium- and iv) high-dose β -asarone groups, which were given β -asarone by intragastric administration at doses of 10, 20 or 40 mg/kg body weight, respectively; v) 3-MA treated group, which was given 3-MA (cat. no. M9281; Sigma-Aldrich; Merck KGaA) by intraperitoneal injection at a dose of 30 mg/kg body weight; and vi) the rapamycin treated group, which was given rapamycin (cat. no. R0395; Sigma-Aldrich; Merck KGaA) by intraperitoneal injection at a dose of 1 mg/kg body weight. The treatments were continuously administered to all mice one per day for 30 days. At the end of the experiment, all mice were sacrificed as described below, for the removal of hippocampal samples, which were subsequently stored at -80°C for flow cytometry, ELISA, immunofluorescence staining, immunohistochemistry, transmission electron microscopy (TEM), reverse transcription-quantitative PCR (RT-qPCR) and western blot analysis.

Immunohistochemical staining of senile plaque formations. A total of 10 mice from each group were deeply anesthetized with 10% chloral hydrate (350 mg/kg, intraperitoneal; no signs of peritonitis were observed) and then transcranially perfused with 0.9% normal saline (30 ml) until colorless liquid flowed from the right atrial appendage. When the run-off became clear, perfusion was performed with 4% paraformaldehyde (30 ml) until the upper limbs became white and stiff. The hippocampus was rapidly dissected from the brains and was placed on ice. Tissues were fixed in 10% formalin at room temperature for 24 h and embedded in paraffin. Paraffin-embedded tissues were subsequently cut into serial 5- μm coronal sections using a microtome. Sections were deparaffinized at 60°C for 1 h, washed in xylene twice for 10 min and rehydrated in a descending alcohol series. Following the quenching of endogenous peroxidase activity for 10 min in PBS containing 3% H_2O_2 at 37°C, sections were heated in antigen retrieval solution (0.01 mol/l citrate buffer, pH 8.0) at 90°C for 10 min, cooled in water and immersed for 5 min in PBS containing 0.3% Triton X-100 at 37°C. Sections were blocked with 5% BSA (cat. no. 810652; Sigma-Aldrich; Merck KGaA) for 30 min at 37°C and the sections were then incubated with anti- $A\beta_{42}$ antibodies (1:50; cat. no. ab10148; Abcam) for 1 h at 37°C, amplified with avidin biotin-peroxidase complex labelling (1:100; cat. no. SV1022; Wuhan Boster Biological Technology, Ltd.), developed with DAB (cat. no. AR1022; Wuhan Boster Biological Technology, Ltd.) and imaged using a U-SPT light microscope (magnification, x200; Olympus Corporation). Data were analyzed using ImageJ version 1.48 software (National Institutes of Health). The average optical density (%) was calculated using the following equation: (Integrated optical density/measurement area) x100.

ELISA analysis of $A\beta_{40}$ and $A\beta_{42}$ levels. The hippocampus was weighed and homogenized with icecold normal saline (1 μl /3 mg) and then centrifuged at 3,000 x g for 10 min at 4°C to obtain the supernatant. $A\beta_{40}$ (cat. no. A226FC) and $A\beta_{42}$ (cat. no. A227FC) levels in the supernatant were determined separately using ELISA kits, according to the manufacturer's instructions (Elixir Canada Medicine Company Ltd.).

RT-qPCR of APP and Beclin-1 mRNA levels. Total RNA was extracted from hippocampus tissues using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The concentration of RNA was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA using a Hifair[®] III 1st Strand cDNA Synthesis SuperMix for qPCR (gDNA digester plus) kit [cat. no. 11141ES60; Yeasen Biotechnology (Shanghai) Co., Ltd.], according to the manufacturer's protocol. Quantitative analysis of the mRNA expression levels of APP and Beclin-1 was performed by qPCR using a Hieff UNICON[®] Power qPCR SYBR Green Master mix [no Rox; Yeasen Biotechnology (Shanghai) Co., Ltd.], according to the manufacturer's protocol, 96-well optical reaction plates and the CFX96 Real-Time PCR Detection system (Bio-Rad Laboratories, Inc.). qPCR was performed using the following conditions: Initial denaturation at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 sec and annealing and extension at 60°C for 30 sec.

