Original Article
Genistein protects against rat hippocampus amyloid-β1-42 neurotoxicity through p-mTOR-dependent autophagy

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Abstract: Deposition of amyloid beta (Aβ) protein is a biomarker of severe Alzheimer's disease (AD), and leads to neural dysfunction and cell function disorders. Genistein (Gen) may exert a significant protective effect against Aβ-induced neurotoxicity, but the underlying mechanism remains elusive. Thus, this study aimed to explore the regulatory mechanism of Gen. Cell viability was evaluated using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. Moreover, apoptosis was analyzed using flow cytometry. The effect of Gen or MHY1485, an activator of the mammalian target of rapamycin (mTOR), on autophagosome formation was observed through transmission electron microscopy. In addition, phosphorylated (p)-mTOR, microtubule-associated protein 1A/1B-light chain 3-phosphatidylethanolamine conjugate (LC3-II), and beclin 1 expression levels were detected via western blotting analysis. Gen significantly increased cell survival rate, which was deceased by Aβ1-42 pretreatment, and decreased the apoptosis induced by Aβ1-42 pretreatment. In addition, Gen reduced Aβ1-42-induced excessive autophagy. Moreover, Gen treatment reduced autophagic vesicle formation, conversion of LC3-I to LC3-II, and beclin 1 expression, which were all increased by Aβ1-42 pretreatment in the neurons. Additionally, Gen markedly suppressed the level of p-mTOR, which was upregulated by Aβ1-42 treatment. Finally, the p-mTOR-specific activator MHY1485 weakened the effect of Gen on cell survival, cell apoptosis, and cell autophagy induced by Aβ1-42 treatment. Taken together, these results suggested that Gen protects hippocampal neurons against Aβ1-42 induced neurotoxicity in rats by decreasing the level of p-mTOR, thus attenuating apoptosis and p-mTOR-dependent autophagy. These findings offer novel insights into the signaling pathway involved in Aβ1-42 toxicity, and the neuroprotective action of Gen.

Keywords: Aβ1-42, Alzheimer's disease, apoptosis, autophagy, genistein, p-mTOR

Introduction

Numerous studies have indicated that neuro-degeneration in Alzheimer's disease (AD), a major form of dementia in the elderly, can be clinically quantified *in vivo* using imaging approaches and fluid biomarkers, such as the accumulation of amyloid-β (Aβ) [1]. Aβ derives from the amyloid precursor protein (APP) after processing by β-site APP-cleaving enzyme 1 (BACE1) and γ-secretase, and has multiple C-termini, mostly ending at residues 40 or 42. Under physiological conditions, Aβ monomers are polymerized to form the soluble Aβ1-42 oligomers, subsequently generating protofibrils and fibrils, which constitute the more toxic form of the peptide, and which are likely to be the

ultimate cause of AD [2, 3]. Recent research suggested that the disruption of autophagy and apoptosis leads to Aβ aggregation in vacuoles and cell death, and that Aβ may affect autophagy and apoptosis during AD pathogenesis [4, 5]. Aβ1-42 oligomers have been reported to induce neuronal degeneration, which is the primary and early event underlying the pathology of AD, which is characterized by the accumulation of aggregated Tau protein, synaptic dysfunction, and eventually neuronal loss in the vulnerable regions of brains [6-8]. Thus, Aβ may play a key role in AD with a strong impact on cell autophagy and apoptosis.

Genistein (Gen) is an isoflavone that has been described as an angiogenesis inhibitor and a

phytoestrogen. Gen can be found in a number of plants, including soybeans, lupin, fava beans, kudzu, and psoralea in food sources, as well as in the medicinal plants, *Flemingia vestita* and *F. macrophylla.* In 2015, a clinical trial demonstrated the neuroprotective functions of Gen against AD, in particular as a moderator of AD-associated cognitive deficits [9]. Gen has also been shown to prevent the impairment of memory and cognition induced by intracerebroventricular administration of Aβ40, streptozotocin, or Aβ25-35 [10]. Furthermore, other studies have revealed that the Aβ peptide-induced inflammatory response of astrocytes could be blocked by Gen in culture or result in the death of cultured SH-SY5Y cells via regulation of many relevant signaling pathways, including the protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathway, which is involved in the negative regulation of cell autophagy and hence is a factor in Aβ-induced autophagy and apoptosis in neurons [11, 12]. mTOR, a ubiquitous protein kinase, is important in autophagy regulation and tau phosphorylation. In patients with severe AD, mTOR expression increases in selected neurons [13]. Furthermore, inhibition of the mTOR phosphorylation (p-mTOR) may induce autophagy, reduce tau protein and Aβ pathology, and ameliorate the associated behavioral deficits, suggesting that p-mTOR and its downstream molecules might be involved in the development of AD through the inhibition of autophagy [14, 15]. However, contradictory findings have also been reported, suggesting that further investigation is required to gain a better understanding of the molecular mechanism of Gen in modulating AD neuropathology.

