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Original article

The production of recombinant amyloid beta (rAβ) and the effect of exosome from stem cell human exfoliated deciduous teeth (SHED) on anti Aβ aggregation activity

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Abstract

Background: Soluble amyloid beta (Aβ) oligomers have been implicated in synaptotoxicity. These oligomers aggregate into large, insoluble fibrillar plaques, which contribute directly to neuronal cell death. The production of recombinant Aβ (rAβ) is essential for advancing research on its aggregation properties. By generating rAβ, researchers can explore novel therapeutic strategies aimed at modulating Aβ aggregation especially exosomes, it is well-known for their therapeutic potential in various diseases, for example Alzheimer's disease (AD). Therefore, preventing Aβ aggregation represents a promising therapeutic strategy for AD, focusing on eliminating different forms of Aβ and mitigating their toxic effects.

Objective: This study aimed to produce and purify rAβ and investigate the anti-amyloid beta aggregation effects of exosomes derived from stem cells of human exfoliated deciduous teeth (SHED).

Methods: rAβ was expressed in *E. coli* Top10 transformed with the pBAD directional TOPO expression-Aβ1-42 vector (pBAD Aβ1-42). rAβ protein was then purified using $Ni^{2}z$ -NTA affinity chromatography. Finally, the Thioflavin T (Th-T) fluorescence assay was performed to evaluate the anti-Aβ aggregation properties of the exosomes.

Results: The *E. coli* pBAD Aβ1-42 produced rAβ protein (~25 kDa) which contained thioredoxin and a histidine tag following induction with 0.002% arabinose. Subsequent purification confirmed the successful isolation of rAβ, as verified by western blot analysis. Furthermore, exosomes demonstrated the ability to reduce Aβ aggregation ~1-fold change, as indicated by the Th-T fluorescence assay.

Conclusion: We successfully expressed and purified functional rAβ proteins, which can serve as valuable tools for assessing the potential of exosomes in preventing Aβ aggregation.

Keywords: Alzheimer's disease, anti Aβ aggregation, exosome, stem cells.

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Alzheimer's disease is a leading cause of dementia, accounting for an estimated 60–80% of cases.[1] Although the primary cause of the disease remains unclear, the predominant hypothesis suggests that amyloid beta (Aβ) aggregation play a crucial role in its histopathology.[2] Aβ is a peptide derived from the amyloid precursor protein (APP) through sequential cleavage by beta-secretase and gamma-secretase, generating monomers composed of approximately 40–42 amino acid residues. These Aβ monomers can further assemble into oligomers, protofibrils, and mature amyloid fibrils.[3] The Aβ oligomers eventually aggregate into large, insoluble fibrillar plaques, which directly cause neuronal death and activate microglia and astrocytes. This activation leads to the release of cytotoxic proinflammatory cytokines and reactive oxygen species, potentially resulting in neuronal damage.[4] Current treatments focus on reducing pathological lesions, with anti-amyloid aggregation therapies , which directly targets the prevention of amyloid plaque formation.[5] Donanemab, a humanized IgG1 monoclonal antibody against the pyroglutamate form of Aβ, has been developed to inhibit its aggregation. Clinical studies have demonstrated that Donanemab reduces amyloid plaques in early-stage patients. However, it has also been associated with reduced brain volume and amyloid-related cerebral edema.[6] Therefore, inhibiting Aβ aggregation represents a promising therapeutic strategy for Alzheimer's disease, with the potential to eliminate various forms of Aβ and mitigate their toxicity.

Nowadays, stem cell-based therapy is emerging as a promising alternative treatment for neurodegenerative diseases, particularly Alzheimer's disease. Several studies have highlighted the therapeutic potential of mesenchymal stem cell (MSC)-derived secretomes, which contain bioactive components that promote neuroprotection and neuroregeneration.[7] Interestingly, the profile of secretory factors within the secretome may vary depending on the source of the stem cells. For instance, stem cells from human exfoliated deciduous teeth (SHED), a type of dental MSC, have demonstrated greater therapeutic potential compared to bone marrow-derived mesenchymal stem cells (BMSCs). Research has shown that the SHED-derived secretome enhances cognitive function in an in vivo Alzheimer's disease model, reduces oxidative stress, and shifts microglia from the pro-inflammatory M1 phenotype to the anti-inflammatory M2 phenotype, thereby decreasing inflammation. In contrast, the BMSC-derived secretome has shown comparatively lower efficacy.[8,9]