All experiments were performed four times. The following primers were used: GAPDH, forward 5'-AGAAGGTGGTGAAGCAGGCATC-3', reverse 5'-CGAAGGTGGAAGAGTGGGAGTTG-3'; APP, forward 5'-TGCAGCAGAACGGATATGAGAAT-3', reverse 5'-GTCAAAAGCCGAGGGTGAGTAA-3'; and Beclin-1, forward 5'-ATACTGTTCTGGGGGTTTTCG-3', reverse 5'-GTCTCTCCTTTTCCACCTCTTC-3'. The primers used in the present study were selected from the PubMed database and were synthesized by Shanghai Jierui Biological Engineering Co., Ltd. Data were analyzed using the relative gene expression ($2^{-\Delta\Delta C_q}$) method (20) and normalized to GAPDH.

Flow cytometric analysis of Beclin-1, LC3A/B and p62. Beclin-1, LC3A/B and p62 expression levels were quantitatively determined using our previously established method (21,22). Hippocampal tissues were prepared as single cell suspensions, and then counted and adjusted to a density of 1×10^6 cells/ml. The cells were blocked with a protein block solution (2% BSA; cat. no. 810652; Merck KGaA) for 20 min and fixed with 1% paraformaldehyde for 20 min, as directed in the instructions for the IntraPrep permeabilization reagent (cat. no. GAS003; Invitrogen; Thermo Fisher Scientific, Inc.). Next, the cells were incubated with rabbit anti-Beclin-1 (1:100; cat. no. sc-11427, Santa Cruz Biotechnology, Inc.), mouse anti-p62 (1:100; cat. no. ab56416; Abcam) and rabbit anti-LC3A/B antibody (1:100; cat. no. 4108; Cell Signaling Technology, Inc.) in the dark at room temperature for 30 min. Subsequently the cells were incubated for 1 h at 37°C with the following secondary antibodies: Anti-rabbit (1:500; cat. no. CW0114S; CoWin Biosciences) and anti-mouse (1:1,000; cat. no. 4408S; Cell Signaling Technology, Inc.) and washed twice with PBS. Finally, the labelled cells were fixed at 37°C overnight in 4% paraformaldehyde and prepared for flow cytometric analysis. The control cells were incubated with secondary antibody alone. Data were collected from 20,000 events for every analysis. FACS data were collected using an ALTRA flow cytometer (Beckman Coulter, Inc.) equipped with EXPOTM32 MultiCOMP software.

Western blot analysis of Beclin-1, LC3A/B, p62 and GAPDH. Total protein was extracted from the hippocampal tissue using phenylmethanesulfonyl fluoride lysis buffer (Sigma-Aldrich; Merck KGaA). The lysates were incubated for 30 min at 4°C and centrifuged at $13,000 \times g$ for 15 min at 4°C. Total protein was quantified using a bicinchoninic acid assay kit (Wuhan Boster Biological Technology, Ltd.) and 100 μg protein/lane was separated by 12% SDS-PAGE (cat. no. P0012A; Beyotime Institute of Biotechnology). The separated proteins were subsequently transferred onto nitrocellulose membranes (0.2 μm ; Bio-Rad Laboratories, Inc.). The membranes were blocked in 5% BSA (cat. no. 810652; Merck KGaA) for 1 h at 4°C. The membranes were washed and incubated with antibodies against Beclin-1 (1:1,000; cat. no. ab62557; Abcam), p62 (1:1,000; cat. no. ab56416; Abcam), GAPDH (1:1,000; cat. no. ab8245; Abcam) and LC3A/B (1:1,000; cat. no. 4108; Cell Signaling Technology, Inc.) for 12 h at 4°C. Following the primary antibody incubation, membranes were washed and incubated with horseradish peroxidase-linked secondary antibodies (1:2,000; cat nos. CW0103 and CW0102; CoWin Biosciences) for 1 h at

room temperature. GAPDH was used as an internal control. The membranes were washed with TBS-20% Tween and visualized using an ECL kit (Bio-Rad Laboratories, Inc.) and a ChemiDoc XRS™ imager (Bio-Rad Laboratories, Inc.). Blots were repeated ≥ 3 times for each condition. Expression levels were quantified using Image-Pro Plus 6.0 analysis software (Media Cybernetics, Inc.) and normalized to GAPDH.

