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ORIGINAL ARTICLE

Neuroprotective Effects of Bornyl Acetate against Okadaic Acid–Induced Cytotoxicity in PC12 Cells Via Beclin-1-Dependent Autophagy Pathway

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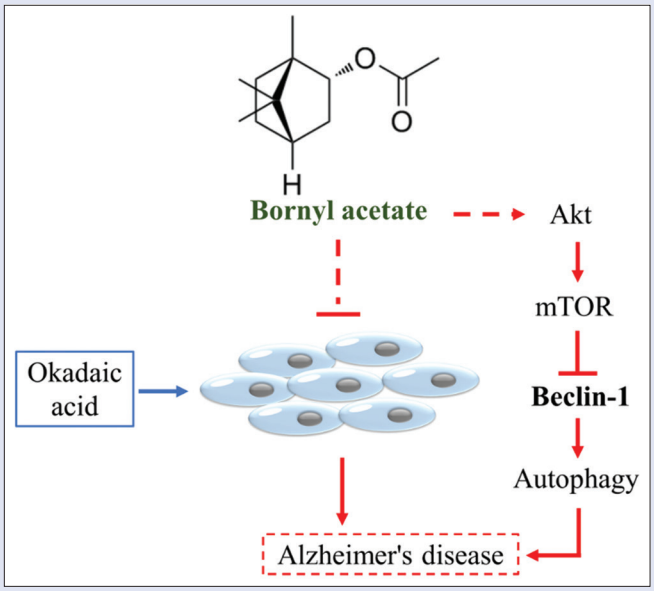
ABSTRACT

Background: β-amyloid (Aβ) deposition and tau protein abnormality are the major pathogenesis of Alzheimer's disease (AD). Autophagy is contributed to eliminating the misfolded proteins or organelles and alleviated cellular injury. Our pre-experimental findings showed that bornyl acetate (BA), the main component of the volatile oil of *Amomum villosum*, has a neuroprotective effect in okadaic acid (OA)–induced PC12 cells. However, the protective mechanism of autophagy that BA relieves OA-induced cellular injury is still unclear. **Objectives:** The purpose of our experiment was to elucidate the mechanism of treatment of AD. **Materials and Methods:** To explore how BA has therapeutic effects on AD, a model of OA-induced PC12 cells was established. The OA modelling induction and BA treatment in cells were evaluated by LDH and CCK-8 methods; ELISA assay was used to detect the tau hyperphosphorylation (p-tau), $A\beta_{42}$ and β-secretase levels;The expression of p-Akt and p-mTOR were detected by western blot analysis; and immunohistochemical, immunofluorescence methods and western blot analysis were used to detect Beclin-1 expression. Autophagosomes in each group were observed by transmission electron microscopy (TEM). 175 nM OA for 48 hr was applied to OA modelling induction. **Results:** Compared to the control group, the OA-induced AD model cells displayed higher levels of p-tau, $A\beta_{42}$ and β-secretase ($P < 0.01$), suggesting that the AD model was successfully established. Compared to the OA model group alone, p-tau, $A\beta_{42}$ and β-secretase levels and autophagy promoter Beclin-1 expression decreased significantly in the BA group ($P < 0.05$), whereas p-Akt and p-mTOR increased ($P < 0.01$). **Conclusion:** BA exhibited a neuroprotection effect against OA-induced cellular injury in the AD model by suppressing the Beclin-1-dependent autophagy pathway, indicating that BA might be an appealing potential strategy to treat AD.

Key words: Alzheimer's disease, autophagy, bornyl acetate, okadaic acid, PC12 cells, tau hyperphosphorylation

SUMMARY

- Aβ deposition and tau protein abnormality are the major pathogenesis of AD. Bornyl acetate (BA), the main component of the volatile oil of *Amomum villosum*, exhibited neuroprotection effect against OA-induced cellular injury in AD model by suppressing the Beclin-1-dependent autophagy pathway, indicating that BA might be an appealing potential strategy to treat AD.



Abbreviations used: Aβ: β-amyloid; AD: Alzheimer's disease; OA: Okadaic acid; BA: Bornyl acetate; Aβ: Amyloid β; TEM: Transmission electron microscopy; 3-MA: 3-methyladenine; FBS: Fetal bovine serum.