Based on these previous findings, we attempted to use rat hippocampal neurons to investigate the protective effects of and underlying mechanism by which Gen regulates neurotoxicity induced by Aβ1-42. In particular, we evaluated the regulatory effects of Gen on cellular functions and the expression of autophagic genes, such as those encoding the microtubule-associated protein 1A/1B-light chain 3 (LC3) and beclin 1, in hippocampal neurons challenged with Aβ1-42 *in vitro*. Furthermore, the mTOR-specific activator MHY1485 was used to investigate whether Gen protects against Aβ1-42-induced neurotoxicity in rat hippocampal neurons through p-mTOR-dependent autophagy. Our study addressed fundamental aspects of the potential of Gen to ameliorate Aβ-induced neuronal cytotoxicity and apoptotic signaling.

Materials and methods

Preparation of Aβ peptides

Aβ1-42 peptides were purchased from US Peptide (Fullerton, CA). As previously described [16, 17], the peptides were dissolved in sterile distilled water to a final concentration of 2 mg/mL and aged for 3 days by incubation in a humidified chamber at 37°C. Subsequently, they were added to the culture medium at the final desired concentration. The final concentration in neurons was 20 μM.

Ethics statement

Eighteen-day-old rats were carefully handled in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, National Academy Press, Washington, DC, USA, revised 1996). And all animal experiments were approved by the Institute of Animal Care and Use Committee of Gannan Medical University.

Hippocampal neuronal culture and treatment

Cultures of dissociated hippocampal neurons were prepared from rats at embryonic day 18 as previously described [18]. Cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 5% fetal bovine serum, 2 mmol/L glutamine (Gibco, Grand Island, NY), penicillin (100 U/mL), and streptomycin (100 μg/mL), and maintained at 37°C and 5% CO_2 in a humid environment. Cultured neurons at 10 days *in vitro* were exposed to 2 μM Aβ1-42 peptides for 24 h, in plating media lacking the additional glutamate, as the positive control group. Subsequently, neurons were treated with Gen or MHY1485 for another 24 h. Cultured neurons treated with only saline were considered as the blank group.

Determination of cell viability

A 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to detect the viability of hippocampal neurons. Briefly, the cells were cultured in medium in 96-well plates at a density of 5×10^4 cells per well for 24 h. Subsequently, they were pretreated with Aβ1-42 (2 μM) for 24 h and were incubated in the absence or presence of Gen (at a final concentration of 25 μM) for another 24 h. A volume of 10 μL of MTT solution was added to the cell media (5 mg/mL) and then cells were

incubated at 37°C for 4 h. The medium was subsequently carefully removed, and 150 μL DMSO was added to each well to dissolve the formazan crystals formed *in situ*. Cell viability was determined by measuring the absorbance of each well at 570 nm using a microtiter plate reader (Biotek, Winooski, VT). Concentration determinations were repeated in three independent experiments, with five replicates per experiment.

Western blotting assay

Whole-cell extracts were prepared in ice-cold (4°C) RIPA lysis buffer (Beyotime, Shanghai, China) for 30 min and sonicated for 20 s. The lysates were then cleared by centrifugation at 18,000 × g for 15 min. The protein concentration was determined using Coomassie R Plus reagent (Pierce, Rockford, IL) and spectrophotometric measurement at 595 nm. Samples were subsequently mixed with an equal volume of a diluting buffer and boiled for 8 min. Proteins in each sample were separated on a gradient sodium dodecyl sulfate-polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA) at 220 mV for 40 min. Following electrophoresis, gels with the resolved proteins were electroblotted to polyvinylidene difluoride membranes (Millipore, Billerica, MA) using a Genie apparatus (Idea Scientific, Minneapolis, MN). The membranes were blocked for 2 h in 5% non-fat milk followed by incubation with a primary antibody overnight on a shaker at 4°C. As primary antibodies, the following were used: anti-rat LC3B (1:2000, Santa Cruz Biotechnology, Dallas, TX,); anti-rat beclin 1 (1:10000, Millipore); and anti-rat p-mTOR (1:5000, Millipore). Subsequently, the blots were washed three times with a washing buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) and incubated with the appropriate horseradish peroxidase -conjugated secondary antibody (ICN Biomedicals, Aurora, OH) at a 1:10000 dilution for 2 h. Blots were placed in an enhanced chemiluminescence detection system (GE Healthcare Bio-Sciences Corporation, Piscataway, NJ) for 3 min and exposed to X-OMAT AR films (Eastman Kodak, Rochester, NY), as described previously (Karmakar et al., 2008). The films were scanned on an EPSON Scanner using Photoshop (Adobe Systems, Seattle, WA) and the optical density of each band was determined using NIH Image J software. All experiments were performed in triplicates and differences in samples from the various groups were analyzed for statistical significance.