Immunofluorescence staining analysis of Beclin-1, LC3A/B and p62. The fixed coronal sections (5- μm) of the hippocampus, aforementioned, were incubated with PBS containing 3% H_2O_2 at 37°C for 10 min to quench endogenous peroxidase activity, heated in antigen retrieval solution (EDTA, pH 8.0) at 90°C for 10 min, chilled in water and then immersed for 5 min in PBS at 37°C. Sections were then incubated with rabbit anti-Beclin-1 antibody (1:50; cat. no. ab62557; Abcam), rabbit anti-LC3A/B antibody (1:50; cat. no. 4108; Abcam) and rabbit anti-p62 antibody (1:50; cat. no. ab56416; Abcam) for 60 min at 37°C. Expression was then amplified with avidin biotin-peroxidase complex labelling and visualized using an IX71 inverted fluorescence microscope (magnification, x400; Olympus Corporation). Data analysis was performed using ImageJ version 1.48 software (National Institutes of Health).

TEM. To further clarify the effect of β -asarone on autophagy, TEM, the standard method for detecting autophagy, was employed (16). Hippocampal tissue from each group were fixed with 2.5% glutaraldehyde in 0.1 mol/l PBS (pH 7.4) at room temperature for 90 min and post-fixed in 1% osmium tetroxide for 30 min. Following washing with PBS, the cells were progressively dehydrated in a 10% graded series of 50-100% ethanol and propylene oxide and embedded in Epon 812 resin. The blocks were cut into ultrathin sections (50-150 μm) with a UC7 vibratome (Leica Microsystems GmbH) and the sections were then both stained with 3% saturated uranyl acetate and 3% lead citrate at room temperature for 30 min. Sections were used to observe dopaminergic neuron morphology under an H-7650 transmission electron microscope (magnification, x30,000; Hitachi, Ltd.), as previously described (11).

Statistical analysis. All statistical analyses were performed with SPSS version 13.0 statistical software (SPSS, Inc.). Data are expressed as the mean \pm SD, and significant differences among different groups were determined by one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. Each experiment was repeated ≥ 3 times. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

β -asarone reduces the formation of senile plaques in the hippocampus. Senile plaques composed of A β peptides are an important criterion for verifying AD (2); inhibiting A β accumulation is a potential strategy for preventing AD. The immunohistochemical staining analysis presented in Fig. 1 demonstrated that the hippocampal tissues of mice in the model group had more senile plaques compared with mice in the normal control group ($P < 0.01$). In addition, the number of senile plaques in the hippocampus of the β -asarone-, 3-MA- and rapamycin-treated groups was significantly

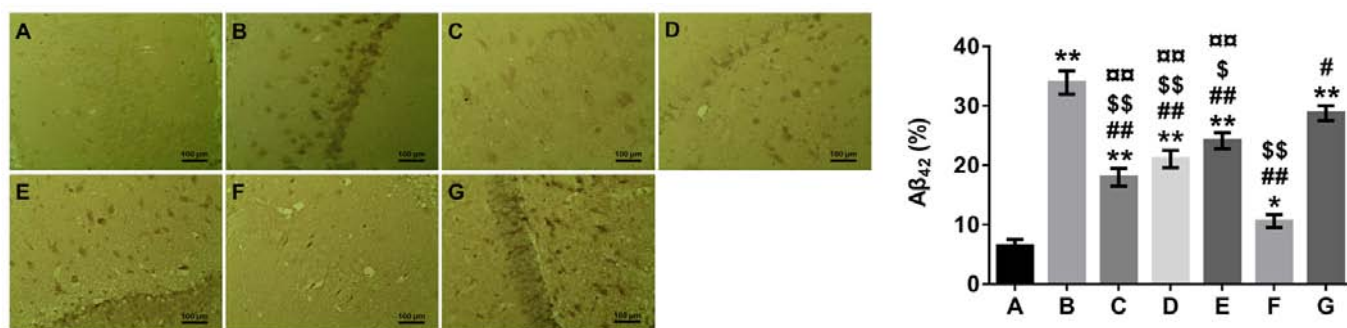


Figure 1. Effects of β -asarone on the formation of senile plaques in the hippocampus of Alzheimer's disease model mice by $A\beta_{42}$ immunohistochemical staining analysis. A, Normal group; B, model group; C, low-dose β -asarone group; D, medium-dose β -asarone group; E, high-dose β -asarone group; F, 3-MA-treated group; and G, rapamycin-treated group. Data are presented as the mean \pm SD of 3 mice. Scale bar, 100 μ m. Data were analyzed using a medical image analysis system. * P <0.05 and ** P <0.01 vs. normal group; # P <0.05 and ## P <0.01 vs. model group; § P <0.05 and §§ P <0.01 vs. rapamycin-treated group; $^{\circ}$ P <0.01 vs. 3-MA-treated group. 3-MA, 3-methyladenine. $A\beta$, amyloid β .