Several studies utilizing in vitro Alzheimer's disease models commonly employ Aβ synthesized through the O-acyl-iso-peptide method, which modifies the protein structure by adding chemical functional groups to enhance solubility. While this method improves protein solubility, it may also interfere with amyloid plaque formation and reduce the protein's toxicity.[10] Additionally, previous studies have shown that recombinant Aβ (rAβ) significantly increases lactate dehydrogenase (LDH) activity in rat primary cortical neurons, leading to neuronal death compared to synthetic Aβ (sAβ).[11] Furthermore, other research has demonstrated that rAβ can induce apoptosis in SH-SY5Y cells.[12] In this study, we aim to establish rAβ as an inducer in an *in vitro* model. The pBAD/TOPO™ ThioFusion™ Expression Kit is used for rAβ production, which enhances solubility by incorporating a fusion protein without altering the native protein structure, thereby preserving amyloid plaque formation. This research investigates the effects of exosomes derived from SHED in preventing amyloid aggregation using an *in vitro* model with rAβ.

Materials and Methods

Construction of Aβ gene and introduction into pBAD directional TOPO expression vector

Amyloid beta sense and antisense strands were annealed and extended to form double-stranded DNA. The resulting double-stranded DNA was PCR-amplified using specific primers for directional cloning into the pBAD TOPO vector. The product was ligated, transformed into E. coli TOP10, and selected on kanamycin-containing medium. Plasmids from positive clones were extracted, sequenced, and compared with the GenBank reference to confirm accuracy.

Expression of recombinant amyloid beta – 6xHis fusion protein

Escherichia coli Top 10 strain transformed with pBAD directional TOPO expression-Aβ1-42 vector (*E. coli* pBAD Aβ1-42) were inoculated into LB broth that contained 50 μg/ml kanamycin and grown overnight at 37 °C with shaking for 200rpm. The overnight culture was inoculated into LB broth and incubated at 37°C for 2–3 hours until it reached the mid-log phase (OD600 ~0.4–0.5). Recombinant Aβ1-

42 protein expression was induced by adding 0.002% arabinose, followed by incubation at 37°C for 6 hours. The cells were then harvested by centrifugation at 4,000 rpm for 20 minutes. The resulting pellet was washed and resuspended in cold PBS, and protein expression was analyzed using SDS-PAGE analysis.

Affinity purification of recombinant amyloid beta – 6xHis fusion protein

Following the induction of recombinant Aβ1-42 protein expression, the protein was extracted and purified under denaturing conditions using Ni²z -NTA affinity chromatography. The purification buffers contained sodium dihydrogen phosphate (NaH_2PO_4), Tris-Hydroxy methyl aminomethane (Tris-base), and urea. Initially, the cells were resuspended in a binding buffer at pH 8.0. The cell suspension was then lysed by sonication on ice. The resulting lysate was incubated with Ni²z -NTA beads at room temperature with shaking for 1–2 hours to allow specific binding of the recombinant Aβ1-42-6xHis fusion protein. After incubation, the beads were washed with wash buffers at pH 6.3. Finally, the bound protein was eluted using an elution buffer at pH 4.5. The purity of the recombinant Aβ1-42-6xHis protein was confirmed by SDS-PAGE analysis.

SDS-PAGE analysis

Expressed or purified recombinant proteins were separated on a 10% SDS-PAGE at 150V for 1 hour using EPS 301 Electrophoresis Power Supply (Cytiva 18-1130-01) in 1X running buffer composing composition of tris-base, glycine and SDS. After, the gel was stained with Coomassie brilliant blue R-250 for 15 minutes, then destained using destain solution approximately 1 hour. Finally, the gel was visualized by an Amersham™ ImageQuant 800 system.

