

Scutellaria baicalensis-derived extracellular vesicles ameliorate Alzheimer's disease through central mitochondrial repair and peripheral gut-brain axis modulation

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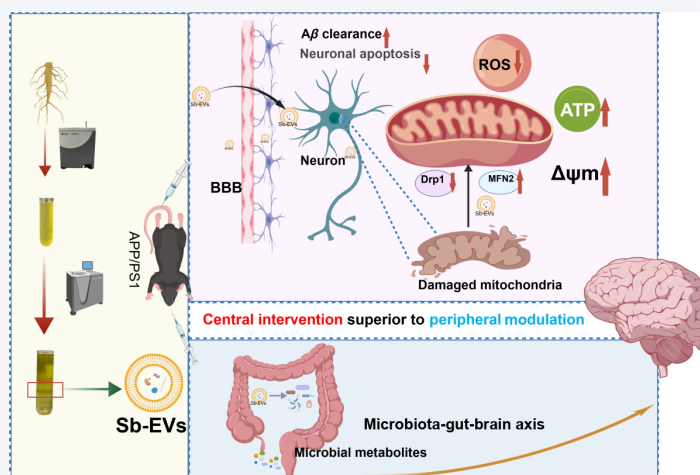
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ABSTRACT: Alzheimer's disease (AD) is a neurodegenerative disorder with increasing prevalence and lack of effective treatments highlighting the urgent need to develop novel therapeutic strategies. *Scutellaria baicalensis*, a traditional Chinese medicinal herb, exhibits anti-inflammatory, antioxidant, and neuroprotective effects against AD. However, the therapeutic potential of its extracellular vesicles (Sb-EVs) remains unexplored. In this study, the effects and underlying mechanisms of Sb-EVs on AD via different administration routes are systematically investigated. The intravenous (i.v.) injection of Sb-EVs effectively crosses the blood-brain barrier (BBB), significantly inhibiting neuronal apoptosis by clearing amyloid-beta ($A\beta$)—induced intracellular reactive oxygen species (ROS) and repairing mitochondrial damage. In contrast, orally administered Sb-EVs is limited by lower brain-targeting efficiency. It improves AD phenotypes via the gut-brain axis by remodeling gut microbiota and modulating metabolic levels. The i.v.-injected Sb-EVs exhibited superior efficacy to that of oral administration by improving cognitive function and attenuating $A\beta$ deposition. Collectively, the potential of Sb-EVs as high-safety, high-efficacy natural nanomedicines for alleviating AD was elucidated.

KEYWORDS: *Scutellaria baicalensis*-derived extracellular vesicles, Alzheimer's disease, blood-brain barrier, mitochondrial dysfunction, gut-brain axis



1 Introduction

The increasing prevalence of Alzheimer's disease (AD) poses a substantial socioeconomic burden. The global population with AD is over 55 million and projected to triple by 2050 [1]. The prevalence of dementia among individuals aged ≥ 65 years in China

is 6.2%; however, only 21% of patients receive a timely diagnosis [2]. Current therapeutic options for AD, including N-methyl-D-aspartate receptor (NMDAR) antagonists and anti-amyloid monoclonal antibodies, are limited by safety concerns and prohibitive costs. Despite over 200 clinical trials conducted as of 2025, the success rate for disease-modifying therapies remains $< 2\%$ [3–5]. Therefore, alternative AD therapies with improved efficacy, safety, and accessibility are urgently required.

AD is a progressive neurodegenerative disorder characterized by the abnormal deposition of amyloid-beta ($A\beta$) [6]. Internalized $A\beta$ targets the outer mitochondrial membrane, impairing oxidative phosphorylation, inducing reactive oxygen species (ROS) overload,

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and triggering the opening of the mitochondrial permeability transition pore. This cascade ultimately leads to cytochrome *c* release and caspase-mediated neuronal apoptosis [7, 8]. Therefore, ameliorating neuronal mitochondrial dysfunction may be a viable therapeutic strategy. For instance, triphenyl phosphonium-modified Cu_{2-x}Se nanozymes effectively mitigate AD pathology by restoring mitochondrial function in neurons [9]. However, effectively crossing the blood-brain barrier (BBB) remains a formidable challenge for targeted mitochondrial interventions in the brain. Additionally, oral therapeutic strategies targeting the gut-brain axis are a promising alternative for AD treatment [10]. Patients with AD exhibit decreased α -diversity of the intestinal microbiota and an increased abundance of pro-inflammatory bacteria [11, 12]. This dysbiosis elevates intestinal barrier permeability, promoting the systemic leakage of endotoxins (such as lipopolysaccharide (LPS)), pro-inflammatory cytokines, and prostaglandins. These factors directly exacerbate neuroinflammation and accelerate AD progression [13–15]. Notably, oral interventions, such as Ganmai Dazao Decoction, have significantly ameliorated cognitive impairment in AD rat models by restoring gut microbiota diversity and reversing microbial metabolite alterations [16]. Therefore, targeting gut microbiota restoration may be a promising and accessible therapeutic strategy for AD intervention.

The classic heat-clearing herb, *Scutellaria baicalensis*, may be a therapeutic candidate for AD because of its antioxidant, anti-inflammatory, and multi-target neuroprotective effects [17, 18]. Baicalin and baicalein effectively ameliorate AD pathology by mitigating neuroinflammation and oxidative stress, boosting mitochondrial activity, and preventing pathological P-Tau accumulation [18–20]. However, similar to numerous natural compounds, these flavonoids are limited by the inability to cross the BBB. As a highly compact, dynamic, and adaptive interface, the BBB considerably restricts their penetration, leading to suboptimal bioavailability within the brain [21]. Plant-derived extracellular vesicles (PEVs) are plant cell-secreted nanovesicles with diameters from 30 to 150 nm. Characterized by a lipid bilayer structure, PEVs harbor diverse bioactive components, such as proteins, lipids, functional nucleic acids (mainly microRNAs, mRNAs, and small RNAs), and small-molecule compounds [22]. As naturally occurring nanovesicles, PEVs exhibit intrinsic targeting properties without requiring prior modification. For example, ginseng-derived extracellular vesicles can cross the BBB and deliver ptc-miR396f directly to the tumor, thereby inhibiting glioma progression [23]. Similarly, Pueraria-derived extracellular vesicles serve as a homogeneous nanopatform for brain-targeted biomacromolecule delivery to ameliorate Parkinson's disease [24]. However, the ability of *S. baicalensis*-derived extracellular vesicles (Sb-EVs) to penetrate the BBB and their therapeutic potential for AD have not yet been systematically investigated.

In this study, Sb-EVs were extracted using differential centrifugation, followed by a comprehensive characterization of their physicochemical features and active ingredients. Subsequently, we investigated their biodistribution and BBB penetration capabilities via distinct administration routes. Furthermore, an *in vitro* BBB model was constructed using bEnd.3 cells to verify whether the Sb-EVs could cross the BBB. Following this, using APP/PS1 mice, we compared the effects of oral gavage and tail vein injection on cognitive behaviors and neuropathological alterations. Finally, to investigate the underlying mechanism of intravenously

injected Sb-EVs, their effects on A β -induced oxidative stress, mitochondrial dysfunction, and apoptosis in HT22 cells were examined. Integrated analysis of gut microbiota and metabolites further provided mechanistic insights into how oral Sb-EVs alleviate AD.

2 Experimental

2.1 Chemicals and reagents

Fresh *S. baicalensis* was purchased from Jingmeiyingrong Trading Co., Ltd. (Shandong, China). Uranyl acetate was obtained from Henan Ruixin Experimental Supplies Co., Ltd. (Henan, China). Fetal bovine serum (FBS) was sourced from Yeasen Biotechnology (Shanghai, China) Co., Ltd. High-glucose Dulbecco's modified Eagle medium (DMEM) was obtained from Thermo Fisher Scientific (MA, USA). Trypsin and Penicillin-Streptomycin were obtained from Meilunbio (Dalian, China). A radioimmunoprecipitation assay (RIPA) lysis buffer, MTT assay kit, JC-1, ATP assay kit, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), and 4',6-diamidino-2-phenylindole (DAPI) were from Beyotime Biotechnology (Shanghai, China). The Annexin V-APC/7-AAD apoptosis detection kit was obtained from Hangzhou Lianke Biotechnology Co., Ltd. (Hangzhou, China). An A β _{1–42} peptide was purchased from Hangzhou Lianke Biotechnology Co., Ltd. (Hangzhou, China). Primary antibodies against Drp1, mitofusin 2 (MFN2), Bcl-2, Bax, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were provided by Proteintech Group (Wuhan, China).

2.2 Extraction of Sb-EVs

S. baicalensis roots were ground in phosphate-buffered saline (PBS) and soaked overnight at 4 °C. The mixture was filtered and sequentially centrifuged at 2000g for 20 min, followed by 10,000g for 60 min at 4 °C to eliminate impurities. To obtain crude Sb-EVs, the supernatant was subjected to ultracentrifugation at 130,000g for 2 h at 4 °C (Optima XE-100, Beckman Coulter). Crude Sb-EVs were dispersed and isolated via a sucrose gradient (8%, 30%, 45%, and 60%) at 130,000g for 2 h. The band located at the 30%–45% interface was harvested, pelleted using ultracentrifugation (130,000g, 2 h), and washed thrice with PBS to obtain Sb-EVs.

2.3 Characterization of Sb-EVs

Nanoparticle tracking analysis (NTA) was used to determine the size distribution of Sb-EVs, and the zeta potential and polydispersity index (PDI) were measured using a Malvern Zeta sizer (Zetasizer Nano ZS, Malvern Panalytical). Morphological characteristics were examined using transmission electron microscopy (TEM, HT7700, Hitachi). The RIPA lysis buffer was used to extract total proteins from Sb-EVs, followed by the addition of the loading buffer and incubation in boiling water for 5 min. Electrophoretic fractionation of protein samples was performed using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) at a fixed potential of 120 V for 1 h, followed by visualization with Coomassie Brilliant Blue R-250 staining. Total RNA was isolated utilizing Trizol reagent and then analyzed using 2% agarose gel electrophoresis. Agarose dissolved in 1× Tris-acetate-EDTA (TAE) buffer was mixed with nucleic acid stain, cast into gels, and loaded with samples and DNA markers. The gels were run at a constant potential of 120 V for 40 min,

followed by band detection using a gel documentation system (Gel Doc XR+, Bio-Rad).

2.4 Stability of Sb-EVs

The stability of Sb-EVs in simulated gastrointestinal fluids was evaluated as previously described by Chen et al. and Gao et al. [25, 26]. Sb-EVs were incubated with simulated gastric fluid at a 1:1 volume ratio in a 37 °C water bath with gentle stirring. Samples were collected at 30-min intervals to monitor changes in morphology and particle size. After 2 h of incubation, the pH of the mixture was adjusted to approximately 6.8. Subsequently, the mixture was combined with simulated intestinal fluid containing 0.1% porcine bile salts at a 1:1 ratio and incubated at 37 °C with gentle stirring. Samples were again withdrawn every 30 min for morphological and size analysis. Additionally, the stability of Sb-EVs in blood was assessed as previously described by Ming et al. [27]. Sb-EVs were mixed with FBS and incubated in a 37 °C water bath with gentle stirring. Samples were collected at 3, 6, 12, 24, and 48 h to monitor any changes in morphology and particle size.

2.5 *In vivo* biodistribution of Sb-EVs in mice

Male C57BL/6 mice (7 to 8 weeks) were randomly assigned into four groups: oral gavage control (i.g.-Free 1,1'-dioctadecyl-3,3',3''-tetramethylindotricarbocyanine iodide (DIR)), intravenous injection control (i.v.-Free DIR), oral gavage experimental DIR-labeled Sb-EVs (DIR@Sb-EVs), and intravenous injection experimental DIR@Sb-EVs. The mice were administered DIR@Sb-EVs or free DIR via oral gavage or tail vein injection, respectively. At designated intervals (3, 6, 12, 24, and 48 h) after administration, the mice were sacrificed. The intestine, heart, liver, spleen, lung, kidney, and brain were harvested. Fluorescence imaging was captured using a multimodal imaging system (Fusion FX6, Vilber). And fluorescence intensity in brain and intestinal tissues was quantified using Kuant software (Kuant 2.4, Vilber Bio Imaging).

