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Mixed oligomers and monomeric amyloid-β disrupts endothelial cells integrity and reduces monomeric amyloid-β transport across hCMEC/D3 cell line as an in vitro blood–brain barrier model

Hisham Qosa [a], Harry LeVine III [b], Jeffrey N. Keller [c], Amal Kaddoumi [a,*]

[a] Department of Basic Pharmaceutical Science, School of Pharmacy, University of Louisiana at Monroe, Monroe, LA, USA
[b] Sanders-Brown Center on Aging, University of Kentucky, Lexington, KY, USA
[c] Pennington Biomedical Research Center, Louisiana State University, Baton Rouge, LA, USA

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ABSTRACT

Senile amyloid plaques are one of the diagnostic hallmarks of Alzheimer's disease (AD). However, the severity of clinical symptoms of AD is weakly correlated with the plaque load. AD symptoms severity is reported to be more strongly correlated with the level of soluble amyloid-β (Aβ) assemblies. Formation of soluble Aβ assemblies is stimulated by monomeric Aβ accumulation in the brain, which has been related to its faulty cerebral clearance. Studies tend to focus on the neurotoxicity of specific Aβ species. There are relatively few studies investigating toxic effects of Aβ on the endothelial cells of the blood–brain barrier (BBB). We hypothesized that a soluble Aβ pool more closely resembling the in vivo situation composed of a mixture of $Aβ_{40}$ monomer and $Aβ_{42}$ oligomer would exert higher toxicity against hCMEC/D3 cells as an in vitro BBB model than either component alone. We observed that, in addition to a disruptive effect on the endothelial cells integrity due to enhancement of the paracellular permeability of the hCMEC/D3 monolayer, the Aβ mixture significantly decreased monomeric Aβ transport across the cell culture model. Consistent with its effect on Aβ transport, Aβ mixture treatment for 24 h resulted in LRP1 down-regulation and RAGE up-regulation in hCMEC/D3 cells. The individual Aβ species separately failed to alter Aβ clearance or the cell-based BBB model integrity. Our study offers, for the first time, evidence that a mixture of soluble Aβ species, at nanomolar concentrations, disrupts endothelial cells integrity and its own transport across an in vitro model of the BBB.

1. Introduction

Alzheimer's disease (AD) is the most common cause of irreversible dementia among the elderly with a rapidly increasing socioeconomic impact [1]. During the last decade, amyloid- and tau-related neuropathologies were considered as the main underlying causes of neurodegeneration, cognitive decline and memory loss associated with AD [2,3]. Amyloid-β (Aβ) peptides are derived from a minor pathway of proteolytic processing of the amyloid-β precursor protein (APP). In AD, amyloid peptides, mainly $Aβ_{40}$ and $Aβ_{42}$, accumulate in the parenchymal tissue and the vasculature of cortical and hippocampal regions of the brain where they assemble and form insoluble plaques [4].

Aβ has multiple different assembly states ranging from monomer to insoluble plaque and all of these states have been identified in the brain of AD patient [5,6]. Two main pools of Aβ have been distinguished in the brain of AD patients, a soluble pool that consists of a mixture of Aβ monomers and soluble oligomers, and an insoluble pool of insoluble oligomers and higher order histologically prominent insoluble Aβ fibrils [7]. Increasing evidence indicates that soluble pool of Aβ is more biologically active than the insoluble Aβ fibrils [8,9]. Moreover, a comparison of pathology with clinical diagnosis of AD brains found a weak correlation between Aβ plaque load and the progression of AD symptoms, in contrast to a better correlation of the soluble pool of Aβ with AD clinical severity [8,10,11].