Western blotting

Expressed or purified recombinant proteins were separated using SDS-PAGE analysis. The proteins were transferred onto a 0.45 μm pore nitrocellulose membrane at 20V for 35 minutes. The membrane was blocked with 5% skim milk at 4°C overnight and subsequently incubated with a primary antibody. The primary antibodies used were either mouse anti-His monoclonal antibody (1:1000, GE Healthcare/Cytiva 27-4710-01) or mouse anti-Aβ monoclonal antibody (1:10000, abcam, ab126649) at room temperature for 2 hours. The following three washes with PBST, the membrane was incubated with a goat anti-mouse HRP-conjugated secondary antibody (1:10000, ImmunoTools, Cat-No: 22549819) at room temperature for 1 hour, followed by three additional washes with PBST. The membrane was then incubated with an ECL substrate solution for 3 minutes and visualized using an Amersham™ ImageQuant 800 system.

Exosome preparation and purification

Methods for exosome isolation and characterization were performed as described previous study. [13] Briefly, SHED cells were cultured to 80% confluence, washed, and incubated in serum-free DMEM. After 48 hours, the conditioned medium was collected, filtered, and concentrated. Exosomes were isolated by differential ultracentrifugation, resuspended in PBS, and stored at “80°C. Concentration was measured by Nanodrop, while size, morphology using NTA, TEM and exosome markers (CD9, CD63, CD81) were analyzed by flow cytometry or western blotting.

Thioflavin T fluorescence assay

Purified recombinant amyloid beta (rAβ) and synthetic amyloid beta (sAβ) were incubated at 37°C for 4 days with 20 μg/ml of exosome that isolation and characterization were performed following the methodology outlined in a previous study [13] in PBS (pH 7.4) to promote Aβ aggregation and anti-Aβ aggregation. A non-aggregated control sample was stored at -80°C. Subsequently, samples containing aggregated or non-aggregated Aβ were mixed with 10 μM Thioflavin T (Th-T) in a final volume of 100 μl. Fluorescence measurements were recorded at an excitation wavelength of 450 nm and an emission wavelength of 490 nm. Background fluorescence was determined using 10 μM Th-T alone. Each experiment was conducted in triplicate, and the average value was reported.

Statistical analysis

All results are expressed as the mean ± standard error of the mean (SEM). Data were analyzed using a t-test and one-way analysis of variance (ANOVA). Differences were considered statistically significant at $P < 0.05$.

Results

The Aβ gene sequencing

After the amyloid beta gene was successfully cloned into the pBAD vector, the plasmids were extracted from *E. coli* and subjected to DNA sequencing. The sequencing results confirmed that the amyloid beta gene sequence was 100% identical to their corresponding reference sequences in GenBank (**Figure 1**).

Expression of recombinant amyloid beta – 6xHis fusion protein in E. coli pBAD Aβ1-42

The E. coli pBAD Aβ1-42 produced recombinant Aβ protein (~25 kDa) which contained thioredoxin and a histidine tag (**Figure 2**) following induction with 0.002% arabinose, as demonstrated by SDS-PAGE analysis (**Figure 3A**, Lane 2). In contrast, no protein expression was observed in the absence of arabinose induction (**Figure 3A**, Lane 1). Western blot analysis further confirmed the expression of recombinant Aβ1-42-6xHis fusion protein using mouse anti-His IgG and mouse anti-Aβ IgG antibodies **(Figure 3B**, Lane 2). No target protein expression was detected in the non-induced E. coli pBAD Aβ1-42 (**Figure 3B**, Lane 1). These results confirm that the E. coli pBAD Aβ1-42 successfully expressed the recombinant Aβ1-42-6xHis fusion protein upon induction with 0.002% arabinose.

Affinity purification of recombinant amyloid Beta – 6xHis fusion protein

E. coli pBAD Aβ1-42 was induced with 0.002% arabinose for 6 hours, and the recombinant Aβ1-42–6xHis fusion protein was subsequently purified using Ni^{2+}-NTA beads. SDS-PAGE analysis was performed to visualize different fractions, including the cell lysate before binding to Ni^{2+}-NTA beads (**Figure 4A**, Lane 1), the cell lysate after binding (**Figure 4A**, Lane 2), the flow-through from the pH 6.3 wash (**Figure 4A**, Lanes 3 and 4), and the eluted fractions (**Figure 4A**, Lanes 5–9), all of which confirmed the presence of the purified protein. Western blot analysis using mouse anti-His IgG and mouse anti-Aβ IgG further validated the identity of the purified recombinant Aβ protein (**Figure 4B**, Lanes 1–3). Strong protein bands at approximately 25 kDa confirmed the successful purification of the target protein. Additionally, the yield of the recombinant amyloid beta–6xHis fusion protein was calculated, with results indicating an approximate yield of 0.15 mg per 1 mL of culture.