Fluorescence-activated cell sorting (FACS) analysis

We performed Annexin V-FITC/PI staining followed by flow cytometry for quantitative determination of the percentage of cells undergoing apoptosis, using the Annexin V-FITC Apoptosis Detection Kit I (Beyotime Biotechnology, Haimen, China). Cells were treated as described above in the cell viability analysis. Briefly, following the treatments, the attached and detached cells were harvested, washed with cold buffer, and digested with trypsin. Subsequently, they were resuspended in 1× binding buffer (0.1 M HEPES/NaOH, pH 7.4, 1.4 M NaCl, 25 mM $CaCl_2$), stained with 5 μL of Annexin V-FITC and 10 μL of propidium iodide (PI) (Annexin V-FITC staining kit, BD Biosciences, CA) according to the manufacturer's instructions. After mixing and incubating the cells in darkness for 15 min at room temperature, cells were detected using FACS analysis (BD Biosciences). Cells were categorized as follows: (1) cells negative for both PI and Annexin V-FITC were considered normal; (2) cells negative for PI negative and positive for Annexin V-FITC were considered early apoptotic; (3) cells positive for both PI and Annexin V-FITC were considered late necrotic; and (4) cells positive for PI and negative for Annexin V-FITC were considered mechanically injured during the experiment. All experiments were conducted in triplicates and analyzed for statistically significant differences.

Transmission electron microscope

To monitor cell autophagy, the ultrastructural changes caused by Gen, MHY1485, or Aβ1-42 in the hippocampal neurons were analyzed by transmission electron microscopy. After collection by centrifugation, cells were fixed in ice-cold 2.5% glutaraldehyde in phosphate-buffered saline (pH 7.3), post-fixed for 30 min in 1% OsO_4 with 0.1% potassium ferricyanide, dehydrated through a graded ethanol series (30-90%), followed by dry acetone, and embedded in Epon. Semi-thin (300 nm) sections were cut, stained with 0.5% toluidine blue, and examined under a light microscope. Ultrathin sections (65 nm) were stained with 1% uranyl acetate and 0.1% lead citrate, and examined with a JEM2000EX transmission electron microscope (JEOL, Tokyo, Japan). From each sample, a total of 20 to 25 micrographs were randomly taken (primary magnification, × 10,000). Subsequ-
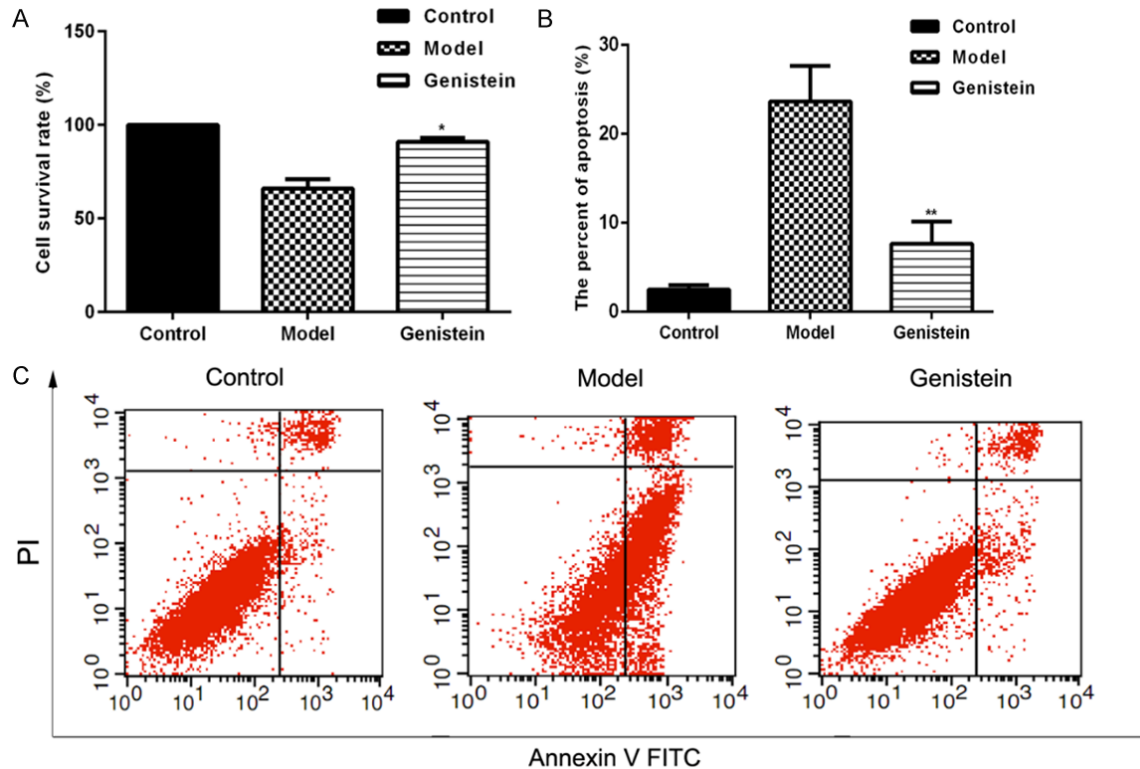
Figure 1. Effect of genistein (Gen) on the number of neurons impacted by Aβ1-42-treatment. A. Cell numbers were counted and cell viability was measured using an MTT assay; B, C. Analysis and percentage of Gen in an Alzheimer's disease (AD) cell model based on detection of apoptosis at 72 h. All experiments were repeated independently three times. *P < 0.05, **P < 0.01, as compared with data from the respective model group at the same time point.