The soluble Aβ pool consists of multiple species of Aβ; however, $Aβ_{40}$ and $Aβ_{42}$ are the most abundant, readily identifiable, and are considered the most important components contributing to the pathology of AD. $Aβ_{40}$ is the most abundant Aβ species in the brain of AD patients, however, $Aβ_{42}$ is the main peptide involved in the soluble oligomers due to its high propensity to aggregate [12]. Moreover, $Aβ_{42}$ oligomers that are initially formed act as a seed that can accelerate the

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid-β; APP, amyloid-β precursor protein; BBB, blood–brain barrier; IDE, insulin degrading enzyme; LRP1, low density lipoprotein receptor-related protein-1; NEP, neprilysin; P-gp, P-glycoprotein; RAGE, receptor for advanced glycation end products; TCA, trichloroacetic acid

* Corresponding author at: Department of Basic Pharmaceutical Science, School of Pharmacy, University of Louisiana at Monroe, 1800 Bienville Dr., Monroe, LA 71201, USA.
E-mail address: kaddoumi@ulm.edu (A. Kaddoumi).

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accumulation of $A\beta_{40}$, which is present in the brain at concentrations several-fold higher than $A\beta_{42}$ [6].

In late-onset "sporadic" AD, extensive studies have suggested that reduced clearance of Aβ from the brain across the blood–brain barrier (BBB) to the periphery significantly contributes to its accumulation in the brain [13]. P-glycoprotein (P-gp) and low-density lipoprotein receptor-related protein-1 (LRP1) at the BBB have been reported to play important roles in Aβ clearance from the brain to the blood [14–16]. On the other hand, the receptor for advanced glycation end products (RAGE) mediates influx of Aβ from blood to brain [17]. In addition to clearance via transport across the BBB, monomeric Aβ is subjected to proteolytic degradation by insulin degrading enzyme (IDE) and neprilysin (NEP) that are expressed in different cellular component of the brain including BBB endothelium [18–22]. Previous in vitro and in vivo studies have demonstrated reduced expression of P-gp in brain capillaries treated with $A\beta_{40}$ or $A\beta_{42}$ monomers, or $A\beta_{42}$ oligomers [23,24]; LRP1 and RAGE gene expressions were reduced in the brain of wild type mice treated with human $A\beta_{42}$ monomers but not with $A\beta_{40}$ monomers [25]. However, none of these studies investigated the effect of Aβ treatment on its own clearance. To our knowledge, studies investigating the effect of more physiologically and pathologically relevant Aβ peptide composition on the clearance of Aβ monomers across the BBB and its degradation are lacking.

Several pathological alterations in BBB function have been observed in AD patients. Disrupted capillary integrity, loss of controlled molecular transport, and uncontrolled solute exchange are among these pathological alterations [26]. Furthermore, available studies have reported loss of tight junction proteins necessary to restrict paracellular transport between blood and brain, which contributes to the pathogenesis of AD [27]. Accumulating evidence suggests that Aβ has disruptive effects on the integrity of the BBB [24,28–30]. In addition, about 80–90% of AD patients develop cerebral amyloid angiopathy (CAA) that is characterized by Aβ accumulation in brain blood vessel walls and associated with compromised BBB function [31]. Available studies have shown that increased levels of soluble Aβ affect integrity of BBB endothelial cells by reducing the expression and re-localization of tight junction proteins [28–30]. However, most of these studies, which investigated the toxicity of specific species of soluble Aβ isoforms against BBB endothelium used high non-physiological or even pathological Aβ concentrations. Thus, evaluating the effect of Aβ on BBB endothelial cells integrity in the presence of more than one Aβ species at nanomolar concentrations provides a more biologically relevant approach to clarify the pathological changes that are associated with Aβ in AD. Accordingly, in this study, we hypothesized that Aβ mixture consisting of nanomolar levels of both $A\beta_{40}$ monomer and $A\beta_{42}$ oligomer exerts greater toxicity against endothelial cells of the BBB than the individual species. To test this hypothesis, the human brain endothelial cell line hCMEC/D3, as an in vitro cell-based BBB model, was used to study the effect of Aβ mixture on the transport of Aβ monomer and Aβ toxic effect on BBB endothelial cells.