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INTRODUCTION

Statistical studies show that Alzheimer's disease (AD) accounts for approximately 50%–70% of senile dementia, which seriously endangers the family and society.[1,2] A study confirmed that β-amyloid (Aβ) deposition and phosphorylated tau (p-tau) accumulation in neurofibrillary tangles could affect the degeneration and loss of neurons, and exacerbate memory and cognitive impairment of AD patients.[3] Significantly, tau is considered an important

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therapeutic target for AD and recently, attention has switched from anti-Aβ to anti-tau therapy in AD treatment. Donepezil, a reversible cholinesterase inhibitor, has been confirmed to reduce p-tau and delay the progression of AD.[4]

Autophagy can help the cell confront the bad environment by regulating autophagy-mediated degradation of proteins and organelles when the cell encounters some environmental changes.[5] It is extremely important to activate autophagy in some diseases characterized by protein aggregation, such as AD.[6] The fusion of autophagosomes and lysosomes can degrade Aβ aggregation.[7] It has been confirmed that the key mechanism behind the accumulation and volume increase of autophagic vesicles in neurons in AD is to impede the process of autophagosome maturation and retrograde access to the neuronal cell.[8] Therefore, the mechanism behind functional dysfunction of endocytosis or the combination of autophagosome and lysosome systems in AD is worth further investigation.[9]

It has been confirmed that *Amomum villosum* shows remarkable pharmacological effects such as reducing dampness and regulating qi, warming and stopping vomit and diarrhea.[10,11] Bornyl acetate (BA) is the main component of the volatile oil of *Amomum villosum*, which is up to 80%.[12] It is reported that BA has an anti-diarrheal effect by inhibiting intestinal smooth muscle spasms.[13] As we know, the brain-gut axis is the neuro-endocrine network connecting the gastrointestinal tract with the central nervous system.[14] It is reasonable to consider that the information about gastrointestinal activities is closely related to the central nervous system.[14] Based on the theory of the brain–gut axis network, we speculated that BA in the volatile oil of *Amomum villosum* has a protective effect on nerve injury.

PC12 cells, originating from pheochromocytoma in the adrenal medulla of rats, secrete neurotransmitters such as catecholamine, dopamine and norepinephrine and are often used as research subjects for neurotoxicity, neuroprotection, neurosecretion, neuroinflammation and synaptogenesis.[15,16] Our preliminarily study found that BA has a neuroprotective effect on okadaic acid (OA)–induced PC12 cells. However, the regulatory mechanism of which BA relieves OA-induced cellular injury is still not completely clear. As we know, OA can inhibit protein phosphatase 2A activity, thereby causing p-tau in the AD brain. Hence, the current study aimed to explore the neuroprotective effects of BA against AD by employing a cell model of OA-induced cellular injury. In addition, we used an autophagy inhibitor (3-methyladenine, 3MA) and autophagy activators (rapamycin) as controls to explore the possible mechanisms of BA in OA-induced PC12 cells by regulating Beclin-1-dependent autophagy.

MATERIALS AND METHODS

Cell culture

PC12 cells, purchased from Cell Resource Center, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, were grown routinely in high glucose Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), maintained at 37°C in an atmosphere containing 5% CO_2 for 48 hr. The media was changed once a day. Cells were plated in 96-welled plates for lactate dehydrogenase (LDH) and CCK-8 assay, 12-welled plates or 6-welled plates for AD cell function assay.

OA induced AD model cells

Different concentrations of 0–250 nM OA (cat. no. P112508; Adamas Reagent, Ltd.) preparation was made according to the method we used previously.[17,18] First, OA was dissolved into PBS containing 0.001% dimethyl sulfoxide (DMSO) and then diluted to the desired concentration with high-glucose DMEM containing 10% FBS. PC12 cells were evenly incubated into 96-welled plates and divided into nine groups in 12 hr, 24 hr and 48 hr, respectively: 50 nM OA group, 75 nM OA group, 100 nM OA group, 125 nM OA group, 150 nM OA group, 175 nM OA group, 200 nM OA group, 225 nM OA group, and 250 nM OA group. Each group had six duplicate wells. After the cells were cultured to a suitable density, the corresponding concentration of OA was added to each group to screen out the most appropriate concentration and time of OA for the next experiment. The absorbance was measured using an LDH kit (cat. no. A020-2-2, Nanjing Jiancheng Institute of Biological Engineering, China) and the probit function of Statistical Package for the Social Sciences (SPSS) calculated that the IC_{50} was 175 nM OA for 48 hr.

