Title: Vitis Vinifera L. Flavones Preserve Mitophagy in the Aβ1-42-Induced Model of Alzheimer's Disease Neurodegeneration

Running Title: VTF Protects AD Neurodegeneration

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Abstract

Introduction: Alzheimer's Disease (AD) is a prevalent neurodegenerative disorder characterized by amyloid-beta (Aβ) accumulation, leading to inflammation, oxidative stress, and impaired synaptic function. This study explores the neuroprotective mechanisms of Vitis vinifera L. flavones (VTF) against Aβ-induced neurodegeneration and its potential as an AD therapeutic.

Methods*:* In an *in vitro* analysis, Aβ1-42 oligomers induced a mitophagy model in SH-SY5Y neuroblastoma cells. Cells were treated with VTF alone and in combination with chloroquine (CQ), a lysosome inhibitor, to assess Aβ1-42-induced mitophagy. Transmission electron microscopy (TEM) and Immunofluorescence (IFC) revealed Aβ1-42 effects on autophagosomes and deposition. Cellular protection against Aβ-induced damage was evaluated using the CCK-8 assay. Western blotting determined the expressions of autophagy–lysosomal pathway proteins (Beclin-1, Atg7, p62, and BACE1) and the LC3-II/LC3-I ratio as an autophagy marker.

Results*:* CQ and VTF demonstrated significant neuroprotection against Aβ1-42-induced neurodegeneration ($P < 0.05$). VTF, alone or with CQ, increased viable cell count (~1.2-fold; $P < 0.05$), indicating reparative capabilities. TEM and IFC showed robust protection by VTF and CQ against Aβ protein deposition and preservation of mitochondrial and autophagosomal structures. VTF and CQ treatments reduced Beclin-1, Atg7, and BACE1 levels, indicating modulation of Mitophagy and autophagy–lysosomal suppression. VTF+CQ maintained LC3-II/LC3-I balance, confirming VTF's role in preserving autophagy ($P < 0.01$).

Conclusions*:* This study unveils VTF's novel neuroprotective role, emphasizing its potential as an AD therapeutic. Future research should extend investigations to *in vivo* models and clinical settings, enhancing understanding of VTF's neuroprotective efficacy.

Keywords: Alzheimer's Disease, *Vitis vinifera L.* flavones, Chloroquine, Aβ1-42-induced neurodegeneration, Mitophagy, Neuroprotective efficacy.

Highlights

- Vitis vinifera L. flavones (VTF) preserve mitophagy in Aβ1-42-induced AD neurodegeneration.
- Significant neuroprotection was observed with VTF and chloroquine against Aβ-induced damage.
- VTF maintains cellular viability and preserves mitochondrial and autophagosomal structures.
- Modulation of autophagy–lysosomal pathway proteins by VTF and chloroquine demonstrated.
- The potential for VTF as a novel therapeutic agent in Alzheimer's Disease warrants further exploration.

1. Introduction

Alzheimer's Disease (AD) is the most common neurodegenerative disease, affecting 18% of China's population (Chan et al., 2013; Jia et al., 2020). The National Aged Population Center of China predicts a significant increase in AD's impact among individuals over 60, reaching approximately 60 million over the next decade. This highlights AD as a major concern in China's public healthcare system (Kolachala, Lopez, Shen, Shayakhmetov, & Gupta, 2021; K. Li et al., 2018; Wang, Fang, Stephenson, Khiabani, & Zamboni, 2008).

AD is characterized by a complex network of pathophysiological pathways influenced by genetic, environmental, and lifestyle factors. Inflammation, heightened oxidative stress, and compromised synaptic function play significant roles in the disease's progression, complicating therapeutic interventions (De-Paula, Radanovic, Diniz, & Forlenza, 2012; X. Li et al., 2023). A nuanced understanding of the molecular intricacies of AD is imperative to develop targeted strategies for this multifaceted neurodegenerative disorder (Skaper, 2012).

Autophagy, driven by the formation of autophagosomes, plays a central role in self-protection during AD progression, eliminating surplus intracellular peptides and damaged organelles (Chung, Hernández, Sproul, & Yu, 2019; Klionsky et al., 2021; Krishnan et al., 2020). A significant focus revolves around initiating amyloid-beta (Aβ) clusters and entangled Tau proteins, considered pivotal biomarkers in AD therapy (Bloom, 2014; Fonseca, Solá, Xavier, Dionísio, & Rodrigues, 2013). Aβ, derived from the amyloid precursor protein (APP), undergoes abnormal aggregation, forming plaques (Salminen et al., 2013). These Aβ clusters, characterized by misfolded proteins, act as a focal point in the observed neurodegenerative cascade in AD. Aβ peptides in AD include Aβ1-42 and Aβ1, the former being a pathogenic variant with 42 amino acids (Aβ1-42), exhibiting an elevated propensity for aggregation and formation of toxic oligomers, contributing significantly to neurodegeneration (Sepulcre et al., 2017). In contrast, Aβ1, a shorter counterpart, is generated during APP cleavage, displaying a comparatively lower inclination for aggregation. The interplay between these forms underscores their distinctive roles within the intricate Aβ cascade, shaping the pathophysiology of AD (Jackson et al., 2016). However, in advanced stages of AD, impaired autophagy may lead to Aβ1-42 and Aβ1 accumulation, exacerbating neurodegenerative processes. Balancing the interplay between Aβ clusters and autophagy dynamics holds potential for targeted therapeutic interventions. Modulating autophagy to enhance Aβ clearance or inhibit Aβ cluster formation presents promising avenues for further exploration in the AD research (Dunys, Valverde, & Checler, 2018).

Several *in vitro* and *in vivo* studies introduced polyphenolic compounds from *Vitis vinifera L.* flavones (VTF), a herb rich in cholinergic neurotransmitters widely used in traditional Chinese medicine (Rodriguez-Mateos et al., 2014). These flavonoids have been shown to prevent damage to hippocampal neurons by inhibiting autophagy and promoting anti-neurodegenerative effects (Benavente-García & Castillo, 2008). Flavonoids from VTF act as oxygen-free radical scavengers and antioxidants, stimulating synaptic plasticity and improving cognitive impairment in AD model mice (Ma et al., 2018). Our latest report demonstrates that VTF can influence the pathological changes of AD by regulating hippocampal neurons via autophagy in APP/PS1 transgenic sedentary Alzheimer model mice. However, it remains unclear whether VTF can prevent hippocampal neuron damage through the inhibition of autophagy and Aβ clustering (Joseph et al., 2023; Lopresti et al., 2023).

In this study, we aim to delve deeper into the anti-neurodegenerative role of the VTF, exploring its relationship with autophagy and Aβ clustering. The investigation focuses on elucidating the *in vitro* mechanism of VTF in protecting neurons from Aβ1-42-induced neurodegeneration. Analyzing

changes in Aβ1-42-induced autophagy-related protein expression in brain tissues will shed light on VTF's potential to suppress excessive autophagy in Aβ1-42-induced SH-SY5Y cells. These findings may offer promising avenues in AD research, indicating that VTF's neuroprotective effects could be attributed to the inhibition of excessive autophagy in Aβ1-42-induced SH-SY5Y cells.