Separately, Sb-EVs were tagged with PKH26 fluorescent dye (PKH26@Sb-EVs), with free PKH26 in PBS serving as the control. Animal grouping and administration routes were performed as described above. Mice were sacrificed 24 h post-administration. Brain tissues were harvested, following immersion fixation in 4% paraformaldehyde (PFA), tissues were processed for paraffin infiltration and subsequent microtomy. The resulting slices were subjected to double-immunofluorescence labeling for NeuN, and images were acquired using confocal laser scanning microscopy (CLSM, STELLARIS 5, Leica Microsystems).

2.6 Cell culture and MTT assay

HT22 and bEnd.3 were procured from American Type Culture Collection (ATCC). Both cell types were cultured in DMEM containing with 10% FBS and kept at 37 °C in a humidified atmosphere with 5% CO₂. To evaluate cell viability, following inoculation into 96-well plates, HT22 and bEnd.3 cells underwent a 12-h incubation until reaching 80% confluency. To test cell viability, they were challenged with different doses of Sb-EVs (0, 0.125 × 10¹¹, 0.25 × 10¹¹, 0.5 × 10¹¹, 1 × 10¹¹, 2 × 10¹¹, 4 × 10¹¹, 8 × 10¹¹, and 16 × 10¹¹ particles/mL) for 24 h. Subsequently, each well received 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution. After a 4-h reaction period, the resulting formazan products were solubilized with 120 μL of dimethyl sulfoxide (DMSO). The plates were incubated on an orbital shaker for 15 min, and the absorbance was measured at 492 nm.

2.7 *In vitro* cellular uptake of Sb-EVs

HT22 and bEnd.3 cells were plated into glass-bottom confocal capsules and maintained for 12 h to achieve approximately 80% confluency. The cells were then incubated with the PKH26-labeled Sb-EVs for 8 h. After washing the cells with PBS, they were fixed with 4% PFA and stained with FITC-phalloidin and DAPI, respectively. Finally, the cellular uptake of Sb-EVs was observed using CLSM.

2.8 Lysosomal escape assay in bEnd.3 cells

bEnd.3 cells were grown in confocal dishes to approximately 80% confluency. Subsequently, the cells were treated with PKH26-labeled Sb-EVs for 8 h. Following the incubation period, the supernatant was aspirated, and the cell monolayer was washed with PBS. Lysosomal and nuclear staining were conducted using LysoTracker Green and DAPI, respectively. Finally, the co-localization of Sb-EVs with lysosomes was visualized using CLSM.

2.9 *In vitro* BBB penetration and HT22 cell uptake

To evaluate the *in vitro* the BBB permeability of Sb-EVs, a BBB model was established as previously described by Wei et al. [28]. The bEnd.3 cells were cultured in the apical chambers of six-well Transwell inserts (Cat. No. 3450-N1, Corning). The growth medium was changed every 48 h, and the trans-endothelial electrical resistance (TEER) was monitored. A TEER value > 200 Ω·cm² indicated the successful establishment of the BBB model. Subsequently, PKH26-labeled Sb-EVs were added to the apical chamber, with free PKH26 as the control. The fluorescence intensity in the basolateral chamber was determined using a multi-mode microplate reader to calculate the BBB permeability rate of Sb-EVs.

A BBB and HT22 cell co-culture system was constructed as previously described by Wei et al. [29]. Particularly, HT22 cells were cultured in the basolateral chamber (lower compartment) beneath the established bEnd.3 monolayer. PKH26-labeled Sb-EVs were added to the upper chamber (upper insert) and co-incubated for 8 h. Following incubation, the incubation medium was aspirated, and the cells were fixed using 4% PFA. The nuclei and cytoskeleton of both the HT22 cells and the bEnd.3 cells were stained with DAPI and FITC-phalloidin, respectively. The penetration of Sb-EVs across the BBB and their subsequent uptake by HT22 cells were visualized using CLSM.

2.10 Evaluation of Sb-EVs effects on Aβ-induced oxidative stress, mitochondrial dysfunction, and apoptosis

Following inoculation into six-well plates, HT22 cells were maintained until they reached 80% confluency. At this stage, the cells were separated into six treatment groups: Control, Model (Aβ-induced), positive control (10 μM Donepezil (DNPZ)), low-dose Sb-EVs (0.5 × 10¹¹ particles/mL), medium-dose Sb-EVs (1.0 × 10¹¹ particles/mL), and high-dose Sb-EVs (2.0 × 10¹¹ particles/mL). Except the Control group, all groups were also treated with 2.5 μM Aβ.

Each experimental condition for HT22 cells was performed in three replicates and maintained for 24 h. Subsequently, the spent supernatant was withdrawn, and the cultures were cleansed using PBS. To evaluate ROS levels, the cells were treated with DCFH-DA and incubated for 30 min at 37 °C, in the dark. The intracellular ROS content was subsequently determined using flow cytometry.

(FACS Celesta, BD Biosciences) and visualized using a CLSM. The mean fluorescence intensity of the CLSM images was quantified using ImageJ software.

The cell culture supernatant (120 μ L) was collected and transferred to a fresh 96-well plate. The lactate dehydrogenase (LDH) working solution was prepared in accordance with the manufacturer's protocols. Each well was supplemented with 60 μ L of the solution. The plate was shielded from light using stannum foil and agitated on a lateral oscillator at ambient temperature for 30 min. Ultimately, the absorbance was read at 490 nm to determine the percentage of LDH leakage.

Following drug intervention, HT22 cells were collected. The cells were then stained with Annexin V-allophycocyanin (APC) and 7-aminoactinomycin D (7-AAD) for 5 min at 25 °C, in the dark. Apoptotic cells were detected using flow cytometry (FCM), and data analysis was conducted using FlowJo software.

To assess mitochondrial function, cells were washed with PBS after treatment. Mitochondrial ROS generation was detected by staining with MitoSOX™ Red (30 min, 37 °C), followed by FCM and CLSM. Mitochondrial membrane potential (MMP) and ATP levels were measured using JC-1 and ATP assay kits, respectively, in accordance with the manufacturers' instructions. MMP was visualized using CLSM, and cellular ATP content was determined via bioluminescence detection on a functional microplate reader.

2.11 Western blotting

Total cellular proteins were isolated from the HT22 line using ice-cold RIPA lysis solution, followed by a bicinchoninic acid (BCA) assay to measure protein concentration. Samples were separated using 10% SDS-PAGE and subsequently blotted onto polyvinylidene difluoride (PVDF) membranes. After nonspecific binding was blocked using 5% bovine serum albumin (BSA), the membranes were incubated with the specific primary antibodies (GAPDH, Bax, Bcl-2, DRP1, and MFN2) overnight at 4 °C. Following sequential rinses in Tris-buffered saline with Tween 20 (TBST), the membranes were treated with secondary antibodies for 60 min at ambient temperature. Finally, protein bands were visualized using an enhanced chemiluminescence (ECL) kit, and their densities were assessed using ImageJ software.

2.12 Evaluation of the alleviating effects of Sb-EVs on Alzheimer's disease

Twenty-eight-week-old male C57BL/6 wild-type (WT; control) and APP/PS1 double transgenic mice (AD model), with body mass of 32 ± 3 g, were supplied by the Animal Experimental Center of Hangzhou Medical College (Hangzhou, China). Animals were maintained in a specific pathogen-free (SPF) facility with *ad libitum* food and water intake.

After a 7-day acclimatization period, APP/PS1 mice were randomly divided into three groups ($n = 6$ per group): (1) AD model group (APP/PS1; administered PBS); (2) Sb-EVs i.v. treatment group (APP/PS1 + i.v. Sb-EVs), receiving 100 μ L of Sb-EVs (2.8×10^{12} particles/mL) via tail vein injection every 2 days; and (3) Sb-EVs intragastric administration (i.g.) treatment group (APP/PS1 + i.g. Sb-EVs), receiving 100 μ L of Sb-EVs (2.8×10^{12} particles/mL) via oral gavage every 2 days. Upon completion of the 31-day drug administration, behavioral tests were conducted from days 32 to 38. On day 39, all mice were sacrificed. Brain tissues, feces, and colonic contents were collected for subsequent analysis.

2.13 Novel object recognition (NOR) test

Cognitive memory was evaluated using the NOR test in a 40 cm $\times$ 40 cm $\times$ 45 cm open field. The protocol included a familiarization phase (10 min exploration of two identical objects) and a testing phase where one familiar object was substituted with a novel one. The recognition index (RI), the preference for the novel object, was quantified as the ratio of novel object exploration to the cumulative time spent with both objects. Locomotor activity (path and total distance) was also tracked. To prevent olfactory interference, the arena and objects were wiped with 75% ethanol after each session.

2.14 Morris water maze (MWM) test

The apparatus consisted of a circular pool (1.5 m in diameter, 0.5 m in height) equipped with an automated image acquisition and behavioral analysis system. The pool was supplied with water kept at a constant temperature of 22 ± 1 °C and titanium dioxide was incorporated into the pool to render the water opaque and white, facilitating tracking. The pool was partitioned into four equivalent sectors as distinct entry locations, with a target platform hidden at a depth of 1 cm within a designated quadrant.

The experiment spanned 5 days and included two phases: the spatial navigation task and the spatial probe trial. For the spatial navigation task (days 1–4), mice were released from the four starting points facing the pool wall. Each mouse was granted a 60-s window to search for the submerged escape platform. If a mouse found the platform and stayed for > 3 s, the duration required to reach the platform was defined as the escape latency. If the mouse did not reach the escape target within 60 s, it was manually led to the platform and permitted to stay for a 10-s interval. The latency was recorded as 60 s. For the spatial probe trial (day 5), after 4 days of training, the platform was removed. Mice were diametrically introduced into the pool from the sector opposite to the former platform location, and parameters including latency to the platform zone, swimming trajectory, frequency of target quadrant crossings, duration of stay in the designated area, and mean velocity were monitored to quantify spatial memory retention. After every experiment, mice were dried to prevent hypothermia and ensure their health throughout the experiment.

2.15 Gut microbiota analysis

Genomic DNA of the microbiota was isolated from cecal samples, and then the V3–V4 regions of the 16S rRNA gene were targeted using polymerase chain reaction (PCR) amplification. Subsequently, sequencing libraries were constructed using the TruSeq® DNA PCR-Free Kit according to the provided protocols. The library quality was assessed using Qubit and quantitative PCR (q-PCR) for quantification. Finally, the qualified libraries were sequenced on an Illumina NovaSeq 6000 platform (Novogene, Beijing, China).

2.16 Untargeted metabolomic analysis of fecal metabolites

Colon contents were collected and placed into Eppendorf tubes. Each sample was homogenized with 500 μ L of 80% methanol, followed by vigorous vortexing. After a 5-min incubation on ice, the mixture was spun at 15,000g for 20 min (4 °C). The resulting supernatant was aliquoted and adjusted to a 53% methanol concentration using liquid chromatography–mass spectrometry (LC–MS) grade water before a final centrifugation step. Finally, the supernatant was obtained and analyzed using the LC–MS system (Thermo Fisher, Germany).

2.17 Statistical analysis

All graphs in this study were generated using GraphPad Prism 10.0 software. All data were obtained from at least three independent experiments and are expressed as the mean $\pm$ standard deviation. The significance of differences between experimental groups was evaluated using one-way analysis of variance, followed by Tukey's post-hoc test. Statistical significance was set at $P < 0.05$ (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, ns, not significant).