Experiment design

The cell culture method was the same as above. The cells were covered with monolayer, digested with 0.25% trypsin, and the digestion was stopped with a DMEM containing serum, and the cell density was adjusted to 1×10^5/mL. The cells were segregated in a 96-welled culture plate or 24-welled plate, and the cells were grown adherently and crossed into a net (24 hr) for the experiment. The cells were divided into eight groups: a normal control group, a model group (175 nM OA), a donepezil group (3.79 μg/mL, national medicine standard H20050978, eisai Pharmaceutical Co., Ltd., China), three BA groups at different concentrations (25, 50, 100 μg/mL BA, cat no. 20347-65-3, was purchased from the national institute of control of pharmaceutical and biological products, China), a 3-MA group (5 nM, cat. no. M9281, Sigma-Aldrich, Germany), and a rapamycin group (100 nM, cat. no. V900930, Sigma-Aldrich, Germany). Except for the normal control group, the model group was only given 175 nM OA for incubation for 48 hr, and the other experimental groups were given the corresponding drug for incubation for 0.5 hr and then 175 nM OA for further incubation for 48 hr for indicator detection.

Cell viability assessment

The cell culture method was the same as above. PC12 cells were cultured in 96-welled plates with cell density of 1×10^5/mL and subsequently divided into seven groups: normal control group, model group (175 nM OA), donepezil group (3.79 μg/mL), four BA groups at different concentrations (25, 50, 75, 100 μg/mL BA). CCK-8 activity was measured after 24 hr incubation with the corresponding drug. The process was as follows: the medium was drained, and 10 μL CCK-8 (cat. no. C0037, Shanghai Biyuntian Biotechnology Co., Ltd., China) was added into each well, and then cultured in the incubator with an atmosphere containing 5% CO_2 for 1 hr at 37°C. The assay was performed at 450 nm on an enzyme-linked immunoassay.

ELISA determination

The cell grouping and drug administration were consistent with the CCK-8 method. The supernatant of PC12 cells was collected, and the activity of p-tau, $Aβ_{42}$ and β-secretase in the supernatant of each group was detected strictly according to the instructions of the corresponding-ELISA kit (cat. no. P261FC, A227FC and B096FC, Elixir Medical Corporation, Milpitas, CA, USA).

Western blot analysis

Cell treatment was the same as above and grouped as follows: normal control group, model group (175 nM), 3-MA group (5 nM), rapamycin group (100 nM), BA group (100 μg/mL). 500 μL PBS was added to each well, the cells were scraped gently, the cells were lysed, the protein supernatant was collected after centrifugation, and the protein concentration was determined by BCA kit (cat. no. P0012S, Shanghai Biyuntian Biotechnology Co., Ltd., China). 40 μg protein samples were added to each well, SDS-PAGE was run at 120 V, the membrane was

transferred, and 5% skimmed milk powder was sealed for 1 hr. Primary antibodies of p-Akt (cat. no. 9275S; Cell Signaling Technology, USA), Akt (cat.no. 9272S; Cell Signaling Technology, USA), p-mTOR (cat. no. ab109268; Abcam, USA), mTOR (cat.no. ab32028; Abcam, USA), Beclin-1 (cat. no. ab62557; Abcam, USA) and GAPDH (cat. no. ab8245; Abcam, USA) were added and incubated overnight at 4°C (all 1: 1,000 dilution). The anti-rabbit secondary antibody (cat no. 7074S, 1: 2000 dilution; Cell Signaling Technology, Inc., USA) was incubated at 37°C after washing the membrane. ECL (cat. no. P0018FS, Shanghai Biyuntian Biotechnology Co., Ltd., China) chemiluminescence was used and GAPDH was used as an internal reference to compare the relative protein expression rates of different groups.

Immunofluorescence analysis

Cell processing and grouping are the same as western blot analysis. Beclin-1 expression in PC12 cells was detected according to the instructions of the immunohistochemical kit (cat. no. P0603, Shanghai Biyuntian Biotechnology Co., Ltd., China). First, we fixed the cell with 4% paraformaldehyde at 4°C for 1 hr, then the primary antibody of Beclin-1 (cat. no. ab62557, 1: 50 dilution; Abcam, USA) was incubated for 1 hr at 37°C, and the corresponding secondary antibody (cat no. 7074S, 1: 200 dilution; Cell Signaling Technology, Inc., USA) was incubated for 30 min at 37°C. Finally, the positive expression rate of Beclin-1 was observed and calculated under an inverted microscope.