2. Materials and Methods

Preparation of flavones from Vitis vinifera L

Vitis vinifera Linnaeus grapes from the *Vitaceae* family were sourced from a reputable Uyghur medicine market in Turpan, Xinjiang Province, China, exactly one week before initiating the experiment. The seeds were meticulously collected in August 2023 from the Gaochang District, Turpan City, located in the northwest region of the city, by P.Z. (GPS coordinates: 42.9225° N, 89.1913° E). The botanical identification of the plant was confirmed as Vitis vinifera L., commonly known as "European grapes" in Chinese (Ōuzhōu pútáo), at the esteemed National Herbarium of China. After collection, the seeds were carefully air-dried to eliminate excess moisture, ensuring optimal extraction efficiency. Subsequently, the dried seeds were finely ground into a powder with a particle size of approximately 20 mg, ensuring uniformity and consistency in the extraction process. The powder underwent meticulous extraction using 95% ethanol as the solvent for a precisely controlled duration of 2 hours, following standard extraction protocols (H. Li, Fu, Deng, David, & Huang, 2020). Following the extraction process, the resulting mixture underwent rotary evaporation under controlled conditions to eliminate the ethanol solvent, resulting in the formation of a crude extract with enhanced purity and concentration. The yield of the crude extract was determined to be 170 g, designated as DCB, and the supernatant obtained after rotary evaporation was carefully preserved for subsequent analysis to prevent the loss of valuable components. To further enrich and purify the extracted flavonoids, a series of sophisticated purification steps were meticulously executed. This purification process involved suspending the crude extract in water, followed by purification with AB-8 resin, a highly efficient adsorbent material known for its excellent selectivity and adsorption capacity for flavonoids. The purification process was carried out using a precise gradient of 5% water and 95% ethanol to achieve optimal separation and purification of the target compounds. Subsequently, a 50% ethanol elution fraction containing highly enriched flavonoids was collected for further processing. Finally, the purified VTF was obtained as a high-quality brown-yellow powder through meticulous vacuum drying at a controlled temperature of 60 °C after freeze-drying of the purified fraction. This final purification step, with a yield of extracts exceeding 92%, ensured the removal of any residual moisture and solvent traces, resulting in the production of highly pure and concentrated flavones ready for further analysis and biological evaluation. As detailed in previous findings (Abdul Manap, Madhavan, Vijayabalan, Chia, & Fukui, 2020), the chemical analysis of VTF extracts showcased a rich presence of flavonoids and stilbenes, with resveratrol (3,5,4'-trihydroxy-trans-stilbene) standing out prominently. Additionally, compounds like Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one), Kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)chromen-4-one), and Myricetin (3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)chromen-4-one) were consistently identified as key components within VTF. This comprehensive examination significantly advances our understanding of their potential health benefits and therapeutic applications.

Preparation of Aβ1-42 oligomers

Aβ1-42 oligomers were prepared following the protocol by Fa et al. (Rezai-Zadeh et al., 2009). Initially, 5 mg of lyophilized Aβ1-42 (Sigma-Aldrich, St. Louis, MO, USA) underwent

equilibration at room temperature for 30 min to prevent condensation upon unsealing. The peptide was then suspended in ice-cold 1,1,1,3,3,3-Hexafluoro-2-Propanol (HFIP) to achieve a 1 mM solution. After brief vortexing, the Aβ1-42/HFIP solution was incubated in polypropylene vials using a glass GasTight Hamilton syringe with a Teflon plug for 2 hours to monomerize Aβ. The subsequent concentration of the Aβ1-42/HFIP solution under vacuum in a SpeedVac centrifuge (800 g, room temperature) produced a clear peptide film. Stringent temperature control (maintained below 25°C) prevented peptide degradation during this process. The Aβ1-42 film was re-suspended in Dimethylsulfoxide (DMSO) containing 10% fetal bovine serum, yielding a concentration of up to 400 μmol/L.

Preparation of Chloroquine (CQ)

In this study, chloroquine (CQ), a lysosome inhibitor, is employed to modulate autophagy. A 50 mM stock solution of CQ diphosphate salt (Sigma-Aldrich, St. Louis, MO, USA) was prepared in were dissolved in DMSO, sterilized by filtration (0.2 micrometers), and stored in aliquots at -20 ºC until use. Work solutions were diluted in 84% (v/v) mouse embryonic fibroblasts (MEFs; Thermo Fisher Scientific, Waltham, MA, USA), with 15.0% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin/streptomycin (P/S; Sigma-Aldrich, St Louis, MO, USA). Cells were treated with CQ in a dose-dependent manner, ranging from 0 to 100 μM.

Cell culture and treatment

The SH-SY5Y human-derived neuroblastoma cell line (ATCC CRL-2266) was sourced from the American Type Culture Collection (ATCC, Manassas, VA, United States) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin/streptomycin (P/S; Sigma-Aldrich, St Louis, MO, USA). The culture conditions were maintained in a constant temperature incubator at 37 °C with 5% CO_2 to optimize cell growth. Regular monitoring for mycoplasma contamination was implemented to preserve the integrity of the cell lines, ensuring that SH-SY5Y cells remained free from potential contamination during experimental procedures. Stock solutions were suitably diluted in DMEM with 1.0% (v/v) FBS to prepare work solutions. The study included the following treatment groups: Control (60 μM PBS), vehicle control (20 μM Aβ1-42 oligomer), VTF (80 mg/L VTF + 20 μM Aβ1-42 oligomer), CQ (40 μM CQ + 20 μM Aβ1-42 oligomer), and VTF+CQ (80 mg/L VTF + 40 μM CQ + 20 μM Aβ1-42 oligomer).

Cell proliferation assays

The induction of cell proliferation by Aβ1-42 was assessed using a Cell Counting Kit (CCK-8 assay, A311-02, Vazyme, Nanjing, China). Initially, cells (5×10^4 cells/well) were seeded in 96-well plates at a predetermined density. After treatments with VTF and CQ, CCK-8 solution was added to each well, followed by incubation. Absorbance was measured at 450 nm using a multimode microplate reader (Thermo Fisher Scientific Inc., MA, United States). Cell viability was quantified as a percentage relative to the absorbance of control cells.

Immunofluorescent assay

The Immunofluorescence (IFC) assay was conducted following established protocols to assess the impact of VTF on Aβ1-42-induced Aβ deposition in SH-SY5Y cells (Abdul Manap et al., 2020). SH-SY5Y cells were seeded at a density of 5×10^4 cells/well in IbiTreat chamber slides (Ibidi

GmbH, Martinsried, Germany) and incubated at 37°C in a humidified 5% CO_2 incubator. Once the cells reached 80% confluency, they were treated with 25 mM Aβ fibrils for 24 hrs. Following treatment, cells were washed with PBS three times, fixed with 4% paraformaldehyde (Aldrich, Steinheim, Germany) for 15 min, and then rewashed with PBS three times. The fixed cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, US), diluted in PBS for 15 min on ice, and washed three times with PBS. To minimize non-specific binding, cells were blocked with 10% bovine serum albumin (BSA; normal goat serum, Abcam, Cambridge, UK) diluted in PBS + 0.1% Tween 20 for 60 min at room temperature. Next, cells were incubated with the primary anti-Aβ1-42 antibody (5 µl, 1:100; Abcam, Cambridge, UK) diluted in 1% blocking buffer (PBS + 0.1% Tween 20 + 1% BSA) overnight at 4°C. Subsequently, cells were washed with PBS + 0.1% Tween 20 three times and incubated with the secondary Cy3-labeled goat anti-rabbit IgGs antibody (3 µl; 1:100, Promega, Madison, WI) diluted in 1% blocking buffer for 60 min at room temperature. Following three washes with PBS + 0.1% Tween 20 in the dark, cells were incubated with fluoro shield mounting medium with DAPI (AB104139, Abcam, Cambridge, UK) for 5 min at room temperature in the dark. Finally, cells were observed under Nikon's NIS-Elements fluorescence microscope (Nikon, Tokyo, Japan). Immunofluorescent signals were captured and analyzed using a fluorescence microscope connected to a computerized image system (Image-Pro Plus V6.0, Silver Spring, MD).