ently, the volume of autophagic vesicles was estimated by morphometry using the Visilog program (Leica TCS SP2 CLSM, School of Medicine Electron Microscopy Center, Shanghai Jiaotong University, Shanghai, China). Autophagic vesicles were classified as early (containing morphologically intact cytoplasm), or late (containing partially disintegrated and electron dense material).

Statistical analysis

Quantitative data were expressed in arbitrary units (%), comparing untreated controls with cells treated with the indicated concentrations of inhibitors or with Aβ1-42 only. All data were presented as mean ± standard deviation (SD) from three or more independent experiments unless otherwise indicated. Statistical comparisons between different treatment groups were conducted with Tukey's multiple comparison test after one-way analysis of variance (ANOVA) using SPSS v. 19.0 software (IBM, Chicago, IL). *P*-values of less than 0.05 were considered to indicate statistically significant differences.

Results

Effect of Gen on Aβ1-42-induced proliferation inhibition and apoptosis

The effects of Gen treatment on the rate of cell survival of hippocampal neurons pre-treated with Aβ1-42 was assessed by the MTS assay. Based on the results, Aβ1-42 treatment could reduce the survival rate of hippocampal neurons when compared with the control group, while this effect could be mitigated by Gen treatment (**Figure 1A**). Moreover, apoptosis was also analyzed using flow cytometry. Compared with the control group, the exposure of hippocampal neurons to Aβ1-42 resulted in a higher percentage of apoptotic cells, while this damage could be protected by Gen treatment (**Figure 1B** and **1C**).