3 Results and discussion

3.1 Isolation and characterization of Sb-EVs

High-purity Sb-EVs were successfully isolated from the roots of *S.*

baicalensis using differential and sucrose density gradient centrifugation. Sb-EVs displayed a representative cup-shaped morphology (Fig. 1(a)), as indicated using TEM. The mean particle size was approximately 129.1 nm (Fig. 1(b)), with a zeta potential of -9 mV (Fig. 1(c)), and a PDI of 0.35 (Fig. 1(d)). A lipidomic analysis was conducted on the Sb-EVs. Phosphatidylcholine (PC, 26.27%), phosphatidylethanolamine (PE, 22.32%), and diacylglycerol (DG, 21.87%) were the three predominant lipid classes in Sb-EVs, collectively accounting for over 60% of the total lipid content (Fig. 1(e)). Subsequently, we extracted total proteins and RNA from the Sb-EVs and subjected them to SDS-PAGE and agarose gel electrophoresis, respectively. The molecular weights of the proteins ranged from 25 to 100 kDa (Fig. 1(f)), and RNA bands were concentrated in the region of ≤ 100 bp (Fig. 1(g)). Therefore,

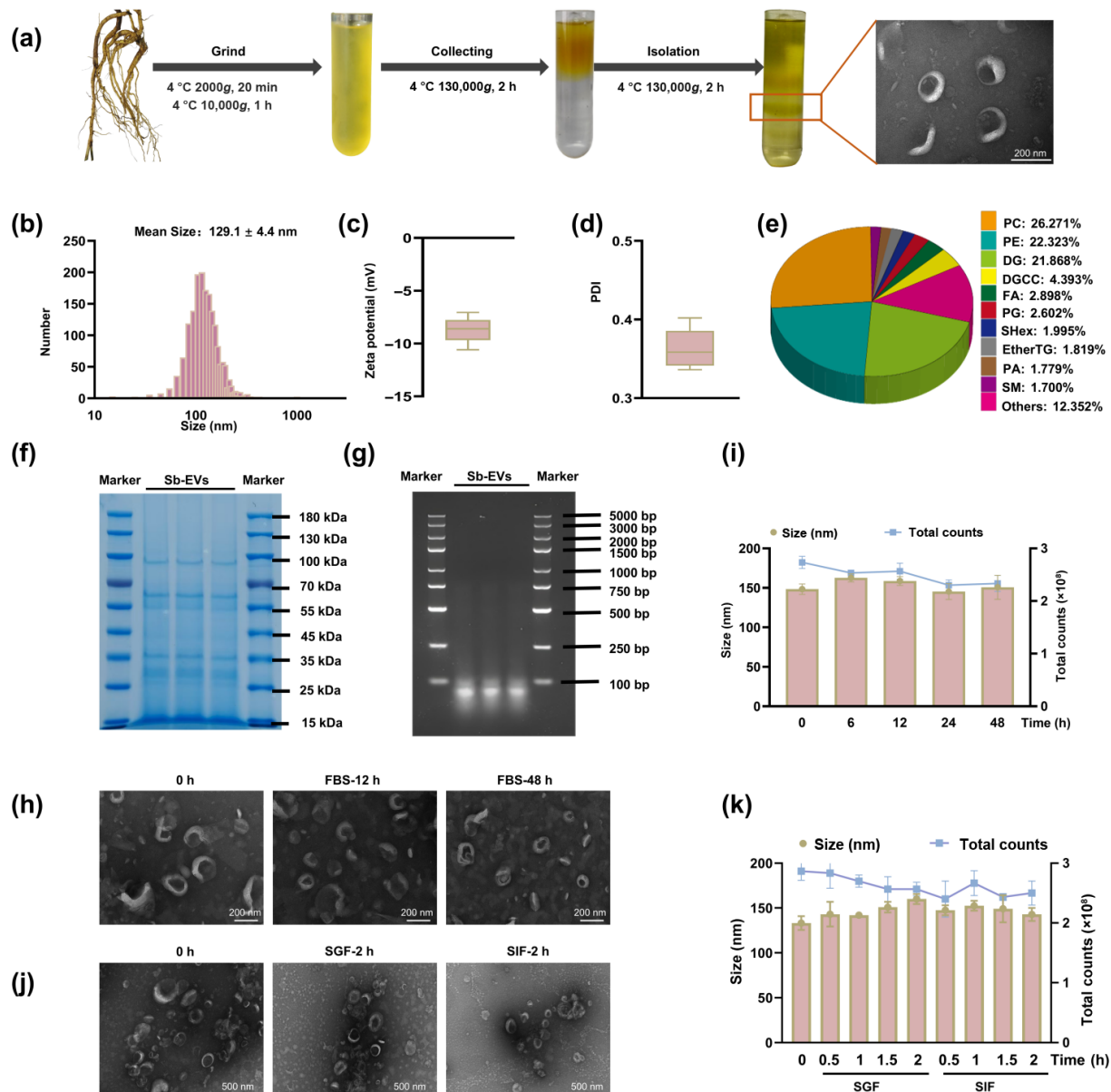


Figure 1 Isolation and characterization of Sb-EVs. (a) Schematic illustration of the isolation process of Sb-EVs and TEM image. (b) Particle size distribution of Sb-EVs determined using NTA. (c) Zeta potential of Sb-EVs measured at 25 °C using a Malvern Zetasizer. (d) PDI of Sb-EVs. (e) Lipidomic analysis of Sb-EVs. (f) SDS-PAGE of total proteins in Sb-EVs. (g) Agarose gel electrophoresis of nucleic acids in Sb-EVs. (h) Morphology of Sb-EVs incubated in simulated blood. (i) Changes in particle size and concentration of Sb-EVs in simulated blood. (j) Morphology of Sb-EVs incubated in simulated gastrointestinal fluid (SGF) and simulated intestinal fluid (SIF). (k) Changes in particle size and concentration of Sb-EVs in SGF and SIF.

the samples were enriched with small RNAs. To evaluate the *in vivo* stability of Sb-EVs following oral and i.v. administration, we monitored changes in their morphology and particle count after treatment with simulated gastrointestinal fluids and blood. No significant changes were observed in the micromorphology, particle size, or particle count of the Sb-EVs after incubation with these simulated fluids (Figs. 1(h)–1(k)). These results indicate that Sb-EVs exhibit excellent stability for both oral and injectable administration.

3.2 *In vivo* distribution of Sb-EVs

To investigate the *in vivo* distribution of Sb-EVs following different administration routes, DIR-labeled Sb-EVs and free DIR were administered to mice via oral gavage and tail vein injection, respectively (Fig. 2(a)). Additionally, a quantitative analysis of their mean fluorescence intensities (MFIs) was conducted (Fig. 2(b)). After oral gavage, the free dye was rapidly metabolized in the gastrointestinal tract, resulting in the diminished fluorescence. In contrast, strong fluorescence was still observable in the intestines of

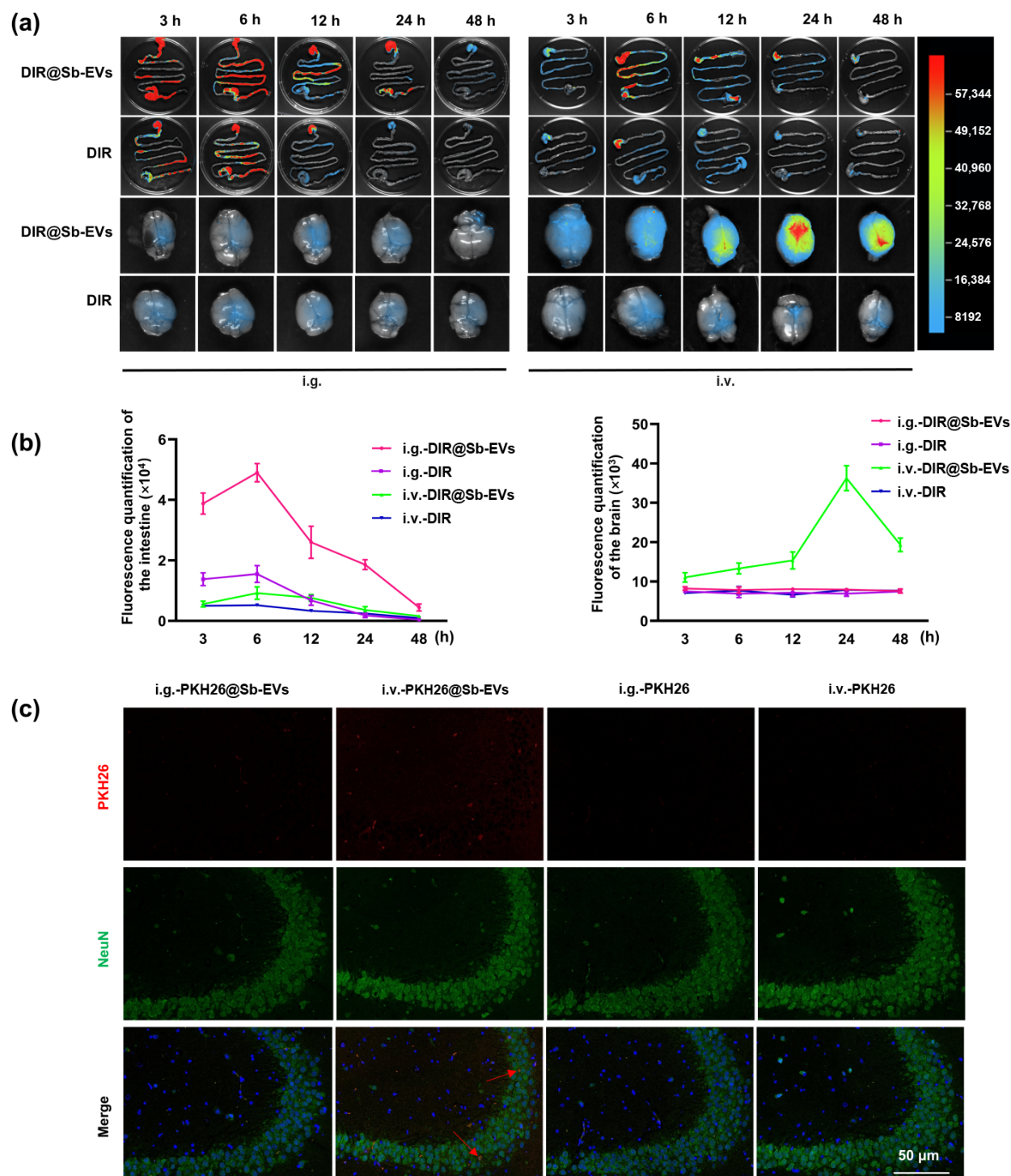


Figure 2 *In vivo* distribution of Sb-EVs. (a) Biodistribution of Sb-EVs in the intestine and brain tissues. (b) MFI of Sb-EVs in the intestine and brain. (c) Colocalization of Sb-EVs with NeuN-labeled neurons in the mouse hippocampus.

mice treated with Sb-EVs up to 24 h after administration, indicating that orally administered Sb-EVs can be retained in the intestine for at least 24 h. However, no fluorescence was detected in the brains of mice treated with either Sb-EVs or free dye via oral gavage, indicating that orally administered Sb-EVs failed to reach the brain. Sb-EVs administered via tail vein injection were also distributed in the intestinal tract via the biliary pathway. Notably, the fluorescence intensity in the brains of mice treated with Sb-EVs via tail vein injection was significantly higher than that of the free dye group, peaking at 24 h after administration. This indicates that i.v. administered Sb-EVs are capable of crossing the BBB.

To validate BBB crossing and neuronal uptake, we administered PKH26-labeled Sb-EVs (PKH26@Sb-EVs) and free PKH26 to mice via oral and i.v. routes. Coronal brain sections were subjected to immunofluorescence analysis (Fig. 2(c)). Red fluorescence was absent from the coronal brain sections of mice treated with free PKH26 or oral PKH26@Sb-EVs. However, after i.v. injection,

PKH26@Sb-EVs were markedly distributed in the hippocampus and showed colocalization with NeuN-positive neurons. This confirms that i.v. administered Sb-EVs can traverse the BBB, accumulate in the hippocampus and cortex, and undergo neuronal uptake.