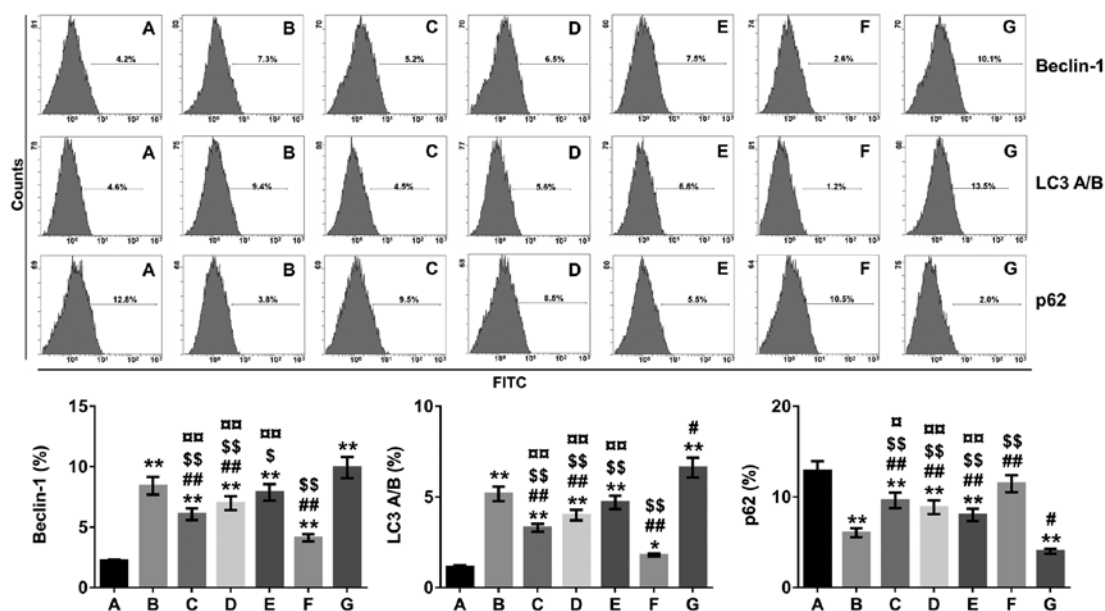


Figure 2. Beclin-1, LC3A/B and p62 expression in the hippocampus of Alzheimer's disease model mice determined by flow cytometry. A, Normal group; B, model group; C, low-dose β -asarone group; D, medium-dose β -asarone group; E, high-dose β -asarone group; F, 3-MA-treated group; and G, the rapamycin-treated group. Data are presented as the mean \pm SD of 7 mice. * P <0.05 and ** P <0.01 vs. normal group; # P <0.05 and ## P <0.01 vs. model group; § P <0.05 and §§ P <0.01 vs. rapamycin-treated group; $^{\circ}$ P <0.05 and $^{\circ\circ}$ P <0.01 vs. 3-MA-treated group. 3-MA, 3-methyladenine; LC3, light chain 3.

reduced compared with the model group (P <0.05 or P <0.01). Furthermore, the number of senile plaques in the hippocampus was higher in the β -asarone- and rapamycin-treated groups compared with the 3-MA-treated group (P <0.01), but lower in the β -asarone- and 3-MA-treated groups compared with the rapamycin-treated group (P <0.05 or P <0.01).

β -asarone decreases hippocampal levels of $A\beta_{40}$ and $A\beta_{42}$. The two main $A\beta$ peptide isoforms produced from APP, $A\beta_{40}$ and $A\beta_{42}$, serve an important role in the pathogenesis of AD because of their strong aggregative ability and neurotoxicity (2). ELISA analysis revealed that hippocampal levels of $A\beta_{40}$ and $A\beta_{42}$ were significantly elevated in the model group compared with the normal control group (P <0.01; Table I). By contrast, a significant decrease in $A\beta_{40}$ and $A\beta_{42}$ levels was observed in the β -asarone-, 3-MA- and rapamycin-treated groups compared with the model group (P <0.05 or P <0.01; Table I).

A significant increase in $A\beta_{40}$ and $A\beta_{42}$ levels was observed in the β -asarone- and rapamycin-treated groups compared with the 3-MA-treated group (P <0.05 or P <0.01), whereas a significant decrease was noted in the β -asarone-treated groups compared with the rapamycin treated group (P <0.01).