Transmission electron microscopy (TEM) assay

For in-depth analysis of cellular ultrastructure and autophagosomes across different groups, transmission electron microscopy (TEM) observations were performed using a CM12 TEM Philips located in Amsterdam, Netherlands. SH-SY5Y cells (3×10^4 cells/well) underwent two washes with 0.2 M PBS (pH 7.4) and were then gently scraped and fixed with 2.5% glutaraldehyde (v/v, in 0.1 M cacodylate buffer, pH 7.4) for 60 min on ice. After thorough washing, cells were post-fixed with 1% OsO_4 (w/v, in 0.1 M cacodylate buffer, pH 7.4) for 60 min at 4°C. Subsequent steps involved dehydration, embedding in Spurr's resin (TAAB Laboratories Equipment Ltd, Aldermaston, England), and examination under TEM at 80 kV.

Western blot analysis

To dissect the cleaved Aβ1-42-induced pathways, we performed Western blot analysis assessing the expression of BACE1, Atg7, p62, LC3-Ⅰ, LC3Ⅱ, and Beclin-1 proteins in various treated SH-SY5Y cell groups. Lysates from 2×10^6 SH-SY5Y cells/well in each group were extracted using RIPA buffer (Roche Diagnostics, Mannheim, Germany). Protein samples were then boiled with a loading buffer and separated using standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) methods. Subsequently, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane, followed by blocking with 5% bovine serum albumin for 30 min at room temperature. The PVDF membrane underwent probing with primary antibodies against Beclin1 (1:2000), p62 (1:2000), Atg7 (1:1000), BACE1 (1:1000), LC3 (1:2000), and GAPDH (1:1000), all purchased from Abcam, Cambridge, UK. Specific antibody binding was detected by incubating the membrane with a goat anti-mouse Immunoglobulin G (IgG) conjugated to biotin (1:2000 dilution) at four °C for 2 hours. Following extensive washing with TBS buffer, 3-39DiAminoBenzidine (DAB) solution was applied for 20 min in the dark, and the membrane was incubated with ExtrAvidin peroxidase (diluted 1:1500) at 4 °C for 60 min. Human GAPDH (36 kDa) Western blots served as controls. To visualize the protein bands, an enhanced

chemiluminescence detection system was utilized, and quantitative analysis was performed using ImageJ software. Table 1 provides comprehensive information about the primary and secondary antibodies employed in IHC staining, facilitating accurate detection and visualization of the target proteins.

Table 1. List of primary and secondary antibodies for immunofluorescence (IFC) and western blot (WB).

Epitope	Spices	Company	Catalogue No.	Dilation	Source
Primary antibodies					
Aβ1-42 (IFC)	Mouse, Rat, Human	Abcam	ab201060	1:100	Rabbit
Beclin1 (WB)	Mouse, Human	Abcam	ab92389	1:2000	Rabbit
p62 (WB)	Mouse, Rat, Human	Abcam	ab91526	1:2000	Rabbit
Atg7 (WB)	Rat, Human	Abcam	ab223380	1:1000	Rabbit
BACE1 (WB)	Mouse, Human	Abcam	ab10716	1:1000	Rabbit
LC3 (WB)	Human	Abcam	ab51520	1:2000	Rabbit
GAPDH (WB)	Human, Mouse, Rat	Abcam	ab8245	1:1000	Rabbit
Secondary antibodies					
Anti-rabbit IgG Cy3-labeled (IFC)	Rabbit	Promega	AP132C	1:1000	Goat
Anti-Mouse IgG HRP-labeled (WB)	Mouse	Abcam	ab6728	1:1000	Goat

Statistical analysis

Statistical analyses were conducted utilizing SPSS software version 26.0 (Chicago, Illinois, USA). Measurement data were expressed as mean ± standard deviation. Graphs were generated with GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA). Statistical comparisons between experimental groups employed either one-way analysis of variance or LSD analysis of variance, depending on the study design. Post-hoc analysis determined the statistical significance (P-value) among groups, with a significance level set at $p < 0.05$ and $p < 0.001$. All charts were created using Prism 8.0 (GraphPad, La Jolla, CA, USA).

3. Results

VTF enhances proliferation in Aβ1-42-induced SH-SY5Y cells

We investigated VTF's protective impact against Aβ1-42-induced cellular stress in SH-SY5Y cells. The CCK-8 assay highlighted a substantial reduction in cell proliferation upon exposure to 20 μM Aβ1-42 oligomers compared to the control group (1.10 ± 0.02 vs. 0.83 ± 0.03; $p < 0.01$). This underscores Aβ1-42 oligomers' neurotoxicity and their hindrance of cell proliferation. Conversely, VTF, CQ, and CQ+VTF demonstrated a marked increase in viable cell count, emphasizing the reparative capabilities of VTF and CQ against Aβ-induced toxicity (Figure 1A; $p < 0.01$). To unravel VTF's impact on Aβ1-42-induced Aβ deposition, IFC measured Aβ optical density values (Figure 1B and 1C). The vehicle control group exhibited a significant increase in IFC Aβ optical density values compared to the control group (Figure 1B, $p < 0.01$). Both the VTF and CQ groups showed decreased optical density values compared to the vehicle control group ($p < 0.05$). Notably, the VTF+CQ group displayed the most substantial reduction in Aβ optical density, with the CQ+VTF group showing a significant decrease (0.007 ± 0.0004 vs. 0.021 ± 0.009; $p < 0.01$). In Figure 1C, Aβ protein expression was prominent outside and within the nucleus of cells. Aβ protein deposition in the control group was the lowest, while the vehicle control group exhibited the highest deposition ($p < 0.01$). Compared to the vehicle control group, the VTF, CQ, and VTF+CQ groups displayed a gradual increase in red fluorescently labeled Aβ protein deposition ($p < 0.01$). Results indicated a significant rise in the red fluorescent labeling area and immunofluorescence Aβ optical density values in the vehicle control group compared to the control group ($p < 0.01$), signifying

substantial Aβ protein deposition. Furthermore, compared to the vehicle control group, the VTF and CQ groups exhibited a reduction in the red fluorescent labeling area and a decrease in Aβ optical density values ($p < 0.05$). The CQ+VTF group demonstrated a significant reduction in Aβ optical density values ($p < 0.01$). These findings collectively suggest that both VTF and CQ can mitigate intracellular Aβ protein deposition, enhancing cell vitality and protecting against Aβ-induced organelle damage. Overall, these results underscore the robust reparative potential of both VTF and CQ in mitigating the aging process and enhancing cellular vitality.

Figure 1.

VTF preserves Aβ1-42-induced mitophagy alterations

To structurally analyze the impact of VTF on hippocampal autophagy, we utilized TEM to examine Aβ1-42-induced alterations in mitochondria and autophagosomes across our study groups (Figure 2). As depicted in Figure 2A, the control group displayed well-defined mitochondria with orderly arranged cristae and minimal autophagosomes. In contrast, Aβ1-42 induction led to disrupted mitochondrial morphology, increased autophagosomes, and cellular stress (Figure 2B). Both VTF and CQ treatments exhibited significant improvements in mitochondrial characteristics, including increased numbers, regular morphology, reduced swelling, enhanced cristae, and fewer autophagosomes compared to the vehicle control group (Figure 2C and 2D, respectively). Notably, the combined treatment of VTF and CQ demonstrated a robust protective effect, preserving mitochondrial integrity and cellular homeostasis (Figure 2E). The TEM results underscored the detrimental impact of Aβ1-42 oligomers, intensifying cell senescence, inducing mitochondrial dysfunction, and promoting aberrant autophagy. Collectively, these findings highlight the multifaceted protective attributes of VTF and CQ, contributing to the maintenance of normal mitochondrial structure, functional homeostasis, autophagy homeostasis, and overall cellular well-being under Aβ1-42-induced cellular stress conditions.