Effect of Gen on Aβ1-42-induced cell autophagy

To detect the effect of Gen on Aβ1-42 induced excessive autophagy, the formation of autopha-
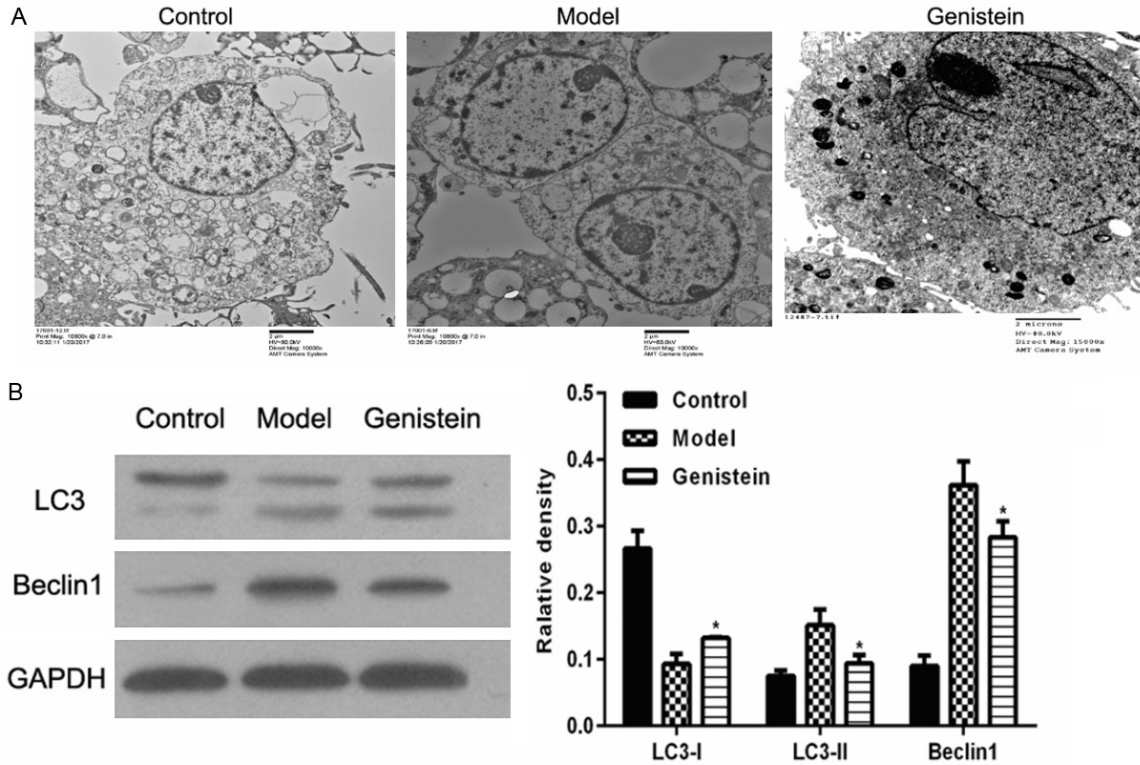
Figure 2. Genistein (Gen) reduces the autophagy increased by Aβ1-42-treated via influencing autophagy-related proteins. A. Images of neurons under a transmission electron microscope (bar: 500 nm). Gen induces the appearance of giant autophagic vacuoles in the Aβ1-42-cotreated group. Inhibition of fusion was reversible. In the positive control group, many vacuoles containing mitochondria formed in the cytoplasm. B. At the indicated time points, cells were lysed and autophagy-related proteins, including LC3-I/II and beclin 1 were detected by western blotting. All experiments were repeated independently three times. *P < 0.05, as compared with the data from the respective model group detected at the same time point.
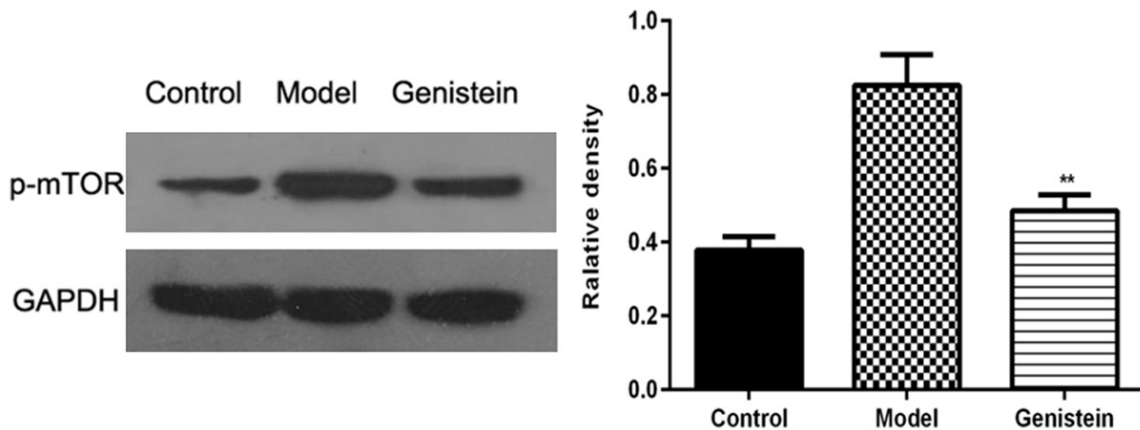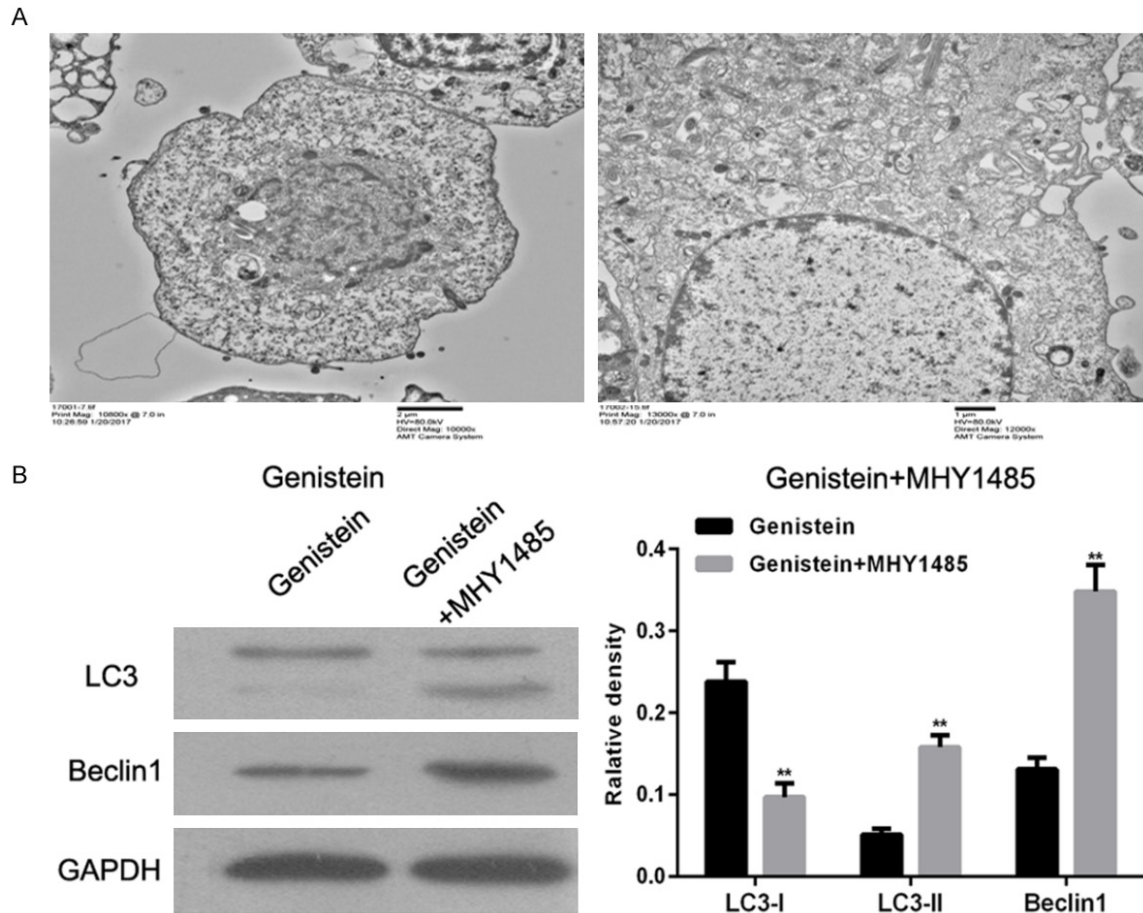