Effect of β -asarone on APP and Beclin-1 mRNA expression levels in the hippocampus. β -asarone treatment also exhibited a notable effect on APP and Beclin-1 mRNA expression levels in the hippocampus of APP/PS1 transgenic mice (Table II). RT-qPCR analysis revealed a significant increase in APP and Beclin-1 mRNA expression levels in the model group compared with the normal control group (P <0.01). In addition, a significant decrease in APP and Beclin-1 mRNA levels was observed in the β -asarone-, 3-MA- and rapamycin-treated groups compared with the model group (P <0.05 or P <0.01; Table II). Furthermore, a significant increase in APP and Beclin-1 mRNA levels was

Table I. A β_{40} and A β_{42} levels in the hippocampus determined by ELISA.

Group ^a	A β_{40} (pg/g tissue weight)	A β_{42} (pg/g tissue weight)
Normal	1.11±0.09	1.44±0.24
Model	9.18±0.38 ^c	9.86±0.19 ^c
Low-dose β -asarone	4.87±0.40 ^{c,e,g,h}	4.46±0.30 ^{c,e,f,h}
Medium-dose β -asarone	5.28±0.45 ^{c,e,g,h}	4.80±0.27 ^{c,e,g,h}
High-dose β -asarone	5.69±0.40 ^{c,e,g,h}	5.60±0.24 ^{c,e,g,h}
3-MA	2.81±0.12 ^{c,e}	3.37±0.20 ^{b,e}
Rapamycin	7.89±0.78 ^{c,d,g}	7.38±0.67 ^{c,d,g}

^an=7 mice/group; ^bP<0.05 and ^cP<0.01 vs. normal group; ^dP<0.05 and ^eP<0.01 vs. model group; ^fP<0.05 and ^gP<0.01 vs. 3-MA treated group; ^hP<0.01 vs. rapamycin treated group. 3-MA, 3-methyladenine; A β , amyloid β .

Table II. APP and Beclin-1 mRNA levels in the hippocampus by reverse transcription-quantitative PCR.

Group ^a	APP	Beclin-1
Normal	1.00±0.00	1.00±0.00
Model	4.21±0.11 ^c	3.81±0.09 ^c
Low-dose β -asarone	1.42±0.04 ^{b,e,f,h}	1.39±0.03 ^{b,e,g,h}
Medium-dose β -asarone	1.77±0.07 ^{c,e,g,h}	1.64±0.05 ^{b,e,g,h}
High-dose β -asarone	2.06±0.08 ^{c,e,g,h}	1.86±0.06 ^{c,e,g,h}
3-MA	1.25±0.05 ^{b,e}	1.15±0.03 ^{b,e}
Rapamycin	2.82±0.13 ^{c,e,g}	3.07±0.11 ^{c,d,g}

^an=7 mice/group; ^bP<0.05 and ^cP<0.01 vs. normal group; ^dP<0.05 and ^eP<0.01 vs. model group; ^fP<0.05 and ^gP<0.01 vs. 3-MA treated group; ^hP<0.01 vs. rapamycin treated group. 3-MA, 3-methyladenine; APP, amyloid precursor protein.

observed in the β -asarone- and rapamycin-treated groups compared with the 3-MA-treated group (P<0.05 or P<0.01), but the opposite was observed in the β -asarone-treated groups compared with the rapamycin-treated group (P<0.01).

Effect of β -asarone on autophagy in the hippocampus. The autophagy inhibitor-3-MA and autophagy activator-rapamycin were used, and β -asarone-treated groups were compared with 3-MA- and rapamycin-groups to determine whether expressions of the autophagy markers Beclin-1 and LC3A/B were altered. The results from Figs. 2-4 demonstrated a significant decrease in the expression of Beclin-1 and LC3A/B accompanied by an increased in the expression of p62 in the low-dose- β -asarone- and 3-MA-treated groups compared with the model group (P<0.05 or P<0.01; Fig. 3; P<0.05 or P<0.01; Figs. 2 and 4), but there was a decrease in p62 expression in the rapamycin-treated group compared with the model group (P<0.05; Figs. 2 and 3; P<0.01; Fig. 4). In addition, Beclin-1 and LC3A/B expression increased, and p62 expression decreased significantly in the high-dose- β -asarone- and rapamycin-treated groups compared with the 3-MA-treated group (P<0.01; Figs. 2-4). However, Beclin-1 and LC3A/B expression was reduced and p62 expression was significantly augmented in the low-dose- and medium-dose β -asarone-treated groups compared with the rapamycin-treated group (P<0.05 or P<0.01; Fig. 3; P<0.01; Figs. 2 and 4).