Immunofluorescence analysis

Cell processing and grouping are the same as western blot analysis. Beclin-1 expression in PC12 cells was detected according to the instructions of the immunofluorescence kit (cat. no. P0176, Shanghai Biyuntian Biotechnology Co., Ltd., China). First, we fixed the cell with 4% paraformaldehyde at 4°C for 1 hr, then the primary antibody of Beclin-1 (cat. no. ab62557, 1: 50 dilution; Abcam, USA) was incubated for 1 hr at 37°C, and incubated with Alexa 488-conjugated anti-rabbit IgG (cat no. 4412S, 1: 200 dilution; Cell Signaling Technology, Inc., USA) at 37°C for 30 min, and the glycerin was sealed. Finally, the samples were observed and recorded under a fluorescence microscope.

Transmission electron microscope

To further explore BA's regulatory effect on autophagy, we observed the formation of autophagosomes in cells via the transmission electron microscope (TEM).[19] Cells from each group were initiated by 2.5% glutaraldehyde in 0.1 mol/L phosphate-buffered serum (PBS) (pH 7.4) at room temperature for 90 min and fixed in 1% osmium tetroxide for 30 min. Next, cells were washed in PBS and progressively dehydrated in a 10% graded series of 50%–100% ethanol and propylene oxide and embedded in Epon 812 resin. Then, the blocks were cut into ultrathin sections (50–150 μm) with a UC 7 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 the formation of autophagosomes in cells under an H-7650 TEM (magnification, x30,000; Hitachi, Ltd.), as previously described.[19]

Statistical analysis

Data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by a Bonferroni *post hoc* test was adopted for multiple comparisons in different groups. And $P < 0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad Prism 8.0.1 software.

RESULTS

AD cell model of OA induction: 175 nM OA for 48 hr

To investigate the effects of BA treatment on the AD cell model, we used OA-induced PC12 cell model. We treated PC12 cells with OA at a concentration of 0–250 nM, and detected the LDH content at 12, 24 and 48 hr by the LDH method, respectively. A higher LDH level in the supernatant may reflect more serious cell injury.[20] Compared with the control group, LDH levels increased in 0–250 nM OA at 12 hr, 24 hr, and 48 hr ($P < 0.01$), respectively. During 12 hr, LDH levels in 200–250 nM OA-treated groups were elevated when compared with that in the 50 nM OA-treated group ($P < 0.05$). Within 24 hr, LDH levels in 150–250 nM OA-treated groups were raised as compared to that in the 50 nM OA-treated group ($P < 0.05$). Followed by 48 hr, LDH level in 125–250 nM OA-treated groups was higher than that in 50 nM OA-treated group ($P < 0.05$). These results indicated that the change of LDH at 48 hr was the biggest relative to the other two time points (12 hr and 24 hr). We used SPSS software to calculate the optimal condition of OA-induced AD cell model, which was 175 nM for 48 hr, and it was applied to the subsequent experiments [Figure 1].

BA exerts an anticytotoxicity effect in OA-induced cell model

CCK-8 determination was then performed to detect cell viability of OA-induced AD cells in each medication group. The results indicated that the cell viability in BA-treated groups (doses of 25, 50, 75, and 100 μg/mL) gradually increased compared to that of the model group alone ($P < 0.01$). However, relative to the normal control group, the cell viability of the model or different concentrations of BA-treated cells showed a significant decrease ($P < 0.01$). Moreover, in comparison to the donepezil group, the cell viability showed no statistical difference in the 100 μg/mL BA-treated cells ($P > 0.05$). These findings suggest that BA could play an anticytotoxic role and promote cell growth [Figure 2].

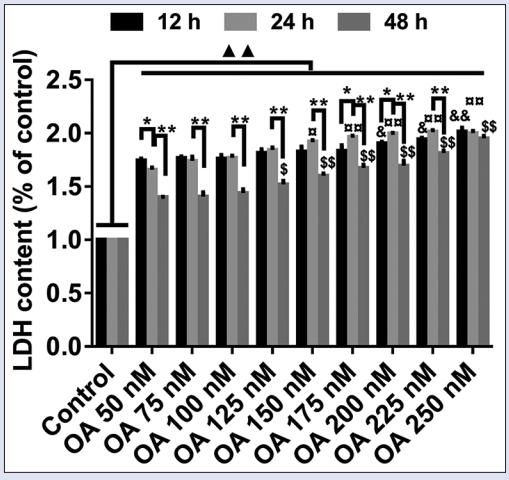

Figure 1: The change of LDH level in different OA concentrations and time period. With the increase of OA concentration from 0 to 250 nM, the content of LDH also increased. The LDH change at 48 hr is the most among 12 hr, 24 hr, and 48 hr. According to the experimental results, the conditions for our final choice of AD cell model are 50 nM OA for 48 hr in PC12 cells. LDH: Lactose reductase; OA: Okadaic acid; nM: nmol/L. ▲▲$P < 0.01$ vs control; *$P < 0.05$, **$P < 0.01$; &$P < 0.05$, &&vs 50 nM in 12 h; ¤$P < 0.05$, ¤¤$P < 0.01$ vs 50 nM in 24 hr; $P < 0.05$, $$P < 0.01$ vs 50 nM in 48 hr ($n = 6$ in each group)