3.3 *In vitro* assessment of BBB penetration and neuronal internalization of Sb-EVs

To verify the potential of Sb-EVs to traverse the BBB and undergo neuronal uptake *in vitro*, we first assessed their cytotoxicity in HT22 and bEnd.3 cells. Sb-EVs exhibited a favorable biosafety profile in both cell lines (Figs. 3(a) and 3(b)). Subsequently, we examined the cellular uptake of Sb-EVs by these two cell types. After an 8-h co-incubation with PKH26-labeled Sb-EVs, significant red fluorescence signals were observed within both cell lines (Fig. 3(c)). These results indicate the efficient cellular internalization of Sb-EVs. Lysosomal escape is crucial for Sb-EVs to penetrate the BBB and

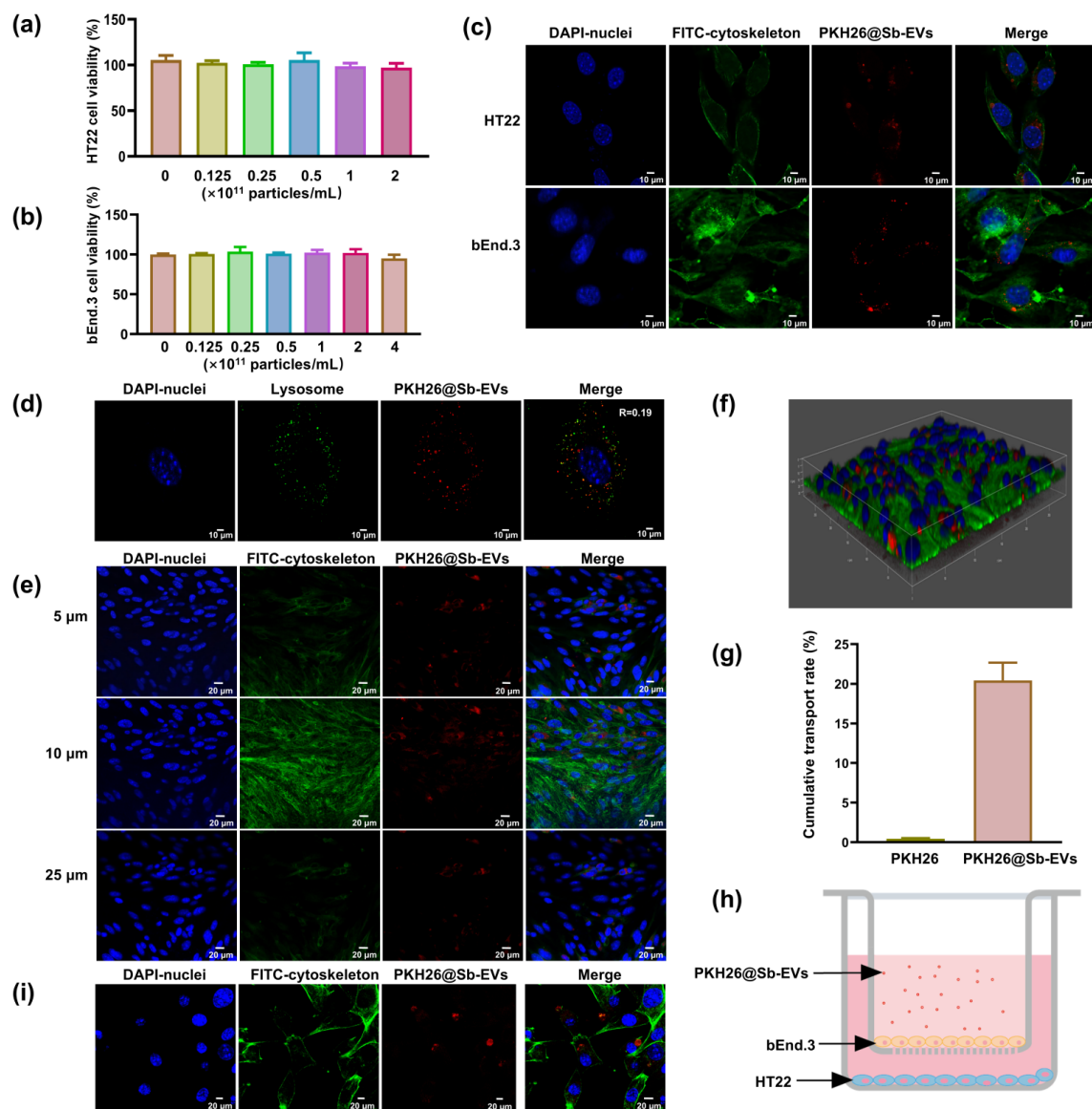


Figure 3 *In vitro* BBB crossing and neuronal uptake of Sb-EVs. (a) Cell viability of HT22 cells. (b) Cell viability of bEnd.3 cells. (c) Cellular uptake of Sb-EVs by HT22 and bEnd.3 cells. (d) Sb-EVs escape lysosomes in bEnd.3 cells. (e) Confocal Z-stack images showing Sb-EVs penetrating the BBB monolayer. (f) 3D reconstruction of Sb-EVs penetrating the BBB monolayer. (g) Transmembrane permeability of Sb-EVs across the *in vitro* BBB model. (h) Schematic illustration of the *in vitro* BBB and HT22 cell co-culture system. (i) Uptake of Sb-EVs by underlying HT22 cells after crossing the *in vitro* BBB.

enter the brain parenchyma [30]. To test the ability of Sb-EVs to penetrate the BBB, the colocalization of PKH26-labeled Sb-EVs with lysosomes was investigated. The red fluorescence of the Sb-EVs did not overlap with the green fluorescence of the lysosomes, indicating that internalized Sb-EVs markedly avoided lysosomal entrapment (Fig. 3(d)). An *in vitro* BBB model was established to further verify the BBB-crossing capability of Sb-EVs. Following a 12-h incubation with PKH26-labeled Sb-EVs, the intact monolayer was subjected to confocal Z-stack scanning and 3D reconstruction. The red fluorescence of the Sb-EVs penetrated the bEnd.3 monolayer from the apical to the basolateral side (Figs. 3(e) and 3(f)), demonstrating that Sb-EVs can successfully traverse the *in vitro* BBB model. The transmembrane efficiency was quantified by calculating the cumulative transport rate of Sb-EVs. The permeation of Sb-EVs exceeds 20% after 8 h of incubation (Fig. 3(g)). To further investigate whether Sb-EVs that cross the *in vitro* BBB can be taken up by neuronal cells, a co-culture system comprising the *in vitro* BBB model and HT22 cells was established (Fig. 3(h)). PKH26-labeled Sb-EVs were added to the apical chamber of the Transwell inserts and incubated for 12 h. Subsequently, the uptake of Sb-EVs by HT22 cells in the basolateral chamber was visualized using CLSM. Distinct red fluorescence signals were observed within the HT22 cells, indicating that Sb-EVs successfully traversed the *in vitro* BBB and were subsequently taken up by the underlying neuronal cells (Fig. 3(i)). Collectively, these *in vitro* experiments further demonstrate that Sb-EVs can cross the BBB and be taken up by neuronal cells, highlighting their potential for treating brain-related disorders.

3.4 Sb-EVs ameliorate cognitive dysfunction in APP/PS1 mice

The effect of Sb-EVs on cognitive deficits in APP/PS1 mice was investigated (Fig. 4(a)). The NOR test, which leverages the innate exploratory preference of rodents for novelty, was used to interpret learning, memory retention, and recognition capacity. Compared with the WT group, APP/PS1 mice showed a markedly lower preference for novel objects with an RI < 40% (Figs. 4(b) and 4(c)). Notably, Sb-EVs restored the exploratory behavior, with the i.v. group achieving a higher RI (70%) than that of the oral gavage group (48%). In the NOR test, no significant differences were observed in total distance traveled across groups (Fig. 4(d)), confirming that the improvements in exploratory behavior were independent of locomotor activity. The MWM was subsequently used to systematically evaluate spatial memory acquisition, retention, and reversal capabilities. During the hidden platform training phase (acquisition phase), APP/PS1 mice failed to locate the platform or exhibited significantly prolonged escape latencies (Fig. 4(e)), indicating profound spatial learning and memory deficits. Conversely, mice treated with Sb-EVs (both i.v. and i.g.) located the platform more rapidly, showing a progressive reduction in latency. The learning curve for the APP/PS1 group remained flat despite repeated trials; however, Sb-EVs effectively rescued spatial learning abilities and enhanced memory function (Fig. 4(f)). In the spatial probe test (probe trial), representative swimming trajectories (Fig. 4(g)) showed that APP/PS1 mice lacked preference for the target platform region, while Sb-EVs intervention markedly improved spatial memory. Specifically, the i.v. Sb-EVs group exhibited a significantly shorter latency to reach the target zone (Fig. 4(h)). Compared with the APP/PS1 mice, Sb-EV-treated mice exhibited a higher frequency of platform crossings (Fig. 4(i)) and

prolonged retention time in the target quadrant (Fig. 4(j)), especially in the i.v. injection group. No significant differences were observed in swimming speed or total distance traveled among all groups (Figs. 4(k) and 4(l)), indicating that these cognitive gains were not confounded by motor or swimming abilities. In summary, both oral gavage and i.v. administered Sb-EVs ameliorated spatial learning and memory deficits in APP/PS1 mice, with the i.v. route demonstrating superior intervention efficacy.

3.5 Neuroprotective effects of Sb-EVs in APP/PS1 mice

Immunofluorescence staining was used to observe the distribution of A β plaques in the hippocampus and cortex of experimental mice. No significant A β deposition was observed in the brains of WT mice, whereas the hippocampus and cortex of APP/PS1 mice exhibited numerous dense plaques with high fluorescence intensity (Figs. 5(a)–5(c)). Notably, after Sb-EVs intervention, the A β fluorescence signals in these brain regions were significantly attenuated, characterized by a marked reduction in both plaque area and number, with the i.v. injection group exhibiting an even more pronounced reduction. Furthermore, we investigated the expression of NeuN, a core marker of mature neurons [31]. Compared with WT mice, APP/PS1 mice exhibited a significant reduction in NeuN signal intensity in the hippocampus and cortex, suggesting a loss of mature neurons (Figs. 5(d)–5(f)). Notably, Sb-EV intervention, particularly via i.v. administration, led to a marked recovery in NeuN expression levels. This indicates that Sb-EVs effectively antagonize A β -mediated neuronal damage and preserve the survival rate of mature neurons. Furthermore, in the CA3 region of the hippocampus, untreated APP/PS1 mice showed disorganized neuronal arrangement, as indicated using hematoxylin and eosin (H&E) staining (Fig. 5(g)). These were characterized by significantly enlarged intercellular spaces, pronounced nuclear shrinkage, and concomitant cell necrosis. In contrast, the morphology and quantity of neurons in Sb-EV-treated APP/PS1 mice were comparable to those of WT mice. These histological findings demonstrate that Sb-EVs effectively alleviate the pathological state of APP/PS1 mice by reducing A β deposition and restoring neuronal morphology, with i.v. injection showing superior therapeutic efficacy over oral gavage.

In AD, neuronal apoptosis is primarily driven by a severe imbalance in mitochondrial dynamics. Specifically, A β hyperactivates the fission protein, Drp1, which overwhelms the protective fusion protein MFN2. This shift causes excessive mitochondrial fragmentation, disrupting the respiratory chain and accelerating cell death [32, 33]. Compared with WT mice, APP/PS1 mice showed a marked upregulation of Drp1 and concomitant downregulation of MFN2, indicating a severe dysregulation of mitochondrial fission and fusion dynamics (Figs. 5(h)–5(j)). In addition, the level of the pro-apoptotic protein, Bax, notably increased, whereas that of the anti-apoptotic protein, Bcl-2, was markedly decreased in the hippocampus of APP/PS1 mice (Figs. 5(k)–5(m)), indicating prominent neuronal apoptosis in this region. Notably, Sb-EV intervention significantly restored the mitochondrial homeostasis and anti-apoptotic capacity of neurons, with the i.v. injection group exhibiting superior therapeutic efficacy. Simultaneously, we performed H&E staining on the major organs (heart, liver, spleen, lung, and kidney) of WT mice following oral and i.v. administration of Sb-EVs. The tissue structures of these organs remained intact with normal cellular morphology. No pathological alterations, such as congestion, edema, inflammatory