TEM was used to analyze the number of autophagosomes (Fig. 5). The number of autophagosomes in the model and rapamycin-treated groups were increased compared with that in the normal control group (P<0.01), whereas the number of autophagosomes in the 3-MA group and in all β -asarone-treated groups was significantly lower compared with the model group (P<0.01).

Discussion

The pathological characteristics of AD include senile plaques, intracellular neurofibrillary tangles, activated microglia and

astrocyte accumulation around A β plaques, and degenerative neurons (23). Senile plaques composed of A β peptides are an important criterion for verifying AD (24). A β_{40} and A β_{42} , the two-main A β isoforms, are produced from the APP by β -secretase and γ -secretase, respectively (2). In addition to the formation of A β oligomers and amyloid deposits, A β_{42} has been linked to AD pathology (25). Inhibiting A β accumulation in the brain may prove to be an important therapeutic strategy for AD. In the present study, the APP/PS1 transgenic mice in the model group exhibited increased intracellular levels of A β_{40} and A β_{42} APP mRNA, and a higher number of senile plaques compared with the normal control group, which suggested increased A β production in the hippocampus of these animals. β -asarone administration in APP/PS1 transgenic mice decreased A β_{40} , A β_{42} and APP levels and the number of senile plaques, suggesting that β -asarone may have a protective effect. These results indicated that β -asarone had a positive effect on reducing A β_{40} , A β_{42} peptide and APP mRNA levels in the senile plaques of APP/PS1 transgenic mice.

Beclin-1, LC3A/B and p62 expression levels have been used as specific markers of autophagy (26). Beclin-1, a key gene in autophagy regulation that was first discovered in mammals, regulates the localization of other autophagy-related proteins to autophagosomes (27). A previous study revealed that Beclin-1 deficiency increases APP and APP-like proteins, accelerates A β accumulation and promotes neurodegeneration in mice, implying that Beclin-1 is involved in the pathogenesis of AD (28). The current study aimed to investigate the effects of β -asarone on A β accumulation and to determine whether APP mRNA reduction was associated with changes in Beclin-1 levels in the hippocampus of APP/PS1 transgenic mice. In the present study, it was demonstrated that β -asarone treatment led to a reduction in APP and Beclin-1 mRNA levels; however, this finding is not consistent with existing research. These results suggested that the inhibition of APP mRNA expression by β -asarone may be correlated with autophagy.

To further investigate the effects of β -asarone on autophagy in APP/PS1 transgenic mice, Beclin-1, LC3A/B

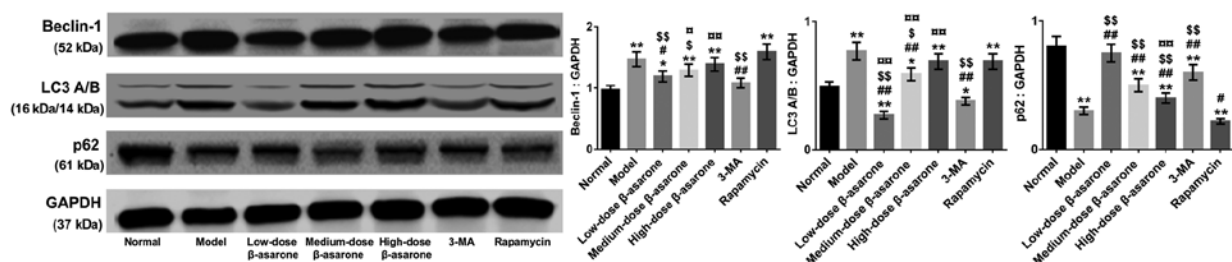


Figure 3. Effects of β -asarone on autophagy-related protein expression (Beclin-1, LC3 and p62) in the hippocampus of Alzheimer's disease model mice determined by western blot analysis. Data are presented as the mean \pm SD of 3 mice. * P <0.05 and ** P <0.01 vs. normal group; # P <0.05 and ## P <0.01 vs. model group; \$ P <0.05 and \$\$ P <0.01 vs. rapamycin-treated group; ^ P <0.05 and ^^ P <0.01 vs. 3-MA-treated group. 3-MA, 3-methyladenine; LC3, light chain 3.