Figure 2.

VTF modulates Mitophagy via the autophagy–lysosomal pathway

In our investigation of the influence of VTF on mitophagy induced by Aβ1-42 in SH-SY5Y cells, we examined crucial proteins associated with the autophagy–lysosomal pathway, including Beclin-1, p62, Atg7, and BACE1 (Figure 3). Western blot analysis revealed a substantial increase in the protein expression of autophagy–lysosomal pathway components, Beclin-1, Atg7, and BACE1, in the vehicle control group compared to the treatment groups (Figure 3A; $p < 0.05$). Upon VTF and CQ treatments, a significant reduction in Beclin-1, Atg7, and BACE1 protein levels was observed in the VTF group, CQ group, and CQ+VTF group compared to the vehicle control group. This suggests that VTF may protect neuronal cells by inhibiting the autophagy-lysosomal pathway. To delve deeper into the VTF+CQ mechanism on the p62 pathway, our analysis of LC3-II/LC3-I levels uncovered excessive autophagy and altered p62 expression across different groups, corroborating our earlier observations. In comparison to the control group, the vehicle control group exhibited significantly elevated levels of LC3-II, coupled with a notable decrease in p62 protein expression (Figure 3B; $p < 0.01$), indicative of heightened autophagy. Intriguingly, the VTF and CQ groups showed a significant increase in p62 protein expression ($p < 0.01$), signifying a lower autophagy level in these drug-treated groups than in the vehicle control group. Figure 3C illustrates the

comparison of LC3-II/LC3-I ratios and p62 expression. The VTF group, CQ group, and CQ+VTF group displayed significantly reduced LC3-II/LC3-I levels compared to the vehicle control groups. However, in contrast, p62 exhibited increased levels in the CQ+VTF-treated groups compared to the vehicle control group (Figure 3C; $p < 0.01$). This supports our hypothesis of excessive autophagy in vehicle control group cells, emphasizing that VTF protects by inhibiting abnormally activated autophagy, thereby preserving nerve cell function. This underscores that both VTF alone and the combined treatment of VTF and CQ upregulate p62 protein expression, modulate mitophagy, and suppress the autophagy–lysosomal pathway involving Beclin-1, Atg7, BACE1, LC3-II, and LC3-II/LC3-I protein levels. While both VTF alone and the combined treatment of VTF and CQ upregulated p62 protein expression, modulated mitophagy, and suppressed the autophagy–lysosomal pathway involving Beclin-1, Atg7, BACE1, LC3-II, and LC3-II/LC3-I protein levels as previously stated, upon closer examination, subtle differences emerged in the expression levels of these proteins between the treatment groups. Further analysis revealed nuanced variations in the regulatory mechanisms underlying the response to VTF alone compared to the combined treatment with CQ, suggesting potential synergistic effects of the combined treatment on autophagy regulation.

Figure 3.

4. Discussion

In this study, we explored the *in vitro* mechanism of VTF in safeguarding neurons against Aβ1-42-induced neurodegeneration. Our results, for the first time, collectively demonstrate that VTF and CQ contribute to maintaining normal mitochondrial structure, functional homeostasis, autophagy homeostasis, and overall cellular well-being under Aβ1-42-induced cellular stress conditions. Our data provides additional support for the neuroprotective role of VTF through the regulation of autophagy, specifically by modulating mitophagy via the autophagy–lysosomal pathway. Notably, our investigation highlights the significant contribution of VTF in exerting anti-neurodegenerative effects by inhibiting excessive autophagy and preserving nerve cell function.

The selection of VTF for investigation in our study was based on their well-documented antioxidant and neuroprotective properties. Many evidence suggests that polyphenolic compounds derived from flavonoids act as effective oxygen-free radical scavengers and antioxidants in AD (Joseph et al., 2023; Lopresti et al., 2023). Moreover, VTF has been reported to exhibit neuroprotective effects by preserving mitochondrial function and regulating autophagy, thereby promoting cell survival and maintaining cellular homeostasis. These properties make VTF an attractive candidate for investigating their potential therapeutic benefits in neurodegenerative diseases, such as AD. Recent studies have unveiled the multifaceted properties of flavonoids, indicating not only their neuroprotective functions but also their anti-inflammatory effects. These compounds, known for their ability to smoothly traverse the blood-brain barrier (BBB), have demonstrated promising outcomes in enhancing learning and memory among mice with cognitive impairments (Kim et al., 2009; Prakash & Sudhandiran, 2015; Spencer, Vafeiadou, Williams, & Vauzour, 2012). Onozuka et al. reported that Nobiletin, a citrus flavonoid, can ameliorate memory impairment and reduce Aβ pathology in a transgenic mouse model of AD. Notably, this effect is attributed to its role in decreasing amyloid beta production through the mediation of presenilin-1 phosphorylation (Abdul Manap et al., 2020; Rezai-Zadeh et al., 2009). Furthermore, our choice of VTF is supported by a growing body of literature highlighting their efficacy in attenuating

neurodegeneration and cognitive decline in preclinical models of AD disease. Therefore, by focusing on VTF in our study, we aimed to contribute to the expanding knowledge of their neuroprotective mechanisms and therapeutic potential in AD and related disorders. Despite existing studies showcasing VTF's ability to promote synaptic plasticity and indirectly influence the expression of cholinergic neurotransmitters, the precise mechanisms, particularly from the perspective of autophagy, have remained elusive (Kim et al., 2009; Prakash & Sudhandiran, 2015; Spencer et al., 2012). Our *in vitro* findings aim to underscore the profound anti-neurodegenerative effects of VTF. Significantly, in comparison with CQ, a prototypical lysosome inhibitor, we underscore the distinctive role of VTF in modulating mitophagy. In the context of AD pathogenesis, the aggregation of Aβ resulting from abnormal APP hydrolysis by β and γ secretases is considered a valuable and prognostic biomarker (Xiao, Ma, Li, Wu, & Yuan, 2017).

The selection of CQ as a lysosome inhibitor in our study was based on its well-documented pharmacological properties and established role in modulating autophagy. CQ acts by disrupting lysosomal acidification and impairing autophagic flux, leading to the accumulation of autophagosomes and the inhibition of protein degradation within lysosomes (Fedele & Proud, 2020; Ke, 2024; Redmann et al., 2017). In AD disease, impaired autophagy and dysfunctional lysosomal degradation contribute to the accumulation of protein aggregates, including amyloid-beta and tau, which are hallmark features of the disease pathology. By inhibiting lysosomal function, chloroquine exacerbates autophagy dysfunction and accelerates disease progression (Varma et al., 2023). Furthermore, CQ has been widely used as a pharmacological tool in preclinical studies to investigate the role of autophagy in neurodegenerative diseases and explore potential therapeutic interventions (Halcrow, Geiger, & Chen, 2021; Rainsford, Parke, Clifford-Rashotte, & Kean, 2015). Its well-characterized mechanism of action and established safety profile make it a valuable tool for dissecting autophagy-related pathways and identifying novel therapeutic targets (Pedrioli, Patani, & Paganetti, 2020; Rainsford et al., 2015). The inclusion of CQ in our study provides a unique opportunity to investigate its synergistic effects with VTF in modulating autophagy and neuroprotection in AD disease models (Caporaso, Gandy, Buxbaum, & Greengard, 1992). By combining CQ's lysosome-inhibiting properties with the antioxidant and neuroprotective effects of VTF, we aim to elucidate the mechanisms underlying their therapeutic potential and advance our understanding of AD pathogenesis and treatment (Furst, 1996; Rainsford et al., 2015).