2. Methodology

2.1. Preparation and characterization of synthetic amyloid-β mixture

Solutions of synthetic $A\beta_{40}$ and $A\beta_{42}$ peptides (AnaSpec, Inc.; CA) were prepared by suspending in 1, 1, 1, 3, 3, 3-hexafluoro-2-propanol (HFIP) (Sigma-Aldrich, MO) each at a concentration of 1 mM and incubated for 1 h at room temperature for complete solubilization. Aβ solutions were aliquoted, HFIP was evaporated overnight and the peptides stored at −20 °C as an HFIP film. For $A\beta_{40}$ monomer, HFIP film was dissolved in media and used immediately for cell treatment or for preparation of Aβ mixture as described below. $A\beta_{42}$ oligomer was prepared as described previously [32]. Briefly, aliquoted $A\beta_{42}$ peptide HFIP-film was suspended in anhydrous DMSO (Sigma-Aldrich, MO) to a final concentration of 5 mM, vortex mixed for 1 min. DMSO solution of $A\beta_{42}$ was diluted with phenol red-free F-12 cell culture media (Gibco, NY) to a concentration of 100 μM, vortexed for 1 min and incubated at 4 °C for 24 h. At the end of incubation period, the $A\beta_{42}$ oligomer solution was centrifuged at 14,000 rpm, 4 °C for 10 min. The supernatant was fractionated at room temperature using size exclusion chromatography (SEC) to separate oligomers from monomers. Two hundred microliters of the supernatant was injected onto a sephadex G-75 column and eluted with 15 ml media at flow rate of 0.5 ml/min. Twenty-four fractions of 0.5 ml were collected after the elution of the first 3 ml. $A\beta_{42}$ oligomer in each fraction was assessed by sandwich ELISA as described previously [33]. 6E10 (aa. 3–8 human Aβ sequence) monoclonal antibody (Covance Research Products, MA) was coated at 5 μg/ml concentration (100 ng/well) on an Maxisorp ELISA plate (Thermo, NY) to capture $A\beta_{42}$ oligomer. Detection was achieved with HRP-conjugated 6E10 antibody at 1 μg/ml (Covance Research Products, MA). Fractions that contained $A\beta_{42}$ oligomers were collected and used immediately after proper dilution to the required concentration for cell treatment or for preparation of Aβ mixtures. Aβ mixtures were prepared by mixing $A\beta_{40}$ monomers at concentrations 0, 50, 100 or 250 nM and $A\beta_{42}$ oligomers at concentrations 0, 25, 50, or 100 nM. Treatment concentrations of $A\beta_{40}$ monomer, $A\beta_{42}$ oligomer and Aβ mixtures were measured using western blot analyses. Forty microliters of the treatment solution and Aβ standards (500, 250, 100, 50, 25 nM) were resolved on a 16% Bis–Tris gel in 3-(N-morpholino) propanesulfonic acid buffer system and transferred onto a 0.45 μm pore size nitrocellulose membrane (Bio-Rad, CA). The membrane was blocked with 5% BSA in TBS buffer (20 mM Tris–HCl, 150 mM NaCl pH 7.5) for 1 h and incubated with 6E10 antibody (1:1000 dilution in TBS buffer containing 5% BSA and 0.05% Tween-20) for 3 h at room temperature. For antigen detection, the membranes were washed and incubated with HRP-labeled secondary anti-mouse (Santa Cruz, TX) at 1:5000 dilution. The bands were visualized using a SuperSignal West Femto detection kit (Thermo Scientific, IL). Quantitative analysis of the immunoreactive bands was performed using a GeneSnap luminescent image analyzer (Scientific Resources Southwest, TX), and band intensities were measured by densitometry analysis. Band intensities were plotted against concentration of standards and the concentration of treatment solution were interpolated from the resulting calibration curve. For all experiments, $A\beta_{42}$ oligomers and Aβ mixtures were prepared in the same way described above using Aβ standards purchased from the same manufacturer (AnaSpec, Inc.).

2.2. Cell culture

Human brain endothelial cells (hCMEC/D3; kindly provided by Dr. P.O. Couraud, Institute Cochin, Paris, France), passages 25–35, were used as a representative model for human BBB. hCMEC/D3 cells were cultured in EBM-2 medium (Lonza, MD) supplemented with 1 ng/ml human basic fibroblast growth factor (Sigma-Aldrich, MO), 10 mM HEPES, 1% chemically defined lipid concentrate (Gibco, NY), 5 μg/ml ascorbic acid, 1.4 μM hydrocortisone, 1% penicillin–streptomycin and 5% of heat-inactivated FBS gold (GE Healthcare Life Sciences, PA). Cultures were maintained in a humidified atmosphere (5% CO_2/95% air) at 37 °C and media was changed every other day.