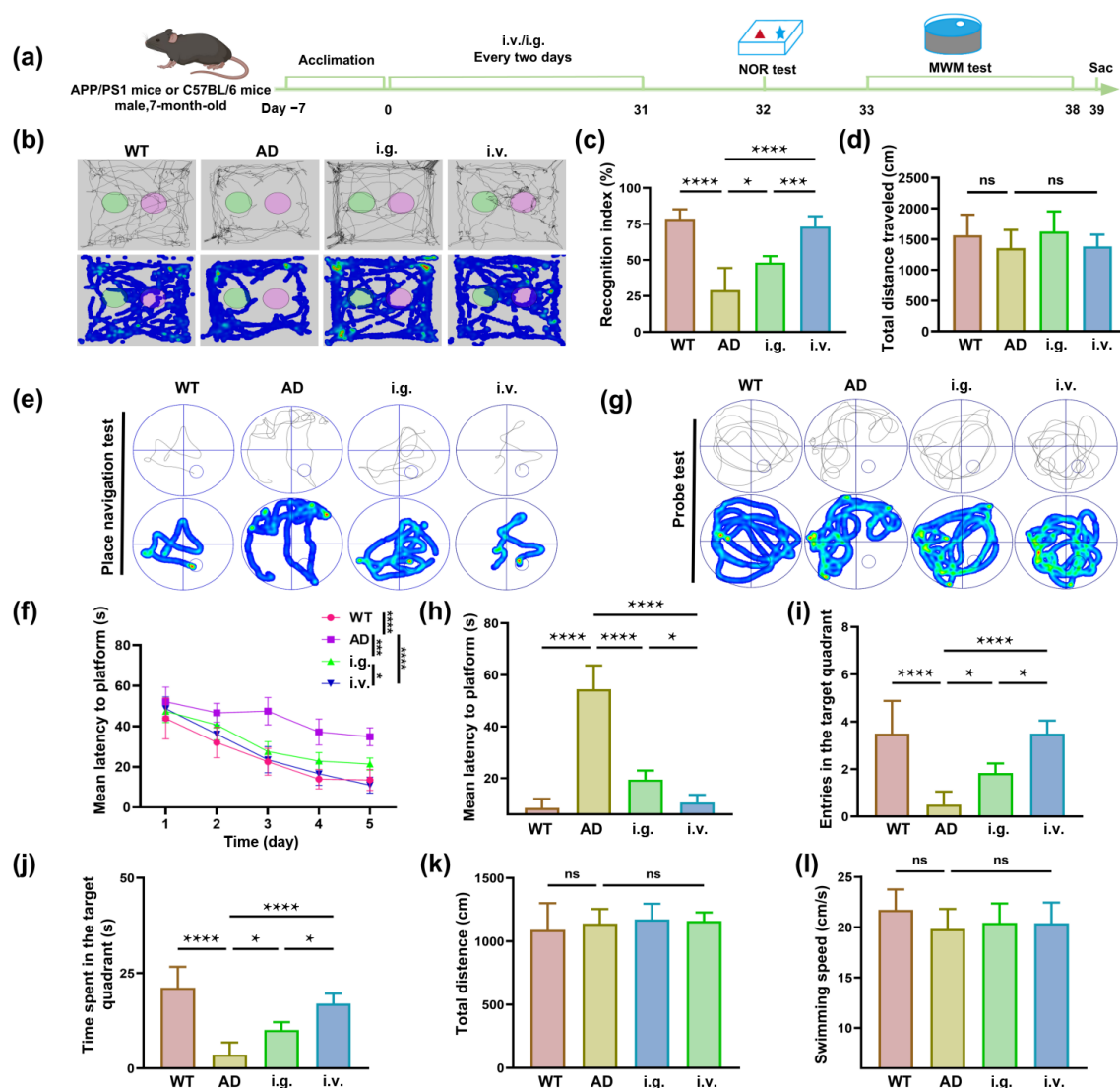


Figure 4 Sb-EVs ameliorate cognitive dysfunction of APP/PS1 mice. (a) The experimental design schematic diagram. (b) Representative exploration trajectories of mice in the NOR test. (c) RI of mice in the NOR test. (d) Total distance traveled by mice during the NOR test. (e) Representative swimming trajectories of mice during the training phase (acquisition phase) of the MWM. (f) Learning curves showing the escape latency of mice during the MWM training phase. (g) Representative swimming trajectories of mice during the probe trial (test phase) of the MWM. (h) Escape latency to reach the target platform during the MWM probe trial. (i) Number of platform crossings during the MWM probe trial. (j) Time spent in the target quadrant during the MWM probe trial. (k) Total swimming distance of mice during the MWM probe trial. (l) Average swimming speed of mice during the MWM probe trial ($n = 6$).

cell infiltration, or degenerative necrosis, were observed (Fig. S1 in the Electronic Supplementary Material (ESM)). These data confirm that Sb-EVs have excellent *in vivo* biosafety. Collectively, Sb-EV intervention can significantly reduce $A\beta$ plaque deposition in the brains of APP/PS1 mice and restore mitochondrial function, thereby increasing neuronal survival rates and alleviating pathological brain lesions. Notably, the intervention effect of i.v. Sb-EVs administration is particularly prominent.

3.6 Sb-EVs alleviate $A\beta$ -induced oxidative stress and neuronal apoptosis

Based on the *in vivo* findings that i.v. injected Sb-EVs can significantly ameliorate cognitive dysfunction as well as brain pathology in APP/PS1 mice, we investigate their effects on $A\beta$ -induced oxidative stress and neuronal apoptosis in HT22 cells. The optimal modeling concentration for investigating the

neuroprotective effects of Sb-EVs against $A\beta$ toxicity was determined to be $2.5 \mu\text{M}$ $A\beta$ using the MTT assay, based on its reduction of HT22 cell viability to approximately 60% (Fig. S2 in the ESM). To determine whether Sb-EVs could effectively alleviate $A\beta$ -induced neuronal oxidative stress, intracellular ROS levels were determined using CLSM. Intracellular ROS levels were markedly elevated following $A\beta$ injury, which was significantly reduced with Sb-EVs treatment in a dose-dependent manner, suggesting that Sb-EVs effectively mitigate $A\beta$ -induced oxidative stress (Figs. 6(a) and 6(b)). These results were further confirmed with the FCM quantitative analysis (Figs. 6(c) and 6(d)). To evaluate the effect of Sb-EVs on $A\beta$ -induced neuronal injury, LDH release into the cell culture supernatant was investigated. Sb-EVs significantly attenuated the LDH release caused by $A\beta$ (Fig. 6(e)). Subsequently, the effect of Sb-EVs on the $A\beta$ -induced decline in cell viability was determined. Medium- and high-dose Sb-EVs significantly improved cell viability (Fig. 6(f)). In addition, $A\beta$ exposure

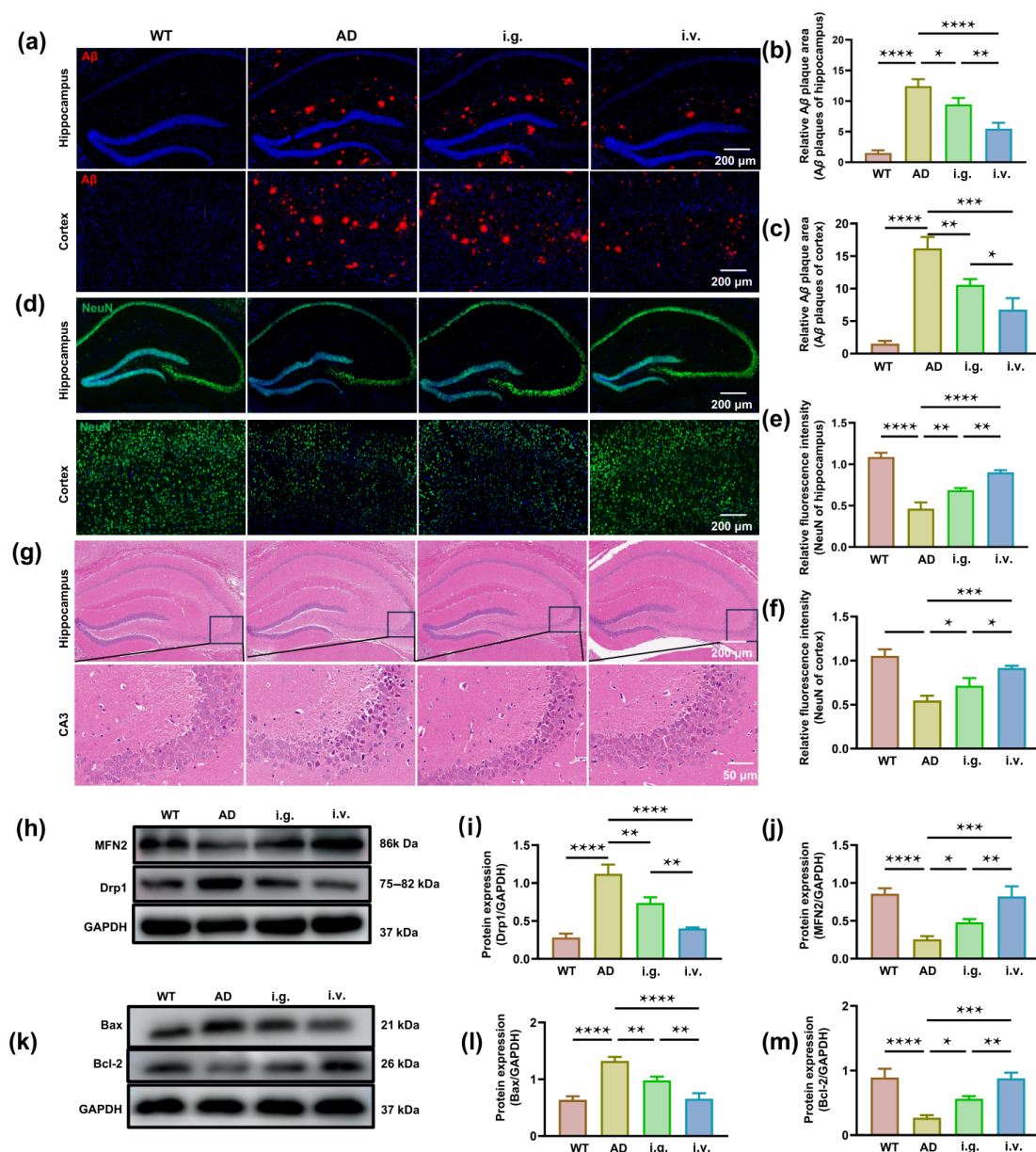


Figure 5 Neuroprotective effects of Sb-EVs in APP/PS1 mice. (a) Immunofluorescence staining of A β in brain tissues. (b) Quantification of A β deposition in the hippocampus. (c) Quantification of A β deposition in the cortex. (d) Immunofluorescence staining of NeuN in brain tissues. (e) Quantitative analysis of NeuN expression in the hippocampus. (f) Quantitative analysis of NeuN expression in the cortex. (g) Representative H&E staining images of brain sections. (h) Representative western blot images of MFN2, Drp1 in mouse brain tissues. Densitometric quantification of (i) Drp1, (j) MFN2. (k) Representative western blot images of Bax, and Bcl-2 in mouse brain tissues. Densitometric quantification of (l) Bax, and (m) Bcl-2, $n = 3$.

significantly induced cellular apoptosis, whereas Sb-EV treatment markedly reduced the apoptotic rate, with the high-dose group reducing the rate to approximately half that of the A β group (Figs. 6(g) and 6(h)). Western blot results further confirmed that Sb-EVs reversed the A β -induced downregulation of the Bcl-2/Bax ratio (Figs. 6(i)–6(k)), indicating effective inhibition of neuronal apoptosis via the upregulation of anti-apoptotic signaling pathways. Collectively, these results indicate that Sb-EVs can significantly attenuate A β -induced neuronal oxidative stress and apoptosis.