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>ref|NM_201414.1| GM Homo sapiens amyloid beta (A4) precursor protein (APP), transcript
variant 3, mRNA
Length=3416

 Score =  233 bits (126),  Expect = 2e-59
 Identities = 126/126 (100%), Gaps = 0/126 (0%)
 Strand=Plus/Plus

Query  1     GATGCAGAATTCCGACATGACTCAGGATATGAAGTTCATCATCAAAAATTGGTGTTCTTT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1983  GATGCAGAATTCCGACATGACTCAGGATATGAAGTTCATCATCAAAAATTGGTGTTCTTT  2042

Query  61    GCAGAAGATGTGGGTTCAAACAAAGGTGCAATCATTGGACTCATGGTGGGCGGTGTTGTC  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2043  GCAGAAGATGTGGGTTCAAACAAAGGTGCAATCATTGGACTCATGGTGGGCGGTGTTGTC  2102

Query  121   ATAGCG  126
             ||||||
Sbjct  2103  ATAGCG  2108
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Figure 1. Analysis of the sequencing data confirmed the identity of the amyloid beta gene. The amyloid beta gene was successfully sequenced, and the results were consistent with the reference sequence

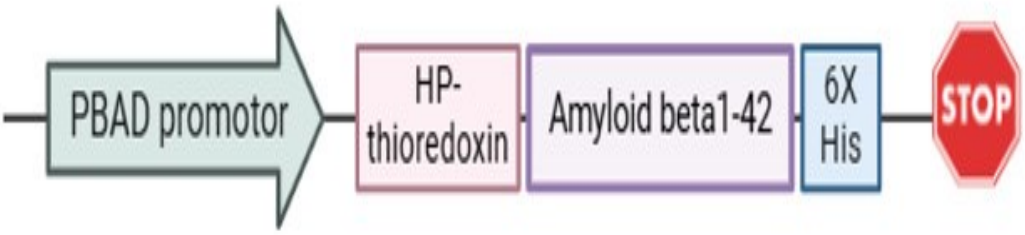

Figure 2. Construction of pBAD directional TOPO expression-Aβ1-42 vector (pBAD202/D-TOPO®/invitrogen).