2.3. Toxicity of synthetic amyloid-β mixture on hCMEC/D3 cells

MTT cytotoxicity assay was performed to select sub-toxic concentrations of Aβ preparations. hCMEC/D3 cells were seeded onto 24-well plate and maintained as described above. At 70% confluence, cells were treated for 24 h with $A\beta_{40}$ monomer, $A\beta_{42}$ oligomers or mixtures of $A\beta_{40}$ monomer and $A\beta_{42}$ oligomers at different concentrations. $A\beta_{40}$ monomer concentration dependent toxicity against hCMEC/D3 cells were studied at the concentrations 0, 50, 100 and 250 nM in the presence or absence of 50 nM $A\beta_{42}$ oligomers. For $A\beta_{42}$ oligomers concentration dependent study, cells were treated with 0, 25, 50 and 100 nM

$A\beta_{42}$ oligomers with or without 100 nM $A\beta_{40}$ monomer. MTT cytotoxicity assays (Trevigen, MD) were performed at the end of incubation period with Aβ preparations as described previously [34].

2.4. Effect of synthetic amyloid-β mixtures on hCMEC/D3 cells monolayer integrity

The barrier integrity of hCMEC/D3 was evaluated by measuring ^{14}C-inulin ([carboxyl-^{14}C]-inulin, M.W.: 5000 Da; American Radiolabeled Chemicals, MO) permeation. Inulin is a marker for paracellular transport across a cell monolayer where its transendothelial transport across intact hCMEC/D3 monolayer is greatly restricted [35]. To prepare hCMEC/D3 cell monolayers, transwell polyester membrane inserts, 6.5 mm diameter with 0.4 μm pores (Corning, NY), were coated with rat tail collagen-IV (150 μg/ml) for 90 min at 37 °C. Cells were plated onto coated inserts at a seeding density of 50,000 cells/cm^2, medium was changed every other day. Trans-epithelial electrical resistance (TEER) was measured using an EVOM epithelial volt-ohmmeter with STX2 electrodes (World Precision Instruments, FL). hCMEC/D3 cell monolayers were used for $A\beta_{40}$ transport and inulin permeation experiments on day 6 of culture [21,35]. On day 6, the TEER value was measured and ranged from 35 to 40 Ω cm^2 that is consistent with previously reported values for this cell line [35]. Cells were treated for 24 h treatment with increasing concentration of monomeric $A\beta_{40}$ in the presence or absence of 50 nM $A\beta_{42}$ oligomer or increasing concentration of $A\beta_{42}$ oligomer with or without 100 nM monomeric $A\beta_{40}$. At the end of treatment, apical to basolateral ^{14}C-inulin permeation coefficient was measured. For inulin permeation, 200 μl of fresh media containing 0.05 mM ^{14}C-inulin was added to the apical chamber and 800 μl of fresh media was added to the basolateral chamber. Cells were maintained in a humidified atmosphere (5% CO_2/95% air) at 37 °C for the time course of the permeability experiment (up to 2 h). Fifty microliter aliquots from the basolateral chamber were collected at 15, 30, 45, 60, 90, 120 min and replaced with 50 μl of fresh media to keep the volume of basolateral chamber unchanged and maintain constant osmotic pressure. After mixing samples with 5 ml of scintillation cocktail, ^{14}C-inulin dpm was measured using a Wallac 1414 WinSpectral Liquid Scintillation Counter (PerkinElmer, MA). Concentrations of ^{14}C-inulin in basolateral side (in dpm/ml) were plotted versus time. ^{14}C-inulin permeation coefficient (P_{app} in cm/s) was calculated from the following equation [36]:

$$P_{app} = (\Delta Q/\Delta t)/(A * C_o) \quad (1)$$

where ΔQ/Δt is the linear appearance rate of the ^{14}C-inulin in the basolateral chamber, A is the surface area of the cell monolayer (0.33 cm^2) and Co is the initial concentration of ^{14}C-inulin (dpm/ml).