Our study shows the potential therapeutic effects of VTF against neurodegeneration, focusing specifically on AD. We found that VTF exhibited promising anti-neurodegenerative properties by modulating mitophagy and suppressing the autophagy–lysosomal pathway. One key finding of our study is the modulation of these findings highlights the potential of VTF as a therapeutic intervention for AD. However, further research is needed to understand the precise mechanisms involved and to assess the safety and efficacy of VTF in clinical settings. Nonetheless, our study provides valuable insights into the neuroprotective effects of VTF, offering hope for the development of novel treatments for neurodegenerative diseases mitophagy by VTF, which plays a crucial role in maintaining mitochondrial homeostasis and cellular health (Moldovan et al., 2020). Mitophagy, the selective degradation of damaged mitochondria, is impaired in AD and other neurodegenerative disorders, leading to mitochondrial dysfunction and oxidative stress. By promoting mitophagy, VTF may facilitate the removal of dysfunctional mitochondria, thereby mitigating oxidative stress and apoptotic signaling pathways implicated in neurodegeneration (Balea Ş et al., 2020). Additionally, VTF suppressed the autophagy–lysosomal pathway, which is dysregulated in AD pathology. By downregulating key autophagy-related proteins like Beclin-1, Atg7, BACE1, LC3-II, and LC3-II/LC3-I, VTF may reduce the accumulation of neurotoxic protein

aggregates associated with AD (Chifenti et al., 2013; He, Peng, Yuan, Xu, & Wei, 2015). However, the molecular mechanisms underlying this modulation, both *in vitro* and *in vivo*, remain elusive. It is particularly unclear whether VTF can prevent hippocampal neuron damage by inhibiting autophagy and Aβ clustering. Here, we observed that VTF preserves Aβ1-42-induced alterations in mitochondrial and autophagosomal structures. Our investigation further delves into the intricate molecular pathways, highlighting VTF's role in modulating mitophagy through the autophagy–lysosomal pathway, involving key players such as Beclin-1, p62, Atg7, and BACE1. Specifically, we emphasize the impact on BACE1, a crucial orchestrator in mitochondrial and autophagosomal processes, demonstrating its elevated levels in the vehicle control group and subsequent attenuation upon VTF treatment (Lee, Ahn, Choi, Kwon, & Yang, 2021; Liu et al., 2023). This dynamic modulation suggests that VTF has the potential to mitigate Aβ production and, consequently, alleviate neurodegeneration. Within the autophagy cascade, Atg7 and Beclin-1 emerge as a pivotal regulator, representing a focal point for understanding VTF's influence on autophagy levels (Caballero & Coto-Montes, 2012; Fîlfan et al., 2017). Our results reveal a significant reduction in Atg7 expression in Aβ1-42-induced SH-SY5Y cells, indicating heightened autophagy. Notably, the subsequent decrease in Atg7 levels following VTF treatment signifies a potential mechanistic facet through which VTF maintains autophagy homeostasis, strengthening its neuroprotective role (Q. Li, Chen, Liu, Yang, & Yang, 2012). Intriguingly, our study unveils a noteworthy increase in p62 expression with VTF treatment. This elevation aligns with our hypothesis that VTF inhibits excessive autophagy, thereby preserving nerve cell function (Caccamo, Ferreira, Branca, & Oddo, 2017). The observed inverse correlation between autophagy and p62 underscores the delicately orchestrated balance potentially sustained by VTF. These findings contribute valuable insights into the molecular underpinnings of VTF's neuroprotective effects in the context of AD, shedding light on its potential therapeutic significance.

Excessive autophagy is a notable concern in Aβ1-42-induced SH-SY5Y cells and AD, emphasizing the crucial need for effective therapeutic interventions. LC3, specifically the LC3-II/LC3-I ratios, emerges as a reliable indicator of autophagy levels (Chai et al., 2022; Heckmann et al., 2019; Pradeepkiran & Reddy, 2020). Our study underscores the significance of this crucial marker, revealing a discernible reduction in LC3-II/LC3-I ratios upon VTF treatment. This substantial attenuation of excessive autophagy further enhances the neuroprotective potential of VTF in the intricate landscape of AD. The nuanced regulation of autophagy levels by VTF not only illuminates the molecular intricacies involved but also unveils promising avenues for therapeutic strategies targeting cellular homeostasis in neurodegenerative conditions. The upregulation of p62 seamlessly aligns with our hypothesis that VTF inhibits excessive autophagy, providing additional evidence of its potential to preserve nerve cell function. This delicate balance between autophagy markers, LC3, and p62, highlights the multifaceted impact of VTF on cellular homeostasis. It not only enriches our understanding of the molecular intricacies involved in neurodegenerative conditions but also lays the groundwork for targeted therapeutic strategies aimed at restoring the autophagic equilibrium (Liu & Li, 2019; Ułamek-Kozioł et al., 2013). In Fig. 4, we illustrate how VTF preserves mitophagy in Aβ1-42-induced AD neurodegeneration. Our findings highlight the significant neuroprotective effect of VTF and chloroquine against Aβ-induced damage. VTF treatment maintains cellular viability and preserves mitochondrial and autophagosomal integrity. Additionally, our study reveals the role of VTF and chloroquine in modulating key proteins in the autophagy–lysosomal pathway. These findings warrant further investigation into VTF's therapeutic potential for AD.

Figure 4.

These findings highlight the potential of VTF as a therapeutic intervention for AD. However, further research is needed to understand the precise mechanisms involved and to assess the safety and efficacy of VTF in clinical settings. Nonetheless, our study provides valuable insights into the neuroprotective effects of VTF, offering hope for the development of novel treatments for neurodegenerative diseases (Balea Ş et al., 2020; Chifenti et al., 2013; He et al., 2015).

Tau, a microtubule-associated protein, plays a crucial role in stabilizing microtubules and maintaining neuronal integrity. However, in AD, tau undergoes pathological changes, including hyperphosphorylation at key sites such as Ser202, Thr205, Ser396, and Ser404 (Rawat et al., 2022). This hyperphosphorylation leads to the formation of paired helical filaments and neurofibrillary tangles (NFTs) (Brion et al., 2001; Ferrer et al., 2005). These tau aggregates disrupt microtubule stability, impair intracellular transport, and interfere with synaptic function, significantly contributing to the cognitive decline observed in AD. The accumulation of NFTs is a hallmark feature of tau pathology and a central focus of therapeutic strategies aimed at treating AD. VTF, as a potential therapeutic agent, presents an intriguing approach to mitigating tau-related neurodegeneration. Preliminary studies suggest that VTF may influence tau phosphorylation pathways, particularly by modulating tau kinases such as GSK3β and CDK5, which are directly involved in tau hyperphosphorylation. By regulating these kinases, VTF could reduce tau hyperphosphorylation and prevent the formation of toxic tau aggregates (Dai et al., 2012). Additionally, VTF may activate cellular mechanisms responsible for tau clearance, particularly autophagy. Autophagy is a vital process for clearing misfolded proteins, including tau aggregates, thereby preventing their accumulation and minimizing subsequent neuronal damage. VTF may enhance autophagic flux, promoting the degradation of tau and helping restore cellular homeostasis (Zhang, Maimaiti, Aili, Yuan, & Xiao, 2022).