BA protects cells against OA cytotoxicity at p-tau, $A\beta_{42}$ and β-secretase levels

We investigated the effect of BA on OA-induced cytotoxicity. p-tau, $A\beta_{42}$ and β-secretase are key elements in AD and we determined their levels via ELISA. Compared to the OA model group alone, the BA-treated and donepezil-treated cells displayed lower levels of p-tau, $A\beta_{42}$ and β-secretase ($P < 0.01$). Compared with donepezil-treated group, the levels of p-tau, $A\beta_{42}$ and β-secretase were higher at doses of 25, 50, 75 μg/mL BA-treated cells ($P < 0.01$). However, the levels of p-tau, $A\beta_{42}$ and β-secretase between the donepezil group and the high dose of BA group (100 μg/mL) were not statistically significant ($P < 0.05$) [Figure 3].

Akt/mTOR signalling pathway was regulated by BA in OA-induced AD cell

To further explore the molecular mechanism of BA in anticytotoxicity induced by OA, we investigated whether autophagy was involved in the progression of AD cell injury regulated by BA. Western blot assay was performed to determine the p-Akt, p-mTOR and Beclin-1 expression in the OA-induced AD cells. The findings displayed that p-Akt and p-mTOR were downregulated and Beclin-1 upregulated in the model group when compared with the control group ($P < 0.01$). In comparison to the model group, p-Akt and p-mTOR increased, accompanied by reduced Beclin-1 in the 3-MA and BA groups ($P < 0.01$); however, these proteins showed the opposite change in the rapamycin group ($P < 0.01$). Furthermore, BA and rapamycin-treated groups decreased the expressions of p-Akt and p-mTOR, while increasing Beclin-1 expression ($P < 0.01$) compared with that of the 3-MA group. These results suggested that BA could activate autophagy by suppressing Akt/mTOR signalling pathway in OA-induced AD cells [Figure 4].

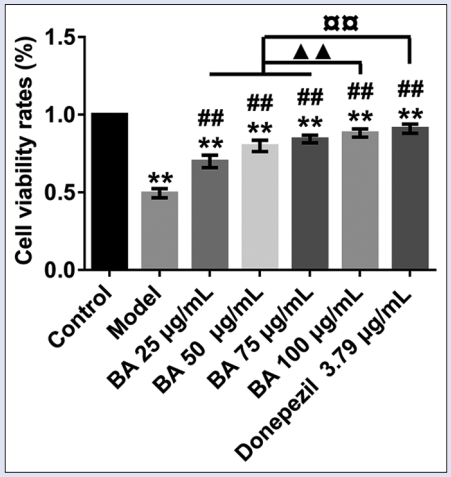

Figure 2: BA elevated the cell viability of PC12 cells after OA damage. The 25, 50, 75, and 100 μg/mL BA all presented the anticytotoxicity effect on OA-induced AD model cells and showed certain dose-effect relationship. OA: Okadaic acid; BA: bornyl acetate. $^{**}P < 0.01$ vs control; $^{\#\#}P < 0.01$ vs model; $^{\blacktriangle\blacktriangle}P < 0.01$ vs 100 μg/mL BA group; $^{¤¤}P < 0.01$ vs donepezil group ($n = 6$ in each group)

Beclin-1 is a target protein of autophagy

To explore the Beclin-1-modulated autophagy, we identified the Beclin-1 which targets autophagy by using immunohistochemistry and immunofluorescence staining. The results showed that Beclin-1 increased when compared with that in the control group ($P < 0.01$). In addition, Beclin-1 in the 3-MA and BA groups was lower than that in the model group, yet Beclin-1 in the rapamycin group was higher ($P < 0.05$). Besides, Beclin-1 was upregulated in the rapamycin and BA groups when compared with that in the 3-MA group ($P < 0.05$) [Figure 5].