2.5. Effect of synthetic amyloid-β mixtures on ^{125}I-$A\beta_{40}$ transport across hCMEC/D3 cell monolayer

The transport of ^{125}I-$A\beta_{40}$ (PerkinElmer, MA) and ^{14}C-inulin, as a marker for paracellular diffusion, were measured across hCMEC/D3 monolayer after treatment with different Aβ preparations. hCMEC/D3 cell monolayers were seeded and maintained in transwell chambers as described above. One day before conducting transport experiments,

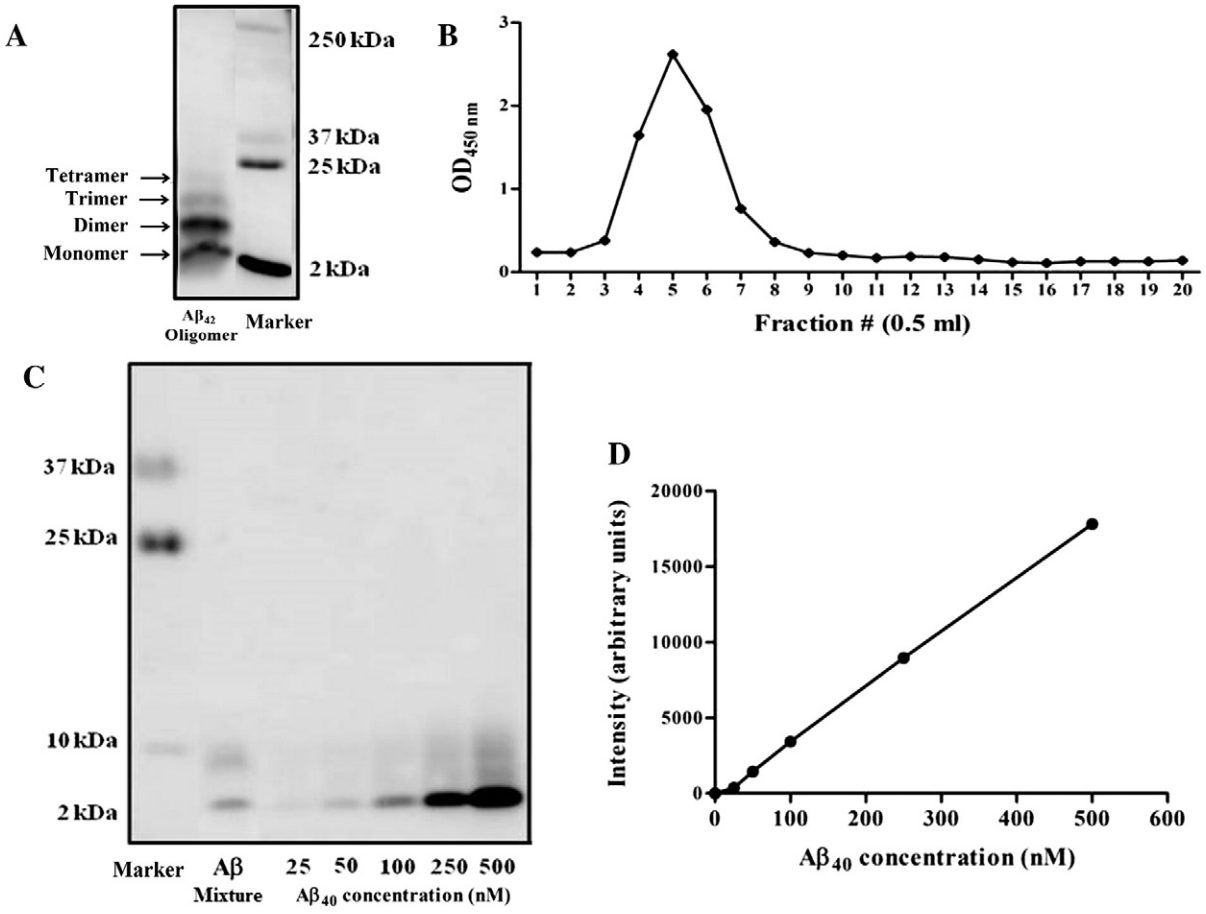

Fig. 1. Preparation of Aβ mixtures. Starting from synthetic $A\beta_{42}$ monomer, a stable oligomer was prepared and fractionated by SEC. (A) A representative blot of crude $A\beta_{42}$ oligomers. (B) SEC profile of $A\beta_{42}$ oligomers as measured by an oligomer-specific ELISA. Fractions 4–8 containing oligomers were collected for further analysis. (C) A representative western blot analysis for purified $A\beta_{42}$ oligomer, Aβ mixture and a calibration curve of monomeric synthetic $A\beta_{40}$ standards to adjust the concentration of each species for cell treatment. (D) Densitometry versus concentration calibration curve that was used to quantify Aβ concentrations.

cells were treated with increasing concentration of monomeric $A\beta_{40}$ in the presence or absence of 50 nM $A\beta_{42}$ oligomer or increasing concentration of $A\beta_{42}$ oligomer with or without 100 nM monomeric $A\beta_{40}$. Basolateral to apical (B → A) transport studies were then performed as described previously [21]. In brief, the transport study was initiated by removing media that contained Aβ mixture and addition of 800 μl of fresh media containing 0.1 nM ^{125}I-$A\beta_{40}$ and 0.05 mM ^{14}C-inulin to the basolateral compartment. At the end of incubation period (6 h), media from both compartments and cells were separately collected for ^{125}I-$A\beta_{40}$ analysis and ^{14}C-inulin measurement [21]. The transport quotients of B → A (^{125}I-$A\beta_{40}$ $CQ_{B \rightarrow A}$) transport were calculated using the following equation [37]:

$$^{125}\text{I-A}\beta_{40}\ \ CQ_{B\rightarrow A} = \frac{\left(\frac{^{125}\text{I-A}\beta_{40}\ \text{in apical compartment}}{^{125}\text{I-A}\beta_{40}\ \text{total}}\right)}{\left(\frac{^{14}\text{C-inulin in apical compartment}}{^{14}\text{C-inulin total}}\right)} \quad (2)$$

where ^{125}I-$A\beta_{40\ total}$ is the total intact cpm in the apical and basolateral compartments, as well as cpm remaining in cells. ^{14}C-inulin total is the total inulin dpm in the apical and basolateral compartments. Trichloroacetic acid (TCA) precipitation [38] was used to measure the amount of intact and degraded ^{125}I-$A\beta_{40}$. Total ^{125}I-$A\beta_{40}$ was determined by counting sample radioactivity. Degraded ^{125}I-$A\beta_{40}$ was measured in the supernatant following precipitation with TCA. To measure degraded ^{125}I-$A\beta_{40}$, one volume of TCA (20%) was added to the sample, and then samples were vortex mixed, incubated in ice for 30 min, and then centrifuged at 14,000 rpm (4 °C) for 30 min. Following centrifugation, the gamma radioactivity of the TCA supernatant containing degraded peptide was measured using a Wallac 1470 Wizard Gamma Counter (PerkinElmer, MA). The intact fraction was calculated by subtracting degraded ^{125}I-$A\beta_{40}$ from total ^{125}I-$A\beta_{40}$.

2.6. Expression of P-gp, LRP1, RAGE, IDE and NEP in hCMEC/D3 cells following amyloid-β treatments

Cells were seeded in 100 mm cell culture dishes (Corning, NY) at a density of 1×10^6 cells per dish. The cells were allowed to grow to 70% confluency before treatment with different Aβ preparations in a humidified atmosphere (5% CO_2/95% air) at 37 °C. Cells were treated for 24 h with control media, 100 nM monomeric $A\beta_{40}$, 50 nM $A\beta_{42}$ oligomers or mixture of 100 nM monomeric $A\beta_{40}$ and 50 nM $A\beta_{42}$ oligomers. At the end of treatment period, RIPA buffer containing complete mammalian protease inhibitor mixture (Sigma-Aldrich, MO) was used to dissolve cells. For western blot analysis of P-gp, LRP1, RAGE, IDE and NEP in hCMEC/D3 cells after Aβ treatment, 25 μg of cellular protein was resolved on 8% Bis-tris gels in 3-(N-morpholino) propanesulfonic acid buffer system and electrotransferred onto 0.45 μm nitrocellulose membrane. Membranes were blocked with 2% BSA and incubated overnight