3.7 Sb-EVs attenuate A β -induced mitochondrial damage

To investigate whether Sb-EVs alleviate A β -induced neuronal oxidative stress and apoptosis by mitigating mitochondrial damage,

mitochondrial superoxide levels were initially assessed. A β -induced HT22 cells displayed dense mitochondrial red fluorescence (Fig. 7(a)), suggesting mitochondrial superoxide accumulation, which was significantly attenuated by Sb-EVs. FCM quantitative analysis further confirmed that Sb-EVs markedly reduced MitoSOX fluorescence compared to that in the A β -induced group (Figs. 7(b) and 7(c)), indicating that Sb-EVs effectively scavenged mitochondrial superoxide accumulation. The JC-1 probe emits red fluorescence within healthy, high-potential mitochondria, but shifts to green-fluorescent monomers in the cytoplasm upon mitochondrial dysfunction and membrane potential depolarization. A β treatment caused a significant decline in the red-to-green fluorescence ratio (Figs. 7(d) and 7(e)), indicating mitochondrial membrane depolarization. Notably, Sb-EVs effectively reversed this

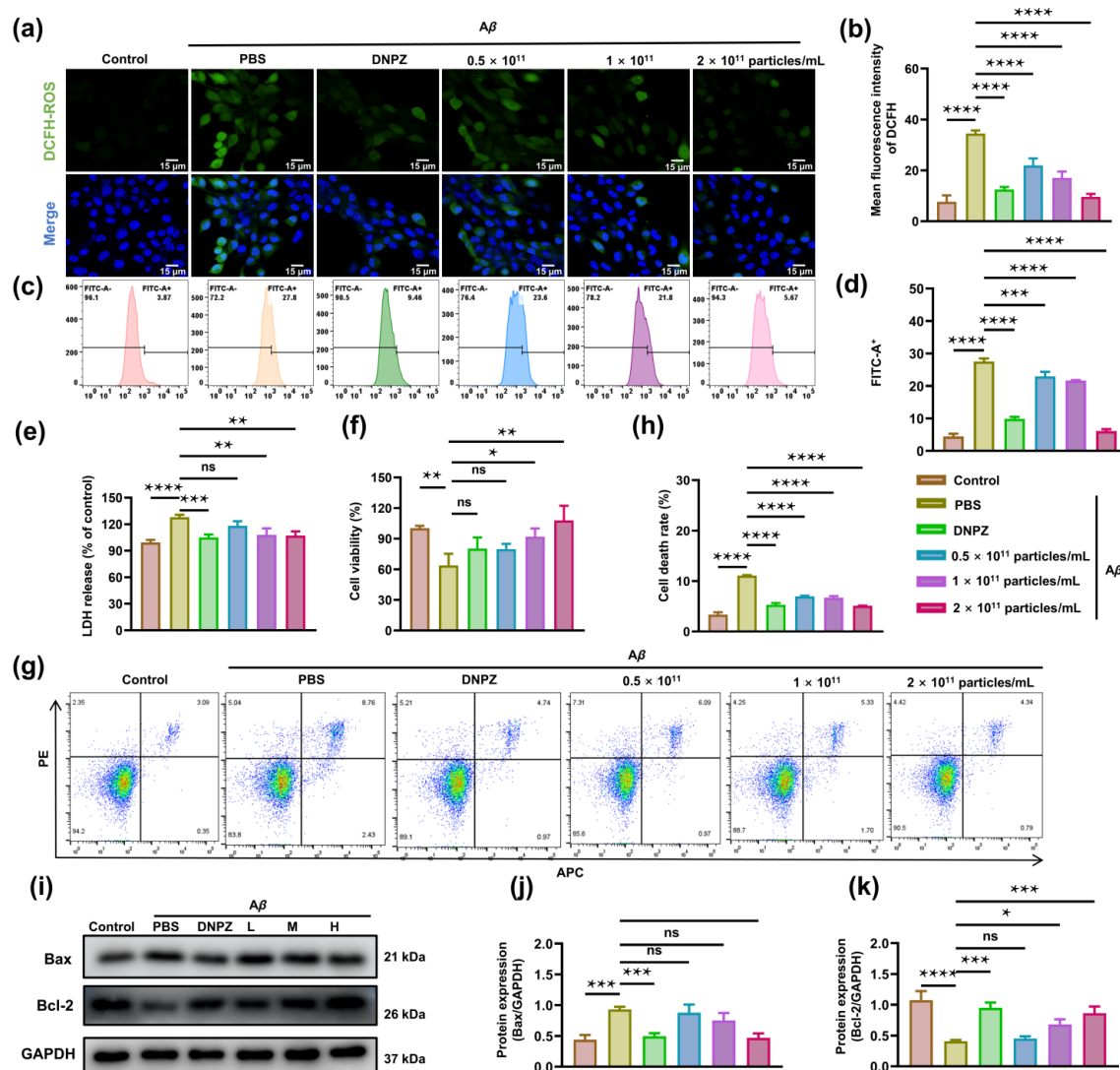


Figure 6 Sb-EVs attenuate A β -induced oxidative stress and apoptosis of HT22 cells. (a) CLSM images of intracellular ROS levels in HT22 cells. (b) Quantitative analysis of ROS fluorescence intensity. (c) FCM analysis of ROS levels in HT22 cells. (d) Quantitative analysis of ROS levels determined using FCM. (e) Relative LDH release in HT22 cells. (f) Cell viability of HT22 cells determined by MTT assay. (g) Apoptosis analysis of HT22 cells using FCM. (h) Quantitative analysis of the apoptosis rate. (i) Western blot analysis of Bcl-2 and Bax protein level. (j) Quantitative analysis of Bax. (k) Quantitative analysis of Bcl-2 ($n = 3$).

shift, rescuing the A β -induced decline in MMP. In addition, A β intervention downregulated the expression of the mitochondrial fusion protein, MFN2, and induced the overactivation of the fission protein, Drp1. These alterations were significantly reversed by Sb-EVs (Figs. 7(f)–7(h)), suggesting that Sb-EVs balanced mitochondrial fusion and fission, thereby alleviating mitochondrial dysfunction. Furthermore, TEM was used to observe mitochondrial morphology. Compared with the control group, A β exposure resulted in fragmented or sparse mitochondrial cristae, and localized outer membrane rupture. In contrast, mitochondria in the Sb-EV-treated group appeared near-normal, characterized by an elliptical shape, uniform matrix, and densely packed, continuous cristae with intact membranes, showing no obvious swelling or vacuolization (Fig. 7(i)). Given that mitochondrial damage directly compromises energy metabolism, intracellular ATP levels were subsequently measured. A β exposure significantly reduced ATP content, which was markedly recovered following Sb-EV treatment, indicating a reversal of energy metabolism deficits (Fig. 7(j)). Consequently, Sb-EVs significantly reversed A β -induced

mitochondrial oxidative damage, dysfunction and metabolic impairment, thereby, exerting neuroprotective effects.

3.8 Sb-EVs modulate gut microbiota dysbiosis in APP/PS1 mice

To investigate the underlying mechanism of orally administered Sb-EVs against AD, we analyzed the gut microbiota of mice. The unique and shared bacterial operational taxonomic units among the different groups are depicted in a Venn diagram (Fig. 8(a)). The Chao1 index and Simpson index indicated that oral administration of Sb-EVs increased the microbial diversity in AD mice (Figs. 8(b) and 8(c)). The WT group distinctly clustered from the AD group, as indicated using PCA (Fig. 8(d)), suggesting significant differences in microbial composition. Compared with the AD group, Sb-EV intervention led to a difference in microbial communities, with the oral gavage group showing a more pronounced deviation, indicating that Sb-EVs significantly reshaped the gut microbiota composition in APP/PS1 mice. Subsequently, the microbial composition at the phylum, family, and genus levels was

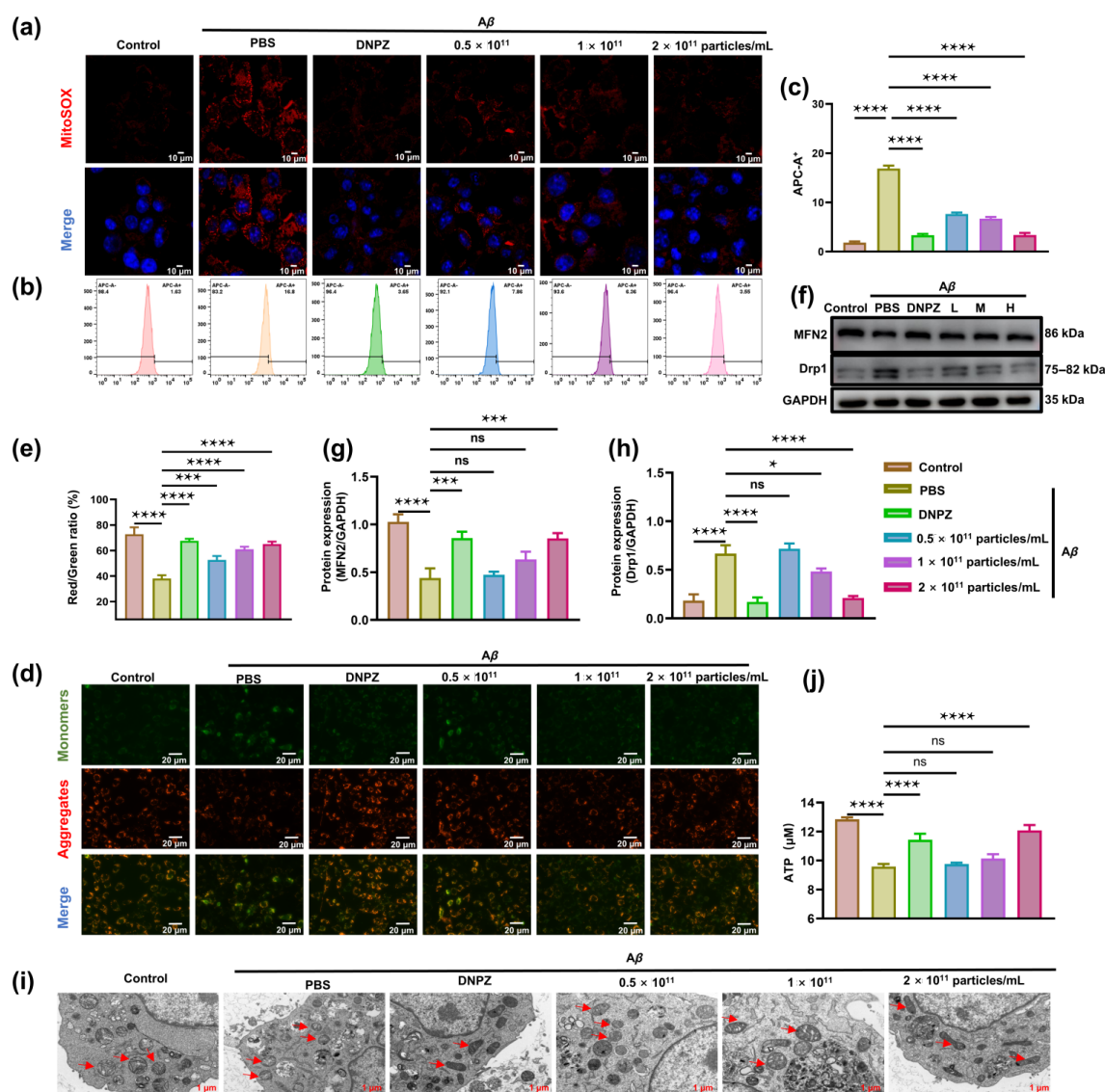


Figure 7 Sb-EVs alleviate Aβ-induced mitochondrial dysfunction. (a) CLSM images of mitochondrial superoxide levels detected using the MitoSOX Red probe. (b) FCM analysis of mitochondrial superoxide levels in HT22 cells. (c) Quantitative analysis of mitochondrial superoxide levels in HT22 cells using FCM. (d) Representative CLSM images showing MMP in HT22 cells. (e) Quantitative analysis of the JC-1 red/green fluorescence ratio in HT22 cells. (f) Representative western blot images of the mitochondrial fusion (MFN2) and fission (Drp1) proteins in HT22 cells. (g) Quantitative analysis of MFN2 (grayscale density). (h) Quantitative analysis of Drp1 (grayscale density). (i) TEM images showing the mitochondrial ultrastructure in HT22 cells. (j) Intracellular ATP levels in HT22 cells ($n = 3$).

systematically evaluated. At the phylum level, the Bacillota phylum includes key butyrate-producing bacteria, such as *Oscillospiraceae*, *Lachnospiraceae*, which are crucial for maintaining intestinal homeostasis [29]. Compared to the WT group, the proportion of Bacillota in APP/PS1 mice was significantly reduced. However, Sb-EVs intervention, particularly via oral gavage, restored its abundance to 1.4 times that of the AD group. Bacteroidota, which plays dual roles in acetate/propionate production and LPS-mediated inflammation, was elevated in the AD model [34, 35]. Furthermore, the relative abundances of Thermodesulfobacteriota and Actinomycetota were significantly increased in APP/PS1 mice. Following the Sb-EV treatment, especially through oral gavage, the abundance of Thermodesulfobacteriota decreased by approximately 2.5-fold, whereas that of Actinomycetota dropped by approximately 5.6-fold (Fig. 8(e)). Heatmap analysis of microbial abundance revealed significant taxonomic shifts at the family level. Specifically, compared with the WT group, the levels of *Oscillospiraceae* and