Moreover, tau pathology is closely associated with neuroinflammation, where the activation of microglia and the release of pro-inflammatory cytokines exacerbate neuronal injury. VTF's potential to modulate neuroinflammation could provide an added benefit in reducing tau-related neurodegeneration by dampening microglial activation and inflammatory responses. Our ongoing and future studies will focus on investigating the precise mechanisms by which VTF influences tau aggregation, degradation, and neuroinflammation (Dai et al., 2012). Gaining a deeper understanding of these processes will provide essential insights into VTF's therapeutic potential and support the development of a novel strategy to mitigate tau-mediated neurodegeneration in AD.

Our study highlights the neuroprotective potential of VTF but acknowledges the limitations of our primarily *in vitro* approach. To better understand VTF's mechanisms and therapeutic implications, we recognize the need for more extensive cellular and molecular *in vitro* investigations. These studies should focus on specific aspects, such as how flavonoids modulate autophagy and interact with key molecular pathways involved in neurodegeneration. Furthermore, structural elucidation of VTF's effects at the cellular level, combined with comprehensive molecular analyses, will offer valuable insights into their therapeutic efficacy. Additionally, while alternative models, such as Aβ-induced mitochondrial dysfunction, could provide complementary evidence, resource constraints and the scope of our study prevented us from exploring them in this research endeavor. Also, our study did not specifically assess alterations in autophagy flow, and we did not include a control experiment to evaluate whether the observed positive effects of VTF could be reversed with the introduction of autophagy inhibitors. Future research endeavors will aim to address these

limitations by incorporating more comprehensive *in vivo* studies, including animal models, and conducting controlled experiments to unravel the intricate dynamics of autophagy modulation by VTF. Furthermore, the complex interplay between various autophagic markers, the specific mechanisms of flavonoids, and the multifaceted nature of AD pathology warrant further detailed exploration in subsequent investigations to enhance our understanding and pave the way for potential therapeutic applications (Funderburk, Marcellino, & Yue, 2010; Nixon, 2007). Exploring additional markers and signaling pathways associated with autophagy modulation by VTF could provide a more comprehensive understanding of its therapeutic potential.

In our previous study, we examined the dose-dependent effects of VTF on autophagy *in vivo* (Zhang et al., 2022). However, we plan to expand these investigations by incorporating additional animal models and cell lines to further explore the interactions between VTF and tau, as well as its broader therapeutic potential in AD. This will help refine our understanding of VTF's dose-dependent effects and potential synergistic interactions with other treatments.

Moreover, extending these investigations to *in vivo* models and clinical studies will bridge the translational gap, offering a more comprehensive assessment of VTF's efficacy in a complex physiological context. Future research will involve advanced molecular assays, including genomic and proteomic analyses, to assess the effects of VTF on cellular and molecular pathology, with a particular focus on tau phosphorylation, aggregation, and clearance mechanisms. Additionally, we plan to employ multi-omics approaches, such as transcriptomics and metabolomics, to gain deeper insights into the systemic impacts of VTF and its potential to modulate key signaling pathways in neurodegeneration. By addressing these key areas, our future studies aim to provide a more detailed and holistic view of VTF's therapeutic potential in AD.

The delineation of VTF's influence on synaptic plasticity and cholinergic neurotransmitters, coupled with its role in autophagy regulation, opens avenues for targeted interventions that address multiple facets of AD pathology. This work sets the stage for continued research aimed at harnessing the full potential of VTF as a promising candidate for neuroprotective strategies in the context of neurodegenerative disorders. By addressing these limitations and discussing avenues for further investigation, we aim to provide a transparent interpretation of our results and contribute to advancing our understanding of neuroprotective compounds for the treatment of neurodegenerative diseases.

5. Conclusion

In conclusion, our study unveils VTF's novel role, alongside CQ, in preserving cellular health under Aβ1-42-induced stress, emphasizing its anti-neurodegenerative effects through mitophagy modulation. While our findings open avenues for further *in vivo* exploration, the study underscores VTF's potential in targeting multiple facets of AD pathology, presenting a promising candidate for future neuroprotective interventions.

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This study was performed at the College of Public Health, Xinjiang Medical University, China.
Authors' contributions

Author Contributions

PZ and HX designed the research study. PZ and HX performed the experiments. PZ analyzed the data. HX drafted the manuscript. All authors contributed to editorial changes in the manuscript. All

authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

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The authors declare no conflict of interest.

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Supplementary material associated with this article can be found in the online version.