Figure 3. Impact of genistein (Gen) on the p-mTOR signaling pathway. At the indicated time points, cells were lysed and autophagy-related proteins, including p-mTOR, were detected by western blotting. Values are expressed as mean ± standard deviation from three independent experiments. **P < 0.01, as compared with the data from the respective model group detected at the same time point.

gosomes was observed using a transmission electron microscope and the expression of the autophagy-related genes encoding LC3B and beclin 1 were examined using western blotting.

As shown in **Figure 2A**, Gen could decrease the number of intracellular autophagosomes that had increased after Aβ1-42 treatment. Furthermore, Aβ1-42 significantly increased the

Figure 4. The effect of Genistein (Gen) on autophagy was negatively impacted by MHY1485. A. Images of neurons under the transmission electron microscope (bar: 500 nm). Both Gen and MHY1485 promotes the appearance of giant autophagic vacuoles in the Aβ1-42-cotreated group. Inhibition of fusion was reversible and the autolysosomes changed into autophagosomes after the addition of Gen, but this was reduced by MHY1485. B. At the indicated time points, cells were lysed and autophagy-related proteins, including LC3-I/II and beclin 1 were detected by western blotting. Values are expressed as mean ± standard deviation from three independent experiments. **P < 0.01, as compared with the data from respective model group detected at same time point.

conversion of LC3-I to LC3-II and the expression of beclin 1, while these effects were reversed by Gen pretreatment (**Figure 2B**).

Gen inhibited the expression of p-mTOR

To study the possible mechanism underlying the effect of Gen on Aβ1-42 induced neurotoxicity, p-mTOR expression was evaluated using western blotting. As shown in **Figure 3**, Aβ1-42 treatment significantly increased p-mTOR expression. This Aβ1-42-induced p-mTOR upregulation was mitigated by Gen pretreatment.