Lachnospiraceae in APP/PS1 mice decreased by 2.5- and 1.5-fold, respectively, whereas *Desulfovibrionaceae* exhibited significant proliferation, with a 2.0-fold increase. After Sb-EV i.v. administration, *Desulfovibrionaceae* showed no downward trend; while *Oscillospiraceae* recovered to levels comparable to those of the WT group and *Lachnospiraceae* increased 1.3-fold relative to that of the AD group. Notably, oral Sb-EV treatment led to a more robust response, with *Oscillospiraceae*, *Lachnospiraceae*, and *Bacteroidaceae* increasing by 3.4-, 1.5-, and 2.4-fold, respectively, while *Desulfovibrionaceae* decreased 2.6-fold compared to the AD group (Figs. 8(f) and 8(g)). Further analysis at the genus level showed that the percentage of *Desulfovibrio* (2.53%) was significantly elevated in the AD group. Conversely, the relative abundance of *Lachnospiraceae_NK4A136_group* (7.55%), *unclassified_Lachnospiraceae* (0.52%), and *[Eubacterium]_xylanophilum_group* (0.078%) were markedly reduced in AD mice. In the i.v. group, no changes in the proportions of *Desulfovibrio*,

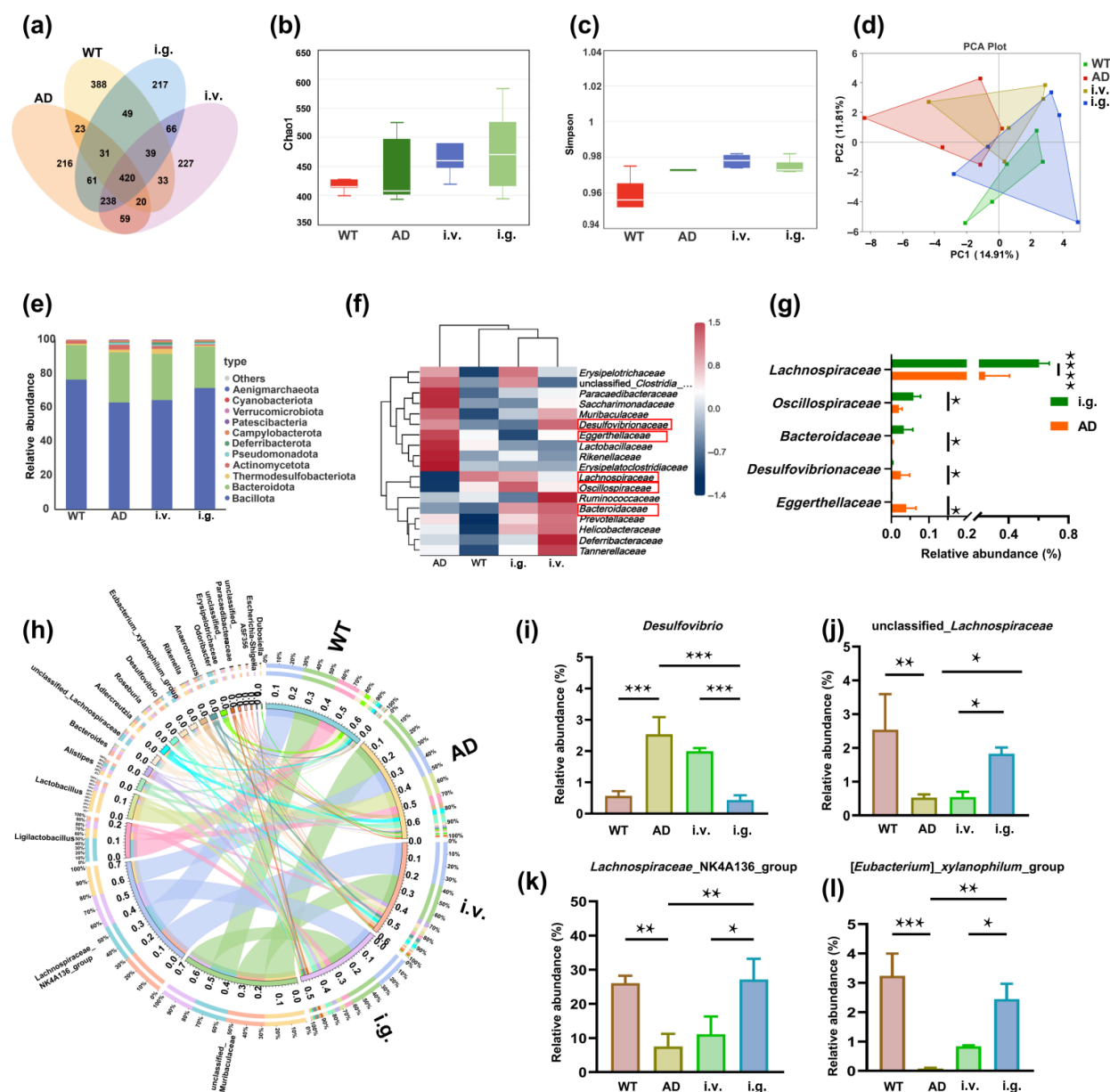


Figure 8 Sb-EVs modulate gut microbiota composition in APP/PS1 mice. (a) Venn diagram. Alpha diversity analysis representing (b) microbial richness (Chao1 index) and (c) diversity (Simpson index), respectively. (d) PCA plot based on Bray–Curtis distance. (e) Stacked bar plot showing the relative abundance of gut microbiota at the phylum level. (f) Heatmap and (g) corresponding quantitative analysis showing the taxonomic shifts at the family level. (h) Overview of microbial abundance distribution at the genus level. Quantitative analysis of key bacterial genera, including (i) *Desulfovibrio*, (j) unclassified *Lachnospiraceae*, (k) *Lachnospiraceae*_NK4A136_group, and (l) *[Eubacterium]*_xylanophilum_group.

*Lachnospiraceae*_NK4A136_group or unclassified *Lachnospiraceae* were observed, but the relative abundance of *[Eubacterium]*_xylanophilum_group recovered to 0.83%. Oral administration of Sb-EVs exerted a more profound effect in ameliorating gut dysbiosis. Specifically, the relative abundance of *Desulfovibrio* was markedly reduced to 0.43% (a 3.5-fold decrease compared to the AD group), whereas the proportion of *Lachnospiraceae*_NK4A136_group, unclassified *Lachnospiraceae* and *[Eubacterium]*_xylanophilum_group increased to 27.15%, 1.83%, and 2.44%, respectively (Figs. 8(h)–8(l)). Collectively, these findings demonstrate that Sb-EV intervention restores gut microbiota homeostasis in APP/PS1 mice by suppressing pathogenic microbes and enriching beneficial taxa. Notably, the therapeutic effect was significantly more pronounced following oral gavage.

3.9 Sb-EVs reshape the gut metabolic profile of APP/PS1 mice

To investigate the effects of Sb-EVs on the gut microbial metabolites of APP/PS1 mice, untargeted metabolomics was performed on colon contents. A Venn diagram illustrating the unique and shared metabolites among groups is shown in Fig. 9(a). PCA demonstrated a clear separation between the WT and AD groups, reflecting a significant shift in the gut metabolic landscape of AD mice. Following intervention with Sb-EVs (both oral and i.v.), the metabolic clusters shifted toward the WT region, suggesting that Sb-EVs can partially restore metabolic homeostasis in AD mice (Fig. 9(b)). Subsequently, enrichment analysis was performed on the top 30 metabolites. The results highlighted a

unique metabolic signature in APP/PS1 mice, characterized by the significant accumulation of pro-oxidative and pro-inflammatory metabolites, including cis-9,10-epoxystearic acid, 11 β -hydroxyprogesterone, canrenone, and 9-hydroxyoctadecanoic acid. In the oral gavage group, these metabolites decreased by 1.95-, 1.3-, 1.4-, and 1.1-fold, respectively, leading to a restoration of the metabolic profile toward that of the WT group (Fig. 9(c)). Volcano plot analysis intuitively demonstrated the significant metabolic remodeling effects of both orally and i.v. administered Sb-EVs on AD mice. Following oral administration of Sb-EVs, the levels of GroPIns, which are closely associated with neuroinflammation and phospholipid degradation, as well as succinyl adenosine, an indicator of energy metabolism impairment, were significantly downregulated. Concurrently, the abundance of 3'-geranyl-chalcone, a metabolite with neuroprotective potential, and neoenactin NL2 was markedly upregulated. In the i.v. injection group, the intervention effectively reversed the abnormal elevation of glutarylcarnitine and (4E,6E)-3-hydroxydeca-4,6-dienoic acid observed in AD mice. Furthermore, this administration route

significantly increased the contents of [12]-shogaol and dethiobiotin (Figs. 9(d) and 9(e)). Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was performed on the non-targeted metabolites, revealing that lipid metabolism was significantly enriched (Fig. 9(f)). APP/PS1 mice showed profound lipid metabolism dysregulation, characterized by a substantial increase in oxidative stress markers (13-OxoODE and 9(S)-HOT) and pro-inflammatory mediators (arachidonic acid (AA) and prostaglandin F2 α (PGF2 α)). Additionally, levels of LysoPC(20:4(5Z,8Z,11Z,14Z)/0:0), 27-hydroxycholesterol, and the stress hormone, corticosterone, were also elevated (Figs. 9(g)–9(j)). Notably, oral gavage of Sb-EVs significantly reversed all the aforementioned metabolic disturbances, whereas i.v. injection only restored three specific metabolites (13-OxoODE, 9(S)-HOT, and corticosterone). These results indicate that Sb-EVs significantly modulate gut microbiota-related metabolic profiles, particularly lipid metabolism. Correlation analysis between the gut microbiota and metabolites (Fig. 9(k)) suggested a close interaction between the gut microecology and metabolic profiles. Furthermore, H&E staining of intestinal sections

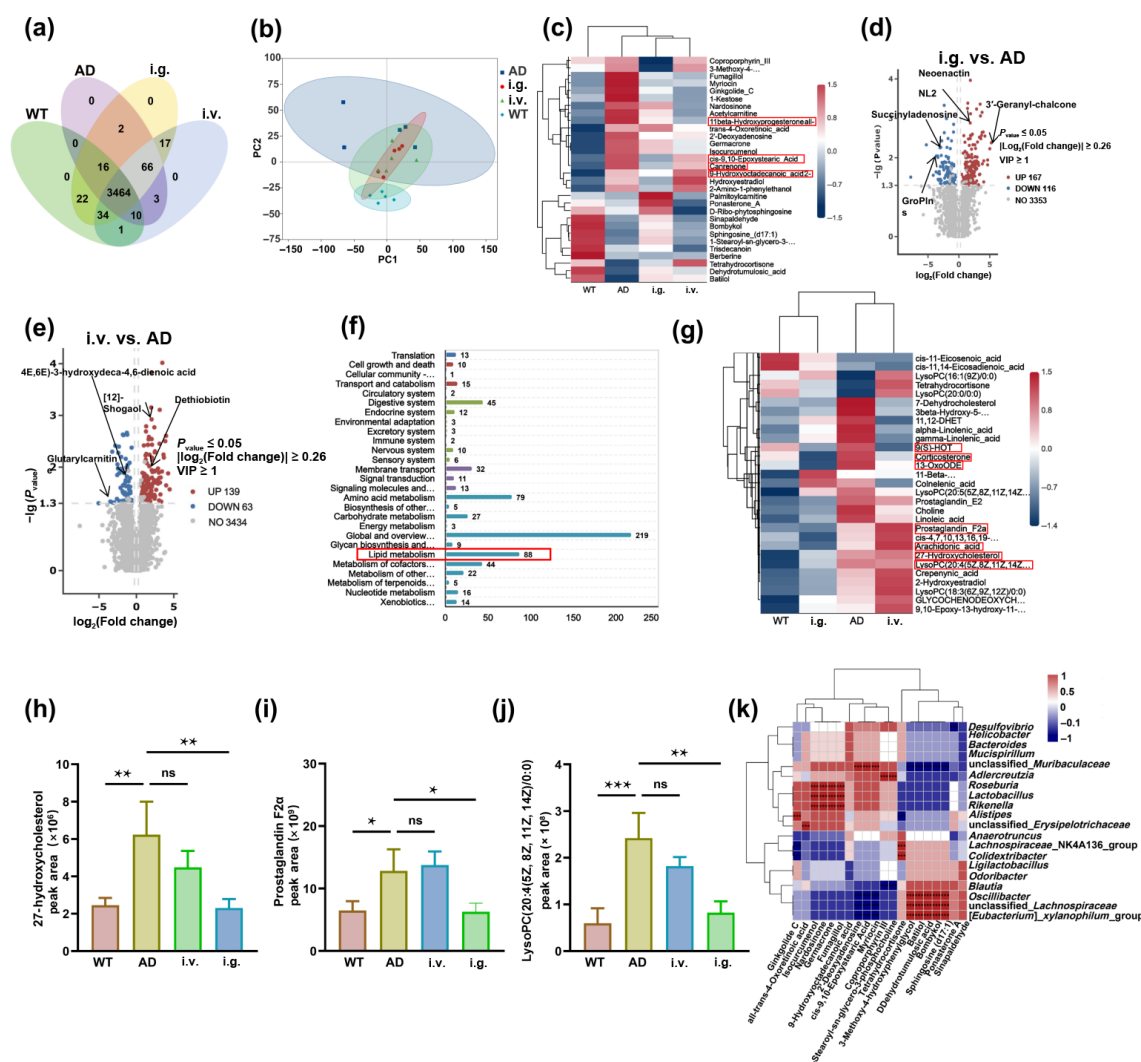


Figure 9 Sb-EVs modulate the gut metabolic profile in APP/PS1 mice. (a) Venn diagram. (b) PCA plot of metabolic profiles. (c) Heatmap representing the enrichment of the top 30 total metabolites. Volcano plots between the APP/PS1 group and either (d) the oral gavage or (e) i.v. treatment groups. (f) KEGG enrichment analysis of the identified metabolites. (g) Heatmap displaying the abundance of specific lipid metabolites. Quantitative analysis of representative metabolites, including (h) 27-hydroxycholesterol, (i) PGF2 α , and (j) LysoPC(20:4(5Z,8Z,11Z,14Z)/0:0). (k) Spearman's correlation heatmap illustrating the association between gut microbiota and differential metabolites.

revealed pronounced inflammation in the AD group, characterized predominantly by inflammatory cell infiltration, which was notably attenuated following Sb-EV intervention (Fig. S3 in the ESM). Collectively, oral administration of Sb-EVs alleviates AD progression in APP/PS1 mice primarily by restoring gut microbiota balance, regulating intestinal metabolites, mitigating gut inflammation, and maintaining intestinal barrier integrity.