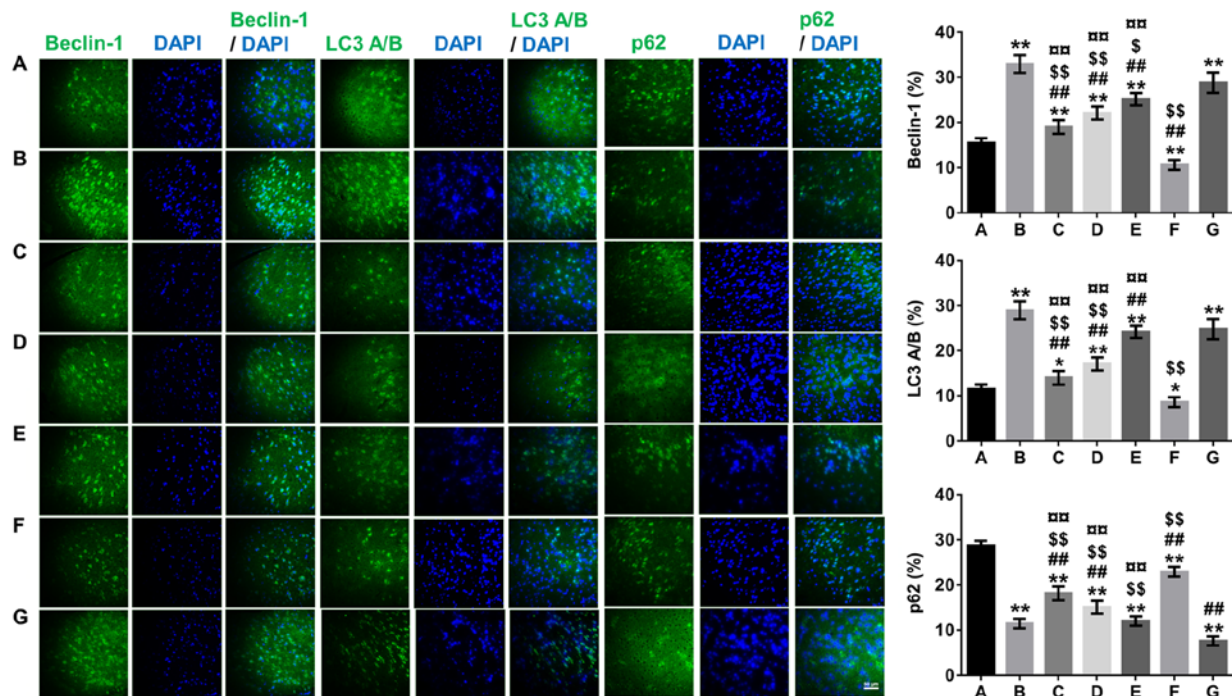


Figure 4. Effects of β -asarone on autophagy-related protein expression (Beclin-1, LC3 and p62) in the hippocampus of Alzheimer's disease model mice by immunofluorescence staining analyses. The samples detected were tissues rather than cells and most of the expressions were normal. Images were captured with an Olympus fluorescence microscope (not taken by confocal microscopy due to experimental conditions) so the images presented are not punctate; magnification, $\times 400$. A, Normal group; B, model group; C, low-dose β -asarone group; D, medium-dose β -asarone group; E, high-dose β -asarone group; F, 3-MA-treated group; and G, the rapamycin-treated group. Data are presented as the mean \pm SD of 10 mice. * P <0.05 and ** P <0.01 vs. normal group; # P <0.01 vs. model group; \$ P <0.05 and \$\$ P <0.01 vs. rapamycin-treated group; ^ P <0.01 vs. 3-MA-treated group. 3-MA, 3-methyladenine; LC3, light chain 3.

and p62 expression levels were detected in the hippocampus by flow cytometry, western blotting and immunofluorescence staining analyses. LC3 staining did not demonstrate punctate LC3A/B fluorescence in our immunofluorescence staining method, and this was consistent with the results of Bel *et al* (29). As an important process that modulates the turnover of proteins, autophagy can serve a protective role in many human diseases that are characterized by toxic aggregation of misfolded proteins by eliminating the aggregated proteins (5). $A\beta_{1-42}$ serves a vital role in the pathogenesis of AD owing to its strong aggregative ability and neurotoxicity (2). Autophagy can protect against AD by clearing $A\beta$ deposition and preserving neuronal function (7). Furthermore, autophagic activity increases when cells experience injury, nutritional deficiency, growth factor deficits or high energy demand (4). However, excessive autophagy can also lead to

autophagic neuron death and apoptosis (5). Previous studies have reported that almost all cells with increased Beclin-1 expression exhibit punctate LC3B fluorescence (30), and autophagy dysfunction or defects cause p62 upregulation (31). Based on the evidence of p62 partial restoration, a previous study has shown that chloroquine is an autophagy inhibitor that further increases LC3A/B expression in rats (32). This indicated that there may be a negative correlation between p62 and LC3A/B expression in autophagy.