Effect of Gen and p-mTOR-specific activator MHY1485 co-treatment on cell survival and cell apoptosis

In order to explore whether Gen protects against Aβ1-42-induced neurotoxicity through p-mTOR-dependent autophagy, the effects of Gen and MHY1485 co-treatment on cell autophagy, cell survival, and cell apoptosis in the hippocampus after pre-treatment with Aβ1-42 were subsequently assessed. As shown in **Figure 4A**, the number of intracellular autophagosomes present after Gen and MHY1485 co-treatment were increased as compared with the cells in the Gen-treated group that had been cultured in the presence of Aβ1-42. The conversion of LC3-I to LC3-II and the expression of beclin 1 after Gen and MHY1485 co-treatment was increased as compared with the cells in the Gen-treated group that had been cultured in the presence of Aβ1-42 (**Figure 4B**). In addition, our results indicated that p-mTOR expression increased significantly when the cells were treated with both Gen and MHY1485 (**Figure 5A**). The cell survival rate was also
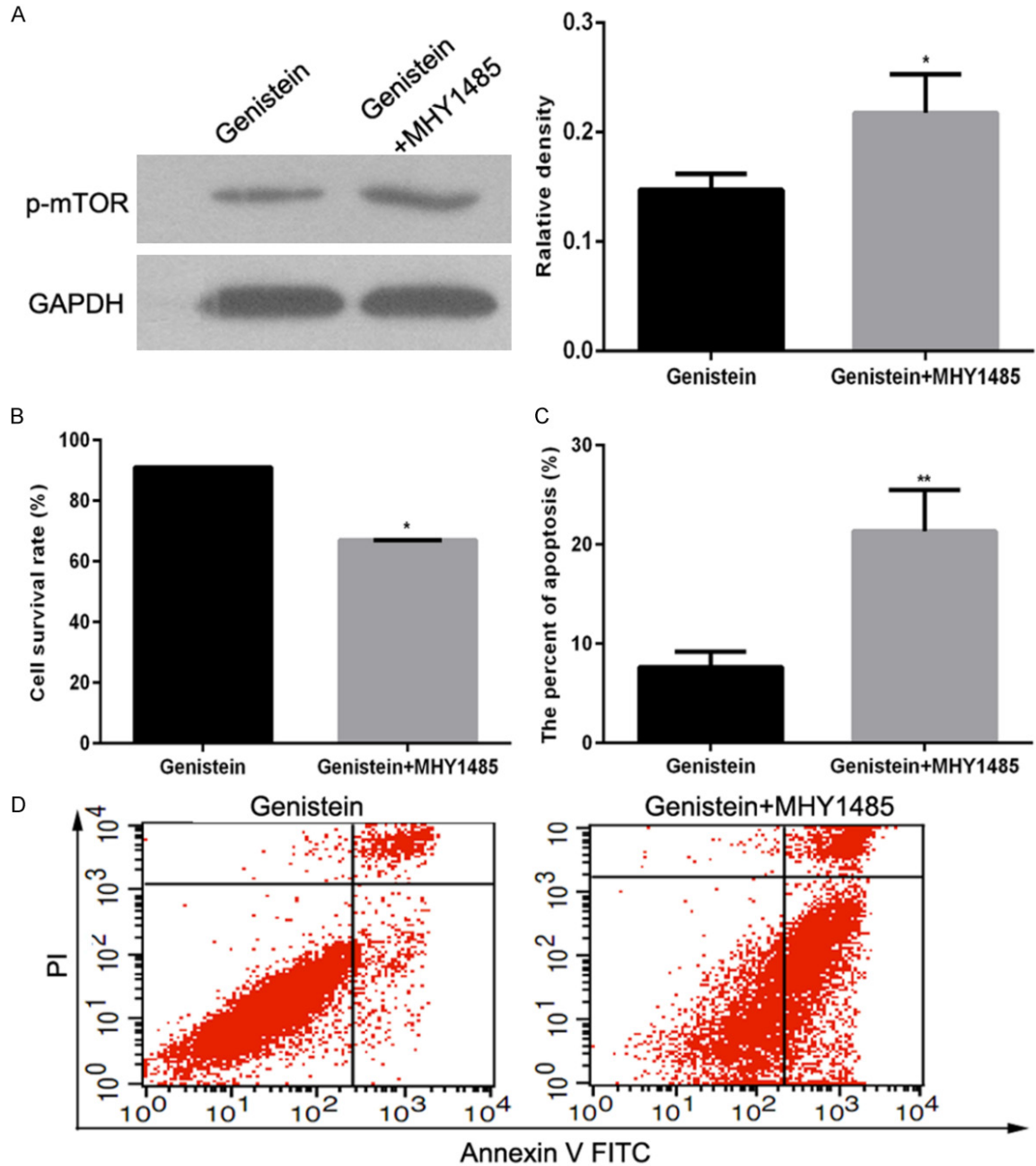
Figure 5. Effect of MHY1485 on pre-treatment of Genistein (Gen) on the number of neurons damaged by Aβ1-42. A. At the indicated times, cells were lysed and autophagy relevant proteins including p-mTOR were detected by western blotting. B. Cell numbers were counted and cell viability was measured using the MTT assay. C, D. Analysis and percentage of rapamycin in AD cell model through detection of cell apoptosis at 72 h. All detections were repeated independently three times. *P < 0.05, **P < 0.01, as compared with the data from the Gen group detected at the same time point.