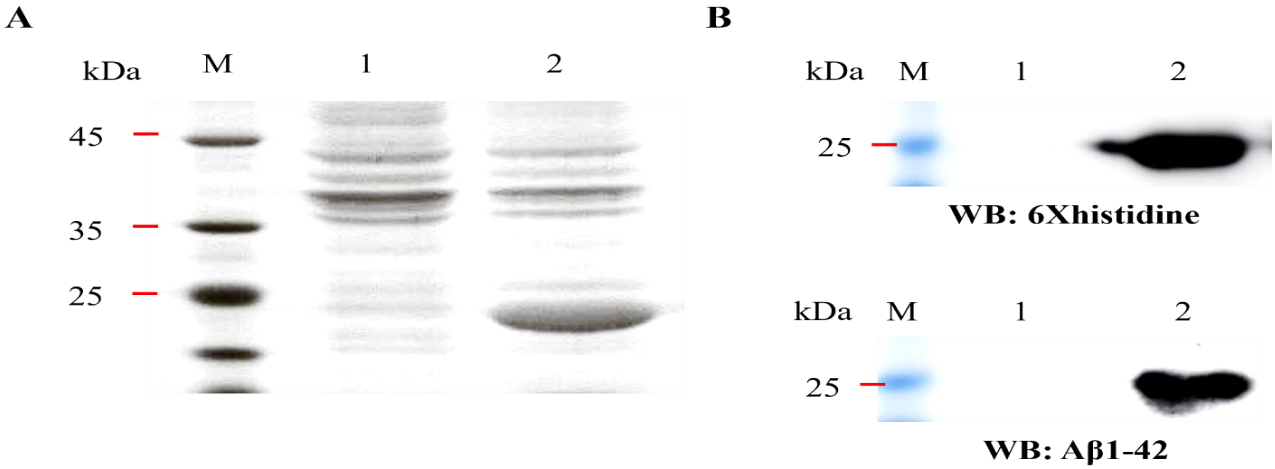

Figure 3. (**A**) Expression of recombinant amyloid beta – 6xHis fusion protein in E. coli pBAD Aβ1-42. SDS-PAGE analysis of E. coli pBAD Aβ1-42 with and without induction using 0.002% arabinose at 37°C for 6 hours. M, protein molecular weight marker; Lane 1, non-induced; Lane 2, induced. (**B**) Western blot analysis of 6xHis-tagged Aβ protein. Lane 1, total protein from non-induced E. coli Top10; Lane 2, total protein from E. coli Top10 induced with 0.002% arabinose.

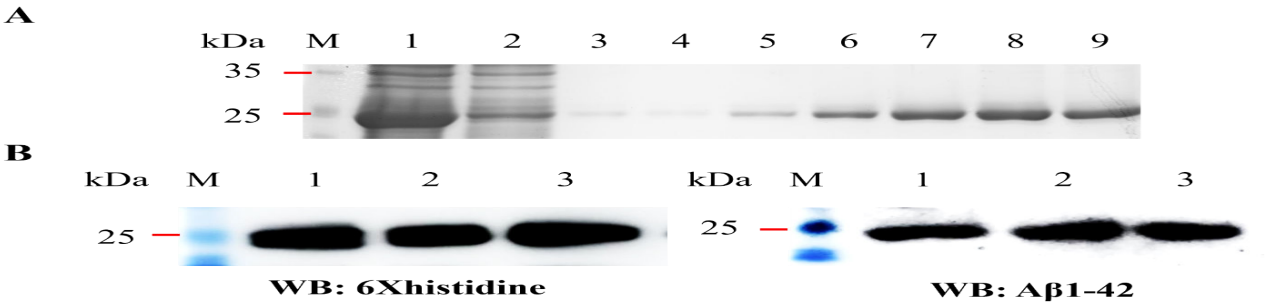

Figure 4. Purification of recombinant amyloid beta – 6xHis fusion protein from E. coli Top10. (**A**) SDS-PAGE analysis of different fractions from *E. coli* Top10 after induction with 0.002% arabinose at 37°C for 6 hours. M, protein molecular weight marker; Lane 1, cell lysate before binding to Ni²z -NTA beads; Lane 2, cell lysate after binding; Lanes 3-4, the flow-through from the pH 6.3 wash; Lanes 5-9, eluted fractions (**B**) Western blot analysis of purified recombinant Aβ1-42-6xHis fusion protein using mouse anti-His IgG and mouse anti-Aβ IgG. Lanes 1–3, eluted fractions from Ni²z -NTA affinity chromatography.

Anti-aggregation activity of exosomes on recombinant amyloid beta – 6xHis fusion protein

A Thioflavin T (Th-T) fluorescence assay was performed to evaluate the anti-amyloid aggregation effect of exosomes. As shown in **Figure 5**, rAβ exhibited a significant increase in protein aggregation approximately 1.84-fold change compared to the non-aggregated rAβ control ($P < 0.001$). Furthermore, when rAβ was co-incubated with 20 µg/mL exosomes, the results indicated that exosomes significantly reduced rAβ aggregation 1.14-fold change results in close to the non-aggregated rAβ control. Similarly, sAβ showed a significant increase in protein aggregation 1.50-fold change compared to the non-aggregated sAβ control ($P < 0.05$), while co-incubation with exosomes led to a reduction in aggregated sAβ approximately 1.00-fold change compared to the non-aggregated sAβ.

Discussion

This study successfully expressed recombinant amyloid beta (rAβ) protein in *E. coli* pBAD Aβ1-42 that contained thioredoxin and a histidine tag. Induction with 0.002% arabinose for 6 hours resulted in the production of an approximately 25 kDa protein. Thioredoxin is well known for enhancing the solubility of fusion proteins, while the histidine tag facilitates purification via affinity chromatography. [14] In this study, Ni^{2+}-NTA affinity chromatography was employed for purification. One major challenge in recombinant protein production using E. coli is the formation of inclusion bodies, which lead to protein insolubility. To address this, purification was performed under denaturing conditions to solubilize the protein and ensure specific binding between the histidine tag and Ni^{2+}-NTA beads. [15]