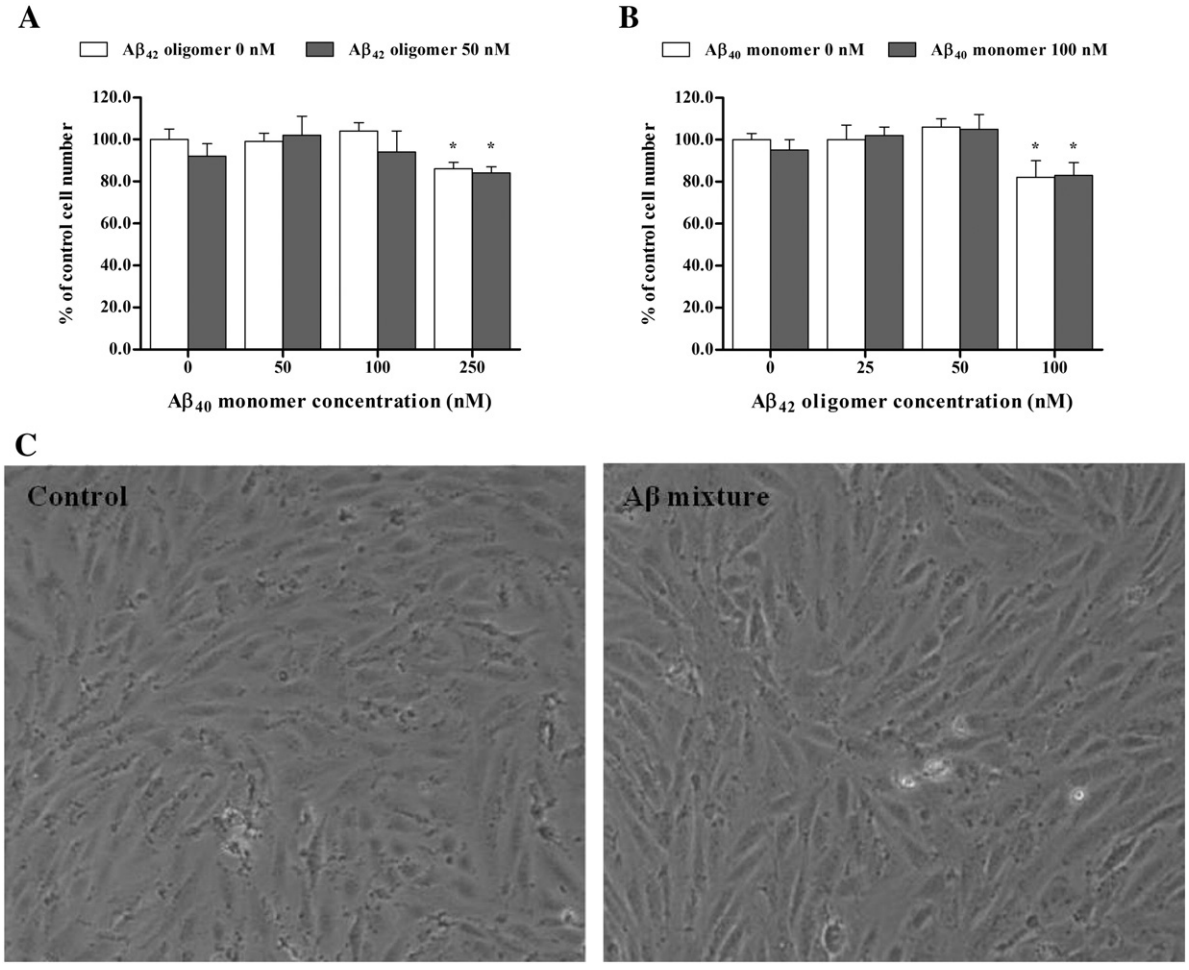

Fig. 2. Cell toxicity assays after treatment with Aβ preparations. (A) Concentration dependent MTT cytotoxcity assay of 0, 50, 100 and 250 nM of monomeric $A\beta_{40}$ with or without 50 nM $A\beta_{42}$ oligomer. (B) Concentration dependent MTT cytotoxcity assay of 0, 25, 50 and 100 nM of $A\beta_{42}$ oligomer with or without 100 nM $A\beta_{40}$ monomer. Significant reductions in cell viability as measured by MTT reduction to formazan were observed only after 24 h treatment with 250 nM monomeric $A\beta_{40}$, 100 nM $A\beta_{42}$ oligomer and their corresponding mixtures. (C) Microscopic images of cells after exposure to control media or Aβ mixture (100 nM monomeric $A\beta_{40}$ and 50 nM $A\beta_{42}$ oligomers) for 24 h. The data are expressed as mean ± SEM of n = 3 independent experiments.

with monoclonal antibodies for P-gp (C-219; Covance Research Products, MA), LRP1 (Calbiochem, NJ), RAGE (Santa Cruz, TX), IDE (Santa Cruz, TX), NEP (Calbiochem, NJ) or GAPDH (Santa Cruz, TX) at dilutions 1:200, 1:1500, 1:200, 1:200, 1:100 and 1:3000, respectively. For antigen detection, the membranes were washed and incubated with HRP-labeled secondary IgG antibody for P-gp, RAGE, NEP and GAPDH (anti-mouse), LRP1 (anti-rabbit) and IDE (anti-goat) (Santa Cruz, TX) at 1:5000 dilution. The bands were visualized and quantified as described above. Three independent Western blotting analyses were carried out for each treatment group.

2.7. Statistical analysis

Unless otherwise indicated, the data were expressed as mean ± SEM. The experimental results were statistically analyzed for significant difference using two-tailed Student's *t*-test for 2 groups, and one-way analysis of variance (ANOVA) for more than two group analysis. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Characterization of Aβ preparations