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Figures

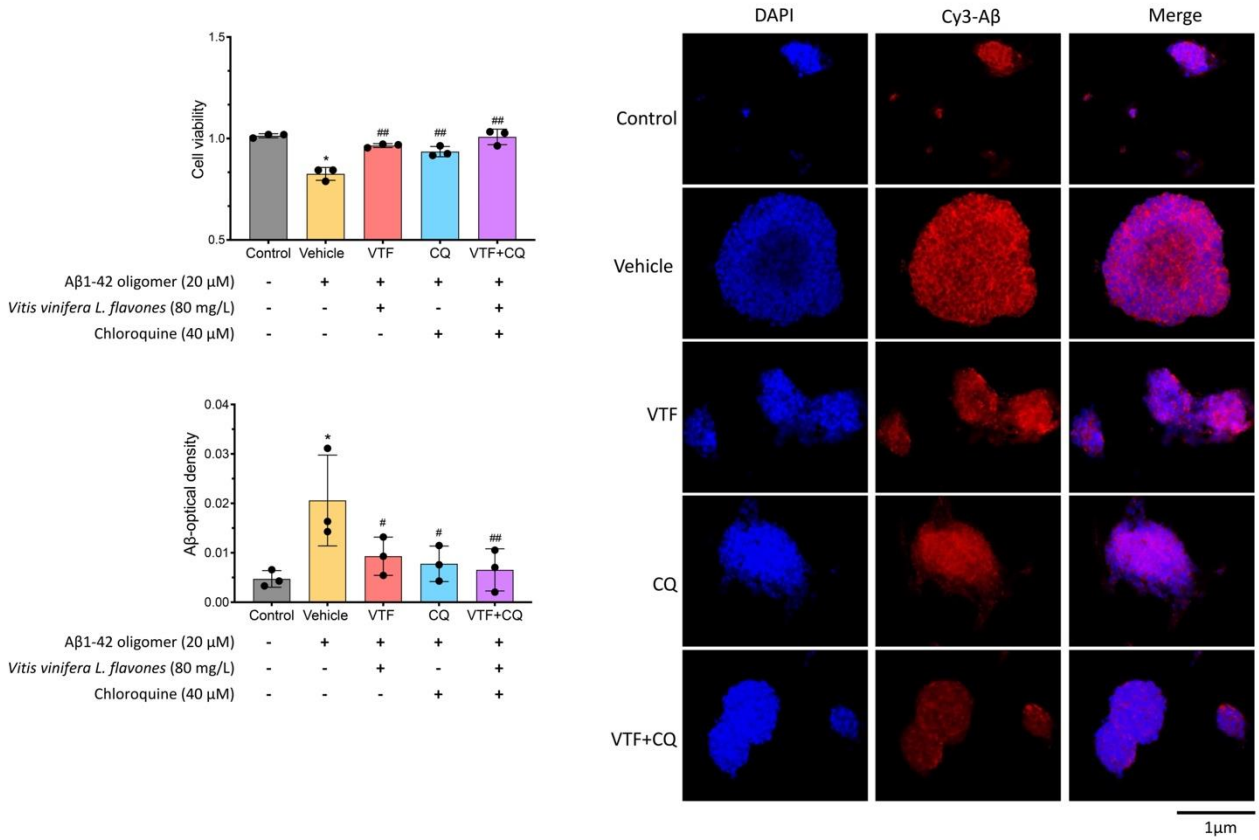

Figure 1. Effect of VTF on Aβ1-42-induced SH-SY5Y cell proliferation. (A) The bar chart presents quantitative results from the CCK-8 assay, illustrating the impact of VTF on the proliferation of SH-SY5Y cells induced by Aβ1-42. (**B).** IFC results show the average Aβ-optical density in all study groups. The vehicle control group exhibits a significant increase in Aβ optical density compared to the control group. The VTF+CQ group displays the most substantial reduction in Aβ optical density, with the CQ+VTF group showing a significant decrease. **C.** Representative IFC images of Aβ1-42 in SH-SY5Y cells under 20X magnification in different study groups. The stains 4' and 6-diamidino-2-phenylindole (DAPI) were used to stain the nuclei (blue), and Cy3 was used to stain the amyloid beta peptides (red). Scale bar: 50 μm. Control cells were treated with PBS, vehicle control cells with VTF (20 μM Aβ1-42 oligomer), CQ cells with 40 μM CQ + 20 μM Aβ1-42 oligomer, and VTF+CQ cells with 80 mg/L VTF + 40 μM CQ + 20 μM Aβ1-42 oligomer. Each group consisted of 5×10^4 SH-SY5Y cells/well. Statistical analysis indicates significant differences: * $p < 0.05$ vs. control, and # $p < 0.05$ and ## $p < 0.01$ vs. vehicle control group.

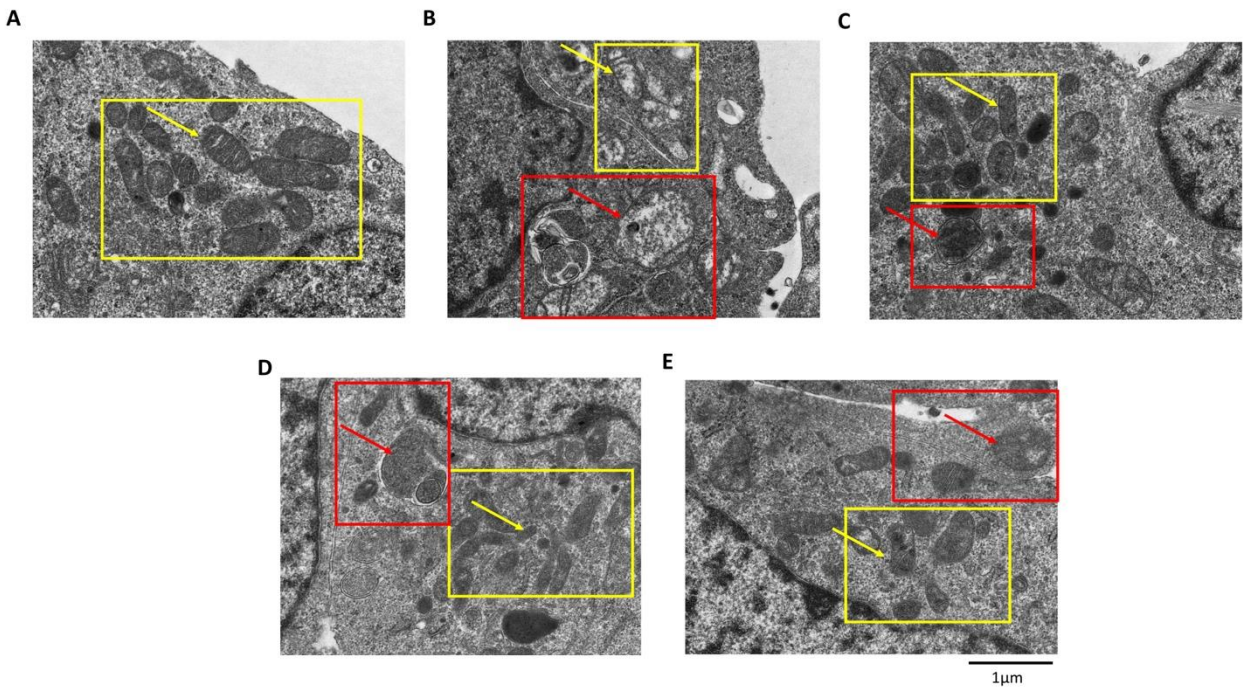

Figure 2. Ultrastructural changes in mitochondria and autophagosomes across treatment groups. The representative TEM images in the control group (**A**), vehicle control Group (**B**), VTF Group (**C**), CQ Group (**D**), and VTF+CQ Group (**E**) reveal well-defined mitochondria with orderly arranged cristae and minimal autophagosomes in the control group. However, Aβ1-42 induction results in disrupted mitochondrial morphology, increased autophagosomes, and cellular stress. In contrast, drug treatment groups (VTF, CQ, and VTF+CQ) exhibit significant improvements in mitochondrial characteristics, emphasizing their protective roles against Aβ1-42-induced changes. Control cells were treated with PBS, vehicle control cells with VTF (20 μM Aβ1-42 oligomer), CQ cells with 40 μM CQ + 20 μM Aβ1-42 oligomer, and VTF+CQ cells with 80 mg/L VTF + 40 μM CQ + 20 μM Aβ1-42 oligomer. The yellow boxes represent the mitochondria zone, while the red boxes represent autophagosomes. The arrows highlight fibril-like aggregates cells. Magnification of 8000X with a 1μm scale bar.