Autophagy was suppressed in the BA-treated cells

TEM is the gold standard for observing autophagy. We verified the study by examining the number of autophagosomes by TEM in all treated group cells. We observed that the organelles in the control group were normal and no autophagosomes were found. However, the formation of autophagosomes was more obvious in the model group. The TEM results showed that the number of autophagosomes was higher in the rapamycin group, whereas that in the BA-treated or 3-MA-treated group was less than in the model group [Figure 6].

DISCUSSION

It has been found that Aβ deposition and tau protein abnormality are the main pathogenesis of AD.[2] Abnormal Aβ aggregation plays a critical role in the pathogenesis of AD, leading to protein phosphorylation in tau neurons, synaptic loss and apoptosis, and ultimately, reduced cognitive performance.[9] Though many Aβ targeted drugs have been developed, there have still been no successful cases through clinical III period tests. Additionally, studies have shown that the richness and diversity of intestinal micro-organisms are significantly decreased in AD patients compared with healthy people.[21,22] Increasing permeability of the intestinal

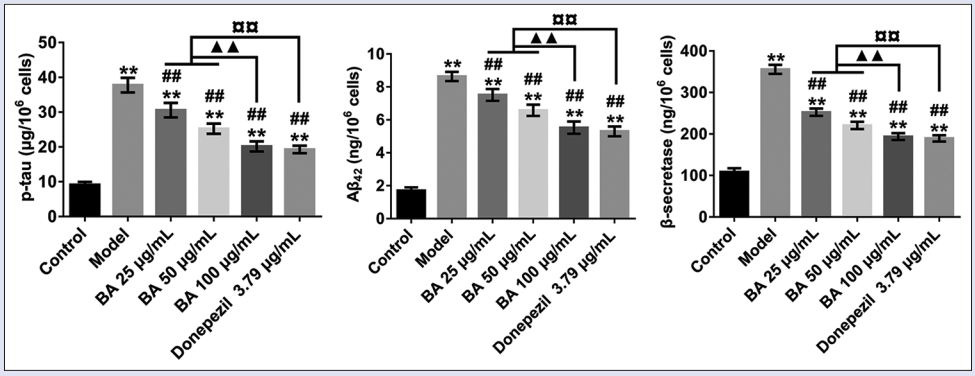

Figure 3: The change of intracellular p-tau, $A\beta_{42}$ and β-secretase levels. In the model group, the p-tau, $A\beta_{42}$ and β-secretase levels increased compared to the control group. However, the p-tau, $A\beta_{42}$ and β-secretase levels gradually reduced with the increase of doses. Aβ: β-amyloid; BA: bornyl acetate. $^{**}P < 0.01$ vs control; $^{\#\#}P < 0.01$ vs model; $^{\blacktriangle\blacktriangle}P < 0.01$ vs 100 μg/mL BA group; $^{¤¤}P < 0.01$ vs donepezil group ($n = 6$ in each group)

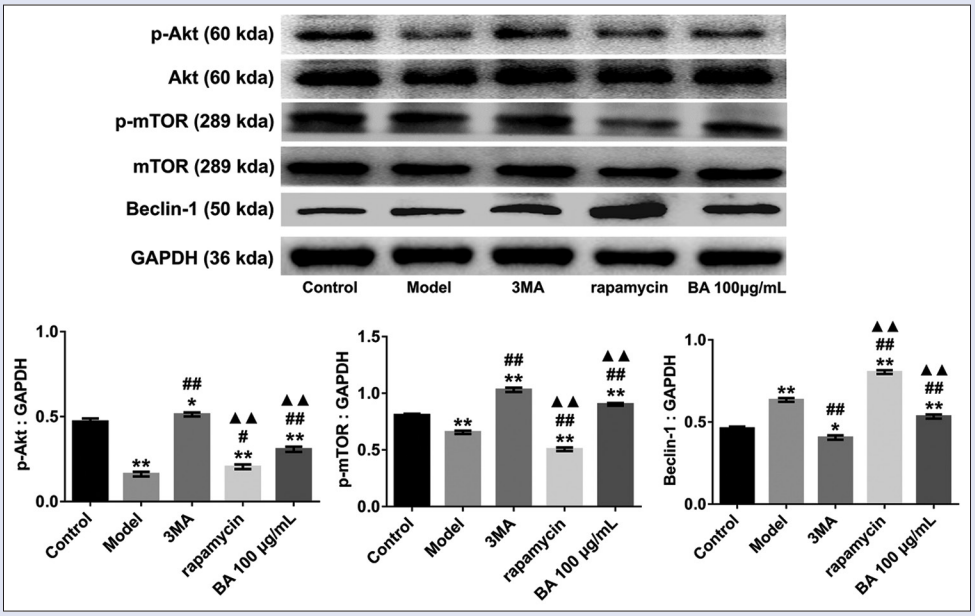

Figure 4: The p-Akt, p-mTOR and Beclin-1 expression in cell damage following OA. In comparison to the model group, p-Akt and p-mTOR increased, accompanied by reduced Beclin-1 in the BA group ($P < 0.01$). OA: Okadaic acid; 3-MA: 3-methyladenine; BA: Bornyl acetate. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs model; $^{\blacktriangle\blacktriangle}P < 0.01$ vs 3-MA ($n = 3$ in each group)