examined and indicated a significant reduction when Gen was combined with MHY1485 (**Figure 5B**). Moreover, apoptosis was also analyzed using flow cytometry, which showed that Gen and MHY1485 co-treatment could increase the percentage of apoptotic cells (**Figure 5C** and **5D**).

Discussion

To date, there has been no report of a successful clinical intervention for AD. Production and aggregation of Aβ, particularly the toxic Aβ1-42 forms, has been known to begin around 40 years of age, causing neurotoxicity and cell

death in the brain [19]. Therefore, cognitive impairment, due to oxidative stress and neuro-inflammation, gradually commence and finally manifest as the disease progresses [20, 21]. By the time AD is diagnosed, which may be 20 years later, the disease may be too advanced for treatment [22-24]. Therefore, it is important to identify safe food ingredients that can help to prevent AD by reducing Aβ production or suppressing oxidative damage. In the present study, we used rat hippocampal neurons to investigate the protective effects and mechanism underlying the regulating effect of Gen on the neurotoxicity induced by Aβ1-42. We firstly found that Gen could suppress Aβ1-42-induced excessive autophagy and the expression of p-mTOR. To explore whether Gen protects against Aβ1-42 induced neurotoxicity through p-mTOR-dependent autophagy, the effect of Gen and MHY1485 co-treatment on cell survival and cell apoptosis in hippocampi pretreated with Aβ1-42 were assessed. In this way, we elucidated the demonstrated that Gen has potential for managing AD, and elucidated the underlying mechanism by which it exerts this regulatory effect.

A previous study has reported that treatment with Gen ameliorated Aβ-induced impairment of short-term spatial memory via an estrogenic pathway in rats [25]. Indeed, estrogen, like Gen, can promote the regeneration of stressed neurons, and protect neurons from death. The protective effect of Gen in PC12 cells entailed attenuation of Aβ25–35-induced apoptosis by interference with p-JNK activation through the mitochondrial pathway [26, 27]. These data were consistent with our present results. The weakening effect of Gen on autophagy through inhibition of p-mTOR was explored in the present study. To verify this evidence in the Aβ1-42-treated AD cell model, important biomarkers of autophagy, such as LC3 and beclin 1, which have been suggested to participate in elongation and formation of autophagosomes, were also assessed by western blotting. Several animal experiments have revealed that autophagosomes positive for marker proteins, such as LC3 and beclin 1, were associated with Aβ production and enrichment in AD [28, 29]. In this experiment, exogenous Gen inactivated autophagy in neurons by decreasing the expression of p-mTOR, LC3-II, and beclin 1 and inducing LC3-I expression. However, these effects were reduced by both Aβ1-42 and MHY1485, further supporting this evidence.

To verify the therapeutic effects of Gen in AD, additional studies may be required to clarify the protective effect of Gen both *in vitro* and *in vivo*. First, more AD cell models should be utilized to validate the present results. Second, more precise results are required to support our hypothesis. For example, other apoptosis-related genes, such as caspase-3 should be evaluated, and the influence of Gen treatment on Aβ1-42 production should be assessed. In addition, it is necessary to compare the various modes of treatment to elucidate the exact mechanism of Gen in AD animal models. Moreover, since the contribution of p-mTOR to cell autophagy has been investigated, the hypothesis that cell proliferation and apoptosis might be influenced by changes in cell autophagy mediated by p-mTOR should also be explored.

In conclusion, we showed that Gen can suppress Aβ1-42-induced cell autophagy through inhibiting the expression of p-mTOR. In addition, our study indicated that Gen plays a protective role against Aβ1-42-induced neurotoxicity through the inhibition of p-mTOR-dependent autophagy. The relationship between Gen and autophagy in Aβ1-42-induced neurotoxicity had not been reported to date. Our study yields insights into the mechanism underlying the regulatory effect of Gen on Aβ1-42-induced neurotoxicity. Furthermore, our study provides a theoretical basis for the clinical use of Gen for the treatment of AD, a disease demonstrating accumulation of Aβ [1].

Acknowledgements

Disclosure of conflict of interest

None.

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