Furthermore, we investigated the potential of exosomes derived from SHED to interact with purified

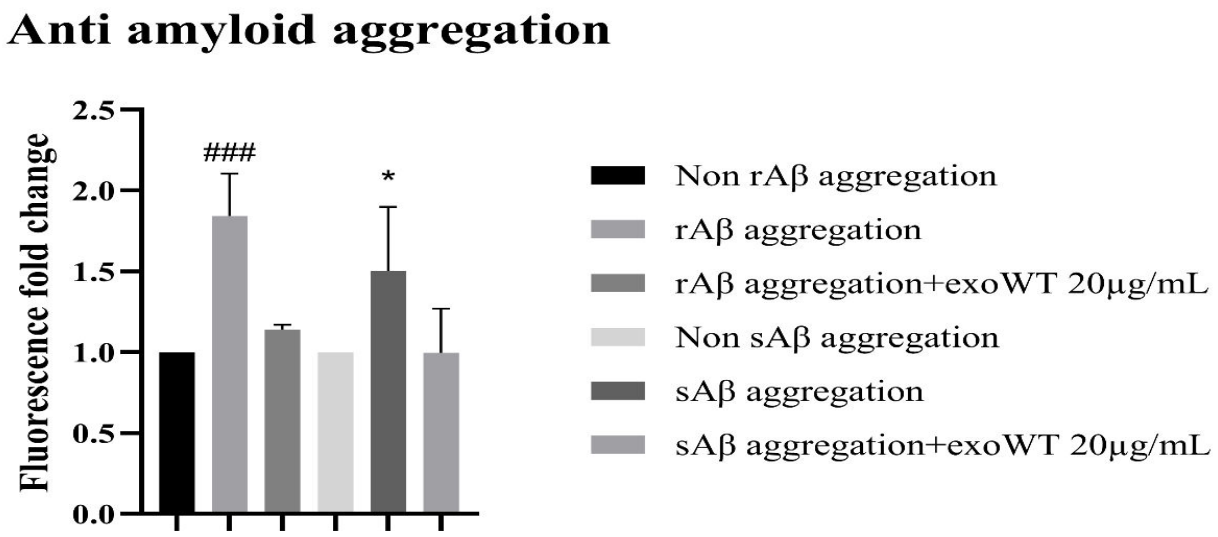

Figure 5. Anti-amyloid beta aggregation activity of exosomes. A Th-T fluorescence assay was conducted using 2.6 μM rAβ and 5 μM sAβ, both with and without exosome co-incubation. The aggregated and non-aggregated samples were then incubated with 10 μM Th-T in PBS, and fluorescence intensity was measured to evaluate the level of amyloid aggregation. (###$P < 0.001$) compared to the non-aggregated rAâ control, (*$P < 0.05$) compared to the non-aggregated sAβ control.

rAβ and sAβ proteins, aiming to modulate oligomer formation into non-toxic species. Using the Thioflavin T (Th-T) assay as following procedure from previous study[16], we assessed fluorescence intensity, which correlates with Th-T binding to amyloid fibrils, indicating aggregation levels. Following a 4-day incubation at 37°C with or without exosomes, we observed anti-aggregation activity against Aβ, The results showed that co-incubation with exosomes reduced protein aggregation to a level equivalent to the non-aggregated control. Previous research has demonstrated that the dental pulp stem cell secretome (DPSC secretome) can proteolytically degrade Aβ in vitro. Additionally, it was found that the DPSC secretome contains the neprilysin (NEP) enzyme, which plays a role in Aβ degradation.[17] Therefore, it is possible that exosomes derived from SHED may also contain NEP enzyme and contribute to Aβ degradation. Therefore, regarding further study aim to compare the toxicity of recombinant and synthetic Aβ aggregates on neurons, clarify the role of exosomes in Aβ-induced neuronal effects, and further investigate the mechanisms underlying Alzheimer's disease.

Conclusion

This study successfully expressed and purified functional recombinant Aβ proteins, which can serve as valuable reagents for evaluating the effects of exosomes on Aβ aggregation inhibition.

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Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data sharing statement

All data generated or analyzed during this study are included in the published article. Additional information is available from the corresponding author upon reasonable request for non-commercial purposes.

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