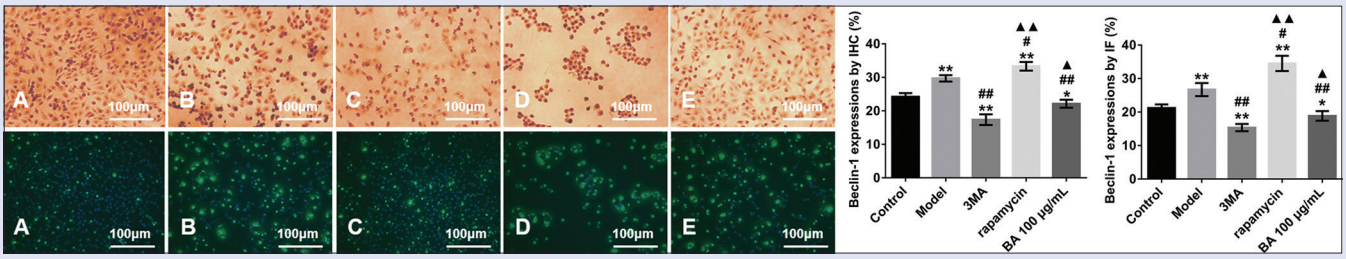

Figure 5: Bornyl acetate decreased Beclin-1 expression by immunohistochemistry and immunofluorescence staining (×200). The results showed that Beclin-1 expression was downregulated in the BA group when compared with that in the model group ($P < 0.01$). 3-MA: 3-methyladenine; BA: bornyl acetate. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs model; $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$ vs 3-MA ($n = 3$ in each group)

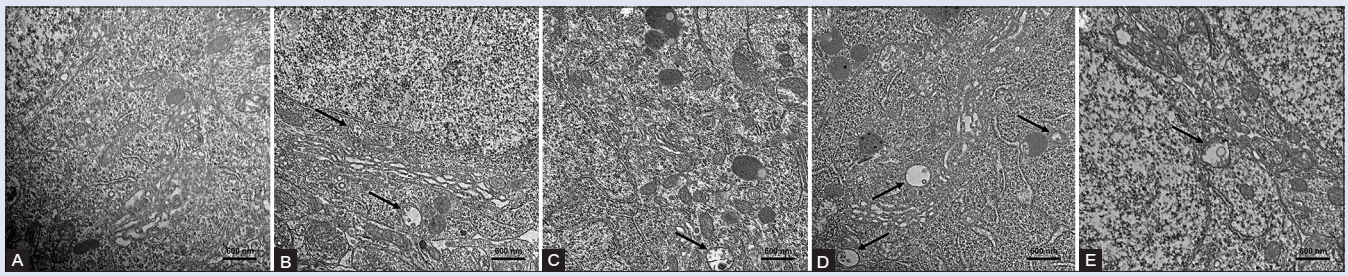

Figure 6: Autophagy was observed via transmission electron microscope (×30000). The results showed that the number of autophagosomes was downregulated in the BA-treated cells when compared with that in the model cells. Bar = 600 nm. The arrows indicated autophagosomes. A: control group, B: model group, C: 3-MA group, D: rapamycin group, E: BA group ($n = 3$ in each group)

barrier and activating the immune occurrents systemic inflammation in AD patients.[23] Aβ can also damage the blood–brain barrier, promote neuroinflammation, and ultimately lead to neurodegeneration. Elevated Aβ aggregation by intestinal micro-organisms is typical of AD occurrence.[24] Intestinal flora has been associated with the development of AD.[25] It is noteworthy that BA, volatile oil extracted from *Amomum villosum*, shows the anti-inflammatory effect and memory improvement pharmacological.[14] Studies have reported that BA can reduce intestinal mucosal inflammation induced by 5-fluorouracil, and enhance the intestinal mucosal barrier by increasing the expression of ZO-1 and occluding.[26] And the neuroprotective effect of donepezil in AD treatment was attributed to its anti-inflammatory action.[6] Therefore,

in this study, we used donepezil as the positive control to evaluate the therapeutic effect of BA on AD.