In the present study, 3-MA was used as an inhibitor of autophagy and rapamycin was used as an activator of autophagy (33,34). It was demonstrated that that Beclin-1 and LC3A/B expression levels in the model group were higher compared with the normal control group. This result suggested that the increased expression of these proteins in the APP/PS1 transgenic mouse may activate autophagy in

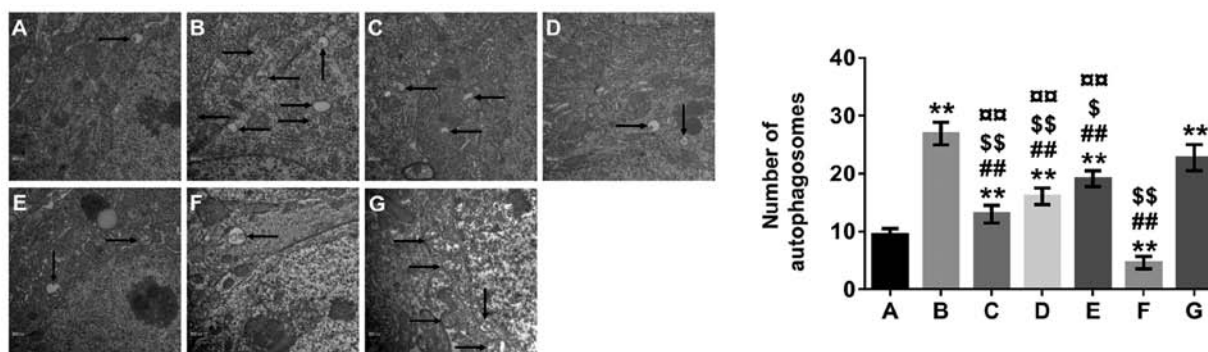


Figure 5. Effect of β -asarone on autophagy in Alzheimer's disease model mice. Transmission electron microscopy (x30,000 magnification) demonstrated that autophagosome formation was barely observed in the normal group. In addition, there were more autophagosomes in the model- and rapamycin-treated groups compared with the 3-MA and β -asarone treated groups. A, Normal group; B, model group; C, low-dose β -asarone group; D, medium-dose β -asarone group; E, high-dose β -asarone group; F, 3-MA-treated group; and G, the rapamycin-treated group. Data are presented as the mean \pm SD of 3 mice. Black arrows indicate autophagosomes. ** $P < 0.01$ vs. normal group; ## $P < 0.01$ vs. model group; \$ $P < 0.05$ and \$\$ $P < 0.01$ vs. rapamycin-treated group; ° $P < 0.01$ vs. 3-MA-treated group. 3-MA, 3-methyladenine.

neuronal cells. In addition, Beclin-1 and LC3A/B expression levels in all β -asarone dose groups and the 3-MA-treated group were lower compared with level in the model group. Together, these results indicated that low-dose β -asarone may suppress autophagic activity by downregulating Beclin-1 and LC3A/B expression. In addition, it also revealed that p62 expression was higher in the 3-MA treated group, near levels seen in the normal control group. By contrast, p62 expression was the lowest in the rapamycin treated group. These results suggested that p62 overexpression may inhibit autophagy. The expression of p62 was higher in all β -asarone dose groups and in the 3-MA treated groups compared with the model group, which indicated that β -asarone may inhibit autophagic activity by upregulating p62 expression. Finally, there were fewer autophagosomes in all β -asarone dose groups compared with the model group. Together, these results suggested that β -asarone inhibited autophagy by decreasing Beclin-1 and LC3A/B expression and increasing p62 expression in the hippocampus of APP/PS1 transgenic mice.

In conclusion, the present study demonstrated that β -asarone could decrease $A\beta_{40}$, $A\beta_{42}$, APP, Beclin-1 and LC3A/B levels and the number of senile plaques, and enhance p62 expression. Collectively, these findings indicated that β -asarone may protect against AD by clearing $A\beta$ accumulation and suppressing autophagic activity, and should be explored as a potential therapeutic agent for AD treatment.

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Availability of data and materials

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

Authors' contributions

MD and LH conceived the study, designed the experiments, analyzed the data and prepared the manuscript. MD, LH and XZ obtained samples for the present study. XZ, MD and LH performed the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures were approved by the Guangzhou University of Chinese Medicine Institutional Animal Care and Use Committee and conformed to the National Institutes of Health Guide for the Care and Use of Animals in Research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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