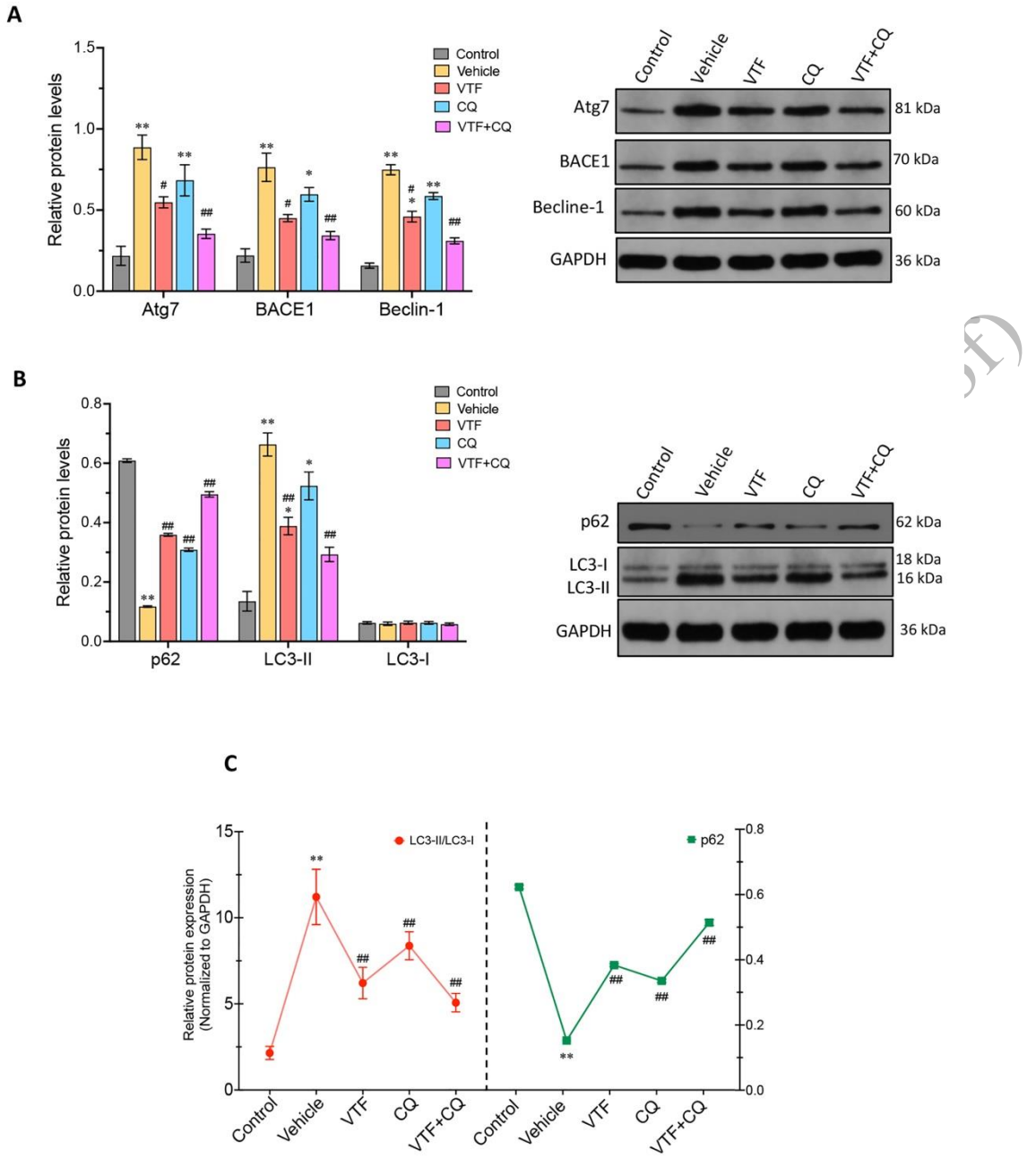

Figure 3. Modulation of mitophagy by VTF in Aβ1-42-induced SH-SY5Ycells. (A). Evaluation of the protein expression levels of key mitophagy modulators (Beclin-1, Atg7, and BACE1) in SH-SY5Y cells treated with Aβ1-42 and subjected to VTF and CQ interventions. The vehicle control group exhibited elevated levels compared to treatment groups, while VTF and CQ treatments reduced the expression of these proteins. (**B).** Assessment of LC3-II and LC3-I ratios along with p62 protein expression in different experimental groups. The vehicle control group displayed excessive autophagy, indicated by increased LC3-II levels, while VTF and CQ treatments reduced these levels. P62 protein expression increased with VTF treatment, suggesting lower autophagy levels in drug-treated groups compared to the vehicle control group. (**C).** Comparison of LC3-II/LC3-I ratios and p62 expression levels among experimental groups. The VTF group, CQ group, and CQ+VTF group exhibited decreased LC3-II/LC3-I ratios, indicating reduced autophagy, while p62 levels increased, especially in the CQ+VTF-treated groups. These findings collectively suggest that VTF modulates mitophagy through the autophagy–lysosomal pathway via the p62 downstream signaling pathway, emphasizing its potential therapeutic role in mitigating Aβ1-42-induced neurodegenerative processes in SH-SY5Y cells. All protein expressions in Western blot were

normalized with GAPDH. The full-length gels are included in Supplementary Figure 1. Control cells were treated with PBS, vehicle control cells with VTF (20 μM Aβ1-42 oligomer), CQ cells with 40 μM CQ + 20 μM Aβ1-42 oligomer, and VTF+CQ cells with 80 mg/L VTF + 40 μM CQ + 20 μM Aβ1-42 oligomer. Each group consisted of 5×10^4 SH-SY5Y cells/well. Statistical analysis indicates significant differences: * $p < 0.05$ and ** $p < 0.01$ vs. control, and # $p < 0.05$ and ## $p < 0.01$ vs. the vehicle control group.

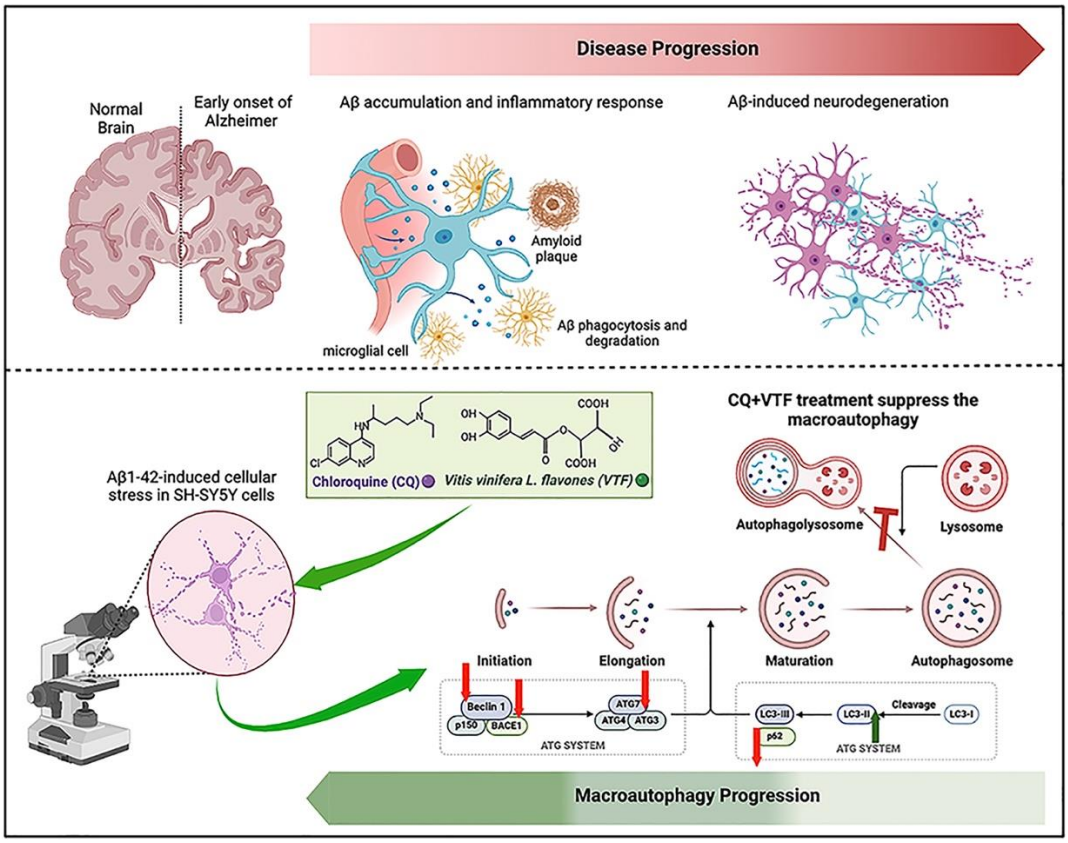

Figure 4. Schematic representation of the neuroprotective effects of VTF in Aβ1-42-induced AD neurodegeneration. This figure illustrates how VTF preserves mitophagy and cellular viability while maintaining the structural integrity of mitochondria and autophagosomes. Furthermore, the role of VTF and chloroquine in modulating proteins within the autophagy–lysosomal pathway is depicted. These findings suggest a potential therapeutic utility of VTF in addressing AD pathology.