In terms of neuropathology, it is widely accepted that intracellular p-tau, neurofibrillary tangles and abundant extracellular amyloid deposition in the brain are the critical symptoms of AD pathogenesis.[8] It has been suggested that OA, an inhibitor of protein phosphatase 2A can trigger p-tau and neuronal death in cells and animals, thus providing an ideal model for the study of AD.[27] According to the Aβ cascade hypothesis, Aβ is produced by APP lysis mediated by β-secretase inhibitor, which represents upstream interference with the Aβ cascade and affects many physiological substrates and functions inside and outside the nervous system.[28] Therefore, the increased expression of p-tau is a typical molecular manifestation of AD, and the significant increase of p-tau expression in PC12 cells after OA treatment may be used as a pathologic feature of the AD model. Our results demonstrated that OA has obvious neurotoxicity on PC12 cells, which can greatly reduce the viability of PC12 cells. Interestingly, the treatment with BA has therapeutic effects on AD cells induced by OA. The viability of PC12 cells induced by OA significantly improved in increasing concentrations from 25, 50, 75 to 100 μg/mL of BA, and the effect of 100 μg/mL BA was almost identical to that of donepezil. Furthermore, we also found that the levels of p-tau, $A\beta_{42}$ and β-secretase in PC12 cells induced by OA were higher than those in the normal control group. However, the levels of p-tau, $A\beta_{42}$ and β-secretase were significantly lower than those in the model group after the intervention of BA or donepezil treatment. These results suggest that BA can reduce the cytotoxicity caused by OA and has a significant neuroprotective effect.

As regards to Beclin-1, a homologous protein of the APG6/VPS30 gene in yeast is a key protein involved in mammalian autophagy.[29] Indeed, recent findings demonstrated the involvement of Beclin-1 phosphatidylinositol phosphate 3 kinase (PI3K) in promoting autophagy-forming complexes, and its role also seems to be associated with the regulation of mammalian autophagy formation and maturity.[30] In the current study, we found that autophagy was overactivated in AD model cells, and Beclin-1 expression was significantly elevated, indicating obvious activation of autophagy flow. This result is consistent with our previous experiments related to the same AD modelling method.[17,18] We also found that Beclin-1-mediated autophagy was activated in OA-induced PC12 cells. After BA intervention, Beclin-1 expression significantly decreased in the BA group and 3-MA group compared with the model group, indicating that BA can alleviate autophagy activation of AD model cells. Interestingly, Wang *et al.* found suppression of autophagy by Aβ-induced PC12 cell injury, which was contrary to findings by Xue *et al.*[31,32] Therefore, the mechanism of autophagy in AD is different in various modeling building drugs in the same cells. As a result, we will focus on the effect of BA and autophagy on neurons in future experiments.

Akt/mTOR signalling pathway is considered to be one of the most direct regulators associated with autophagy occurrence and development.[32] Akt is known to be selectively activated by PI3K. Previous reports have suggested that the inductor Akt is involved in the activation of mTOR.[33] Inevitably, mTOR activity regulated the formation and maturation of autophagosomes through the Akt/mTOR–related autophagy pathway.[34] To further clarify the mechanism of BA, this study also detected the phosphorylated proteins of Akt and mTOR. Using autophagy inhibitors and autophagy activators as controls, the results showed that BA could activate the expressions of p-Akt and p-mTOR, and it was presumed that BA could slow down the autophagy activity by activating the Akt/mTOR pathway.

CONCLUSION

In conclusion, our study provides evidence that BA is a novel therapeutic to AD as it regulates Beclin-1-dependent autophagy. Our results indicated that BA protected against OA-induced cellular injury by reducing the expressions of p-tau, $A\beta_{42}$ and β-secretase and inhibiting the Beclin-1-dependent autophagy by modulating Akt/mTOR signalling pathway. Therefore, our findings suggested that BA is a potential treatment strategy for OA-induced AD cell injury.

Authors' contribution

LH and MD conceived the study, designed the experiments, analyzed the data and prepared the manuscript. LH, XZ, ZZ, WN and MD selected the subjects and obtained samples for the present study. XZ, MD and LH performed the experiments. The authors read and approved the final manuscript. All authors declare that all data were generated in-house and that no paper mill was used.

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Conflicts of interest

There are no conflicts of interest.

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