4 Discussion

This work focused on elucidating the intervention effects of Sb-EVs administered via oral gavage and tail vein injection on AD progression in APP/PS1 mice, thereby seeking potential clinical interventions. A major obstacle in treating AD is the BBB, which severely restricts drug delivery to the central nervous system (CNS), thus representing a critical bottleneck in the development of effective neurological therapies. In this study, orally administered Sb-EVs exhibited prolonged retention within the gastrointestinal tract but failed to penetrate the brain tissue *in vivo*. In contrast, i.v. administered Sb-EVs successfully entered the brain. Using an *in vitro* BBB model, we further verified that Sb-EVs could traverse the BBB and were taken up by neurons. Orally administered Sb-EVs must sequentially cross the two biological barriers, the intestinal epithelial barrier and BBB [36]. Consequently, their effective delivery to cerebral target regions is significantly hindered, resulting in minimal or even undetectable CNS exposure. Following i.v. injection, Sb-EVs successfully reached cerebral regions. PEVs have intrinsic targeting properties. They can utilize specific ligands, such as surface glycoproteins, to selectively engage receptors on brain microvascular endothelial cells, promoting receptor-mediated transcytosis. Additionally, they can directly fuse with the plasma membrane, momentarily opening transmembrane channels to facilitate efficient delivery via endocytosis or membrane fusion [22]. These mechanisms enable PEVs to traverse the BBB effectively without the need for additional chemical modification. Consistent with our findings, extracellular vesicles derived from *Momordica charantia* (bitter melon) and *Panax* exhibit exceptional BBB permeability [37, 38]. Compared with synthetic nanocarriers that typically require complex surface engineering and functionalization to achieve targeting capabilities [39, 40], PEVs offer a naturally targeted and more streamlined approach [9, 41]. The intrinsic brain-targeting ability of Sb-EVs, achieved without chemical modification, is accompanied by superior biosafety, minimal immunogenicity, and low cytotoxicity. These attributes render them a highly promising natural vehicle for crossing the BBB and treating CNS disorders.

During AD progression, accumulation of A β in the brain triggers mitochondrial dysfunction, resulting in excessive ROS production, oxidative stress, and ultimately neuronal death [42]. Sb-EVs effectively alleviated A β deposition and NeuN-positive neuronal damage in brain tissues, ultimately improving cognitive deficits in AD mice. Notably, i.v. administered mice exhibited superior AD pathology alleviation compared with that of the orally administered mice. This enhanced efficacy may be attributed to the ability of i.v.-administered Sb-EVs to traverse the BBB and directly target neurons within the brain, thereby significantly improving AD-related symptoms. Gao et al. [43] demonstrated that T807/TPP-modified red blood cell membrane nanoparticles effectively mitigated AD by restoring neuronal mitochondrial function, thereby protecting neurons from apoptosis. Consistently, our results demonstrated that Sb-EVs significantly mitigated A β -

induced oxidative stress and mitochondrial impairment and inhibited apoptosis. The robust efficacy of Sb-EVs likely stems from their unique structural and molecular composition, which distinguishes them from isolated active compounds. Primary flavonoids derived from *S. baicalensis* (such as baicalein and baicalin) possess antioxidant, anti-inflammatory, acetylcholinesterase (AChE) inhibitory, and anti-amyloid aggregation activities [44]. However, their poor aqueous solubility, low bioavailability, and limited BBB permeability significantly hinder their application in the treatment of neurological disorders [45, 46]. Sb-EVs are natural nanovesicles capable of encapsulating a diverse array of bioactive molecules for cross-kingdom regulation [47, 48]. In addition to their intrinsic neuro-targeting capabilities and high cellular affinity, the microRNAs, proteins, and lipids within PEVs all serve as vital bioactive components with distinct regulatory and delivery functions [22, 49, 50]. For example, miRNA5266 from bitter melon-derived extracellular vesicles alleviate ischemic brain injury by reduced MMP-9 expression [38]. Photosynthesis-related and antioxidant proteins derived from spinach-derived thylakoids promote the polarization of pro-inflammatory M1 macrophages toward an anti-inflammatory phenotype, thereby suppressing and remodeling the hyper-inflammatory microenvironment in rheumatoid arthritis [51]. PE, one of the main lipids in Sb-EVs, deficiency leads to decreased mitochondrial membrane potential, impaired respiratory chain supercomplex assembly, and reduced oxygen consumption capacity [29]. Consequently, it is highly likely that Sb-EVs exert their multi-faceted neuroprotective effects through the synergistic interplay of this diverse repertoire of bioactive constituents, thereby offering a highly promising nanoscale therapeutic strategy for AD intervention.

Furthermore, clinical investigations have highlighted that gut microbiota dysbiosis is intrinsically linked to the progression of AD. This dysbiosis not only disrupts the local intestinal environment but also influences CNS functions by modulating metabolites [52]. Dietary supplementation with a *Lactobacillus* and *Bifidobacteria* mixture modulated brain metabolites in the cerebral cortex and hippocampus, effectively enhancing neurotransmission and improving memory [53]. However, the therapeutic efficacy of probiotics is substantially hindered by their low survival and colonic colonization rates [54]. Encapsulation and bioengineering strategies can effectively enhance their viability and colon-targeting capabilities [55, 56], however, the high costs and complex manufacturing procedures associated with these techniques limit their widespread application. As novel prebiotics, PEVs selectively promote the proliferation of probiotics. For example, in colitis models, *M. charantia*-derived extracellular vesicles enrich the gut microbiota with *Muribaculum* and *Alistipes* [26], whereas honeysuckle-derived extracellular vesicles provide therapeutic effects by suppressing *Escherichia-Shigella* and *Akkermansia* overgrowth and promoting beneficial *Eubacterium* and *Turicibacter* growth [57]. Additionally, *Platycodon grandiflorum*-derived extracellular vesicles enhance the efficacy of triple-negative breast cancer immunotherapy by reducing harmful *Bacteroides* and increasing beneficial *Lactobacillus* [58]. In this study, orally administered Sb-EVs, significantly optimized the microbial composition and metabolic profile of AD mice. At the family level, *Lachnospiraceae*, *Oscillospiraceae*, and *Bacteroidaceae* are core probiotic clusters within the intestinal microecosystem. These taxa have robust carbohydrate-degradation capabilities, fermenting dietary fibers to produce short-chain fatty acids (SCFAs), such as butyrate [59]. As the primary energy source for intestinal epithelial

cells, SCFAs strengthen the intestinal barrier function and perform potent anti-inflammatory effects, serving as a critical metabolic cornerstone for maintaining immune homeostasis and metabolic health [60]. However, the proportions of these beneficial taxa were significantly reduced in APP/PS1 mice. Conversely, the abundance of potentially pathogenic families, including *Desulfovibrionaceae* and *Eggerthellaceae*, was significantly higher in the AD model. At the genus level, the relative abundance of *Desulfovibrio* was significantly increased in the gut of APP/PS1 mice. The metabolites of these pathogens, such as hydrogen sulfide (H_2S) and LPS, can further exacerbate systemic inflammation by compromising the intestinal barrier, thereby accelerating the progression of AD-like pathology. Conversely, beneficial SCFA-producing genera, including *Lachnospiraceae*_NK4A136_group, unclassified_*Lachnospiraceae*, and the [*Eubacterium*]*_xylanophilum_group*, showed significantly decreased relative abundance in APP/PS1 mice. Notably, orally administered Sb-EVs effectively corrected the gut microbiota dysbiosis in APP/PS1 mice. Dysbiosis of the gut microbiota drives an imbalance in intestinal metabolites, which amplifies systemic and neuroinflammatory responses, impairs $A\beta$ clearance, and promotes Tau phosphorylation, thereby exacerbating the overall development of AD [61]. In this study, AA and PGF2 α levels were significantly elevated in APP/PS1 mice, indicating profound lipid metabolism disorders and inflammatory cascades. As a key product of the cyclooxygenase (COX) pathway, the increase in PGF2 α levels not only signifies enhanced COX-2-mediated neuroinflammation but also reflects intense lipid peroxidation of AA under oxidative stress [62]. Our findings suggest that Sb-EVs may block further neuronal damage by inhibiting the AA metabolic pathway and scavenging free radicals. Furthermore, the levels of LysoPC(20:4(5Z,8Z,11Z,14Z)/0:0), 27-hydroxycholesterol, and the stress hormone, corticosterone were elevated in APP/PS1 mice. Elevated 27-hydroxycholesterol, a marker of cholesterol overload, drives neuroinflammation and $A\beta$ pathology, whereas fluctuations in corticosterone indicate heightened physiological stress [63]. Notably, Sb-EVs intervention significantly normalized these abnormal metabolic markers. Therefore, orally administered Sb-EVs ameliorate AD progression in APP/PS1 mice by modulating the gut microbiota and its associated metabolic profiles.

5 Conclusions

In summary, this study elucidates that i.v.-administered Sb-EVs can cross the BBB and directly act on neurons. This process effectively alleviates AD by reducing $A\beta$ aggregation-induced overproduction of ROS, mitigating mitochondrial dysfunction, and inhibiting neuronal apoptosis. Conversely, although orally administered Sb-EVs show limited brain penetration, they indirectly ameliorate AD phenotypes through the gut-brain axis, reshaping gut microbiota and associated metabolites. Notably, the therapeutic efficacy of i.v. delivery was significantly superior to that of oral gavage in attenuating AD pathology. These results provide a robust theoretical foundation for the use of Sb-EVs in AD management and a novel, non-toxic, and effective strategy for the intervention of neurodegenerative disorders.

Electronic Supplementary Material: Supplementary material (further details on the biosafety of Sb-EVs, the cytotoxicity of $A\beta$, and H&E stained images of intestinal tissue from AD mice under

different treatments) is available in the online version of this article at <https://doi.org/10.26599/NR.2026.94909027>.

Data availability

Additional data related to this paper may be requested from the corresponding author upon request.

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Declaration of competing interest

All the contributing authors report no conflict of interests in this work.

Author contribution statement

Y. Y.: Data curation, formal analysis, investigation, methodology, writing – original draft, funding acquisition. X. L. Y.: Data curation, formal analysis, investigation, methodology, writing – original draft. W. T. D., Y. X., S. Z., and X. Z. M.: Formal analysis, software, validation, visualization, supervision. F. L. C., S. Y. G., and J. S.: Conceptualization, funding acquisition, project administration, writing – review & editing.

Informed consent

Not applicable.

Ethics statement

The animal experiments were approved by the Committee on Ethical Use of Animals of Hangzhou Medical College (Ethical code: ZJCLA-ACUC-20011131).

Use of AI statement

None.

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