To study the toxicity of Aβ species on hCMEC/D3 cells, $A\beta_{40}$ monomer and $A\beta_{42}$ oligomers were first prepared and characterized. These Aβ species were selected because $A\beta_{40}$ is the most abundant Aβ peptide in the brains of AD patients, and soluble $A\beta_{42}$ oligomers are the initial seeds for forming higher order assemblies, and they have proved to have high toxicity [6]. Fig. 1A and C confirmed $A\beta_{42}$ oligomers formation; oligomers include SDS-stable dimer, trimer, tetramer; no large protofibrils were detected. Fig. 1B shows a representative SEC profile where $A\beta_{42}$ oligomers, as measured by a total oligomer-specific ELISA, were detected in fractions 4–8. Subsequently, immunoblotting assay was utilized to estimate the concentrations of both, the monomer and oligomers. The concentrations of all Aβ species were calculated based on a calibration curve of Aβ monomer standards (Fig. 1C and D).

3.2. Assessing toxicity of Aβ preparations against hCMEC/D3

Cell viability assays were performed initially to confirm that Aβ preparations used in the treatment of hCMEC/D3 cells were not toxic at the applied concentrations after 24 h of treatment. MTT cytotoxicity assay with hCMEC/D3 cells demonstrated that cell treatment did not affect the viability of hCMEC/D3 after 24 h exposure to Aβ preparations except at high concentrations of $A\beta_{40}$ monomers and $A\beta_{42}$ oligomers added separately or as a mixture. MTT assay results from cells treated with $A\beta_{40}$ monomers at 50 and 100 nM, $A\beta_{42}$ oligomers at 25 and 50 nM, or mixtures of these concentrations were comparable to control treated cells (Fig. 2A and B). However, MTT assay showed that the viability of hCMEC/D3 decreased by 14–17% following treatment with 250 nM $A\beta_{40}$ monomer, mixture of 250 nM $A\beta_{40}$ monomer and 50 nM $A\beta_{42}$ oligomer, 100 nM $A\beta_{42}$ oligomer and mixture of 100 nM $A\beta_{42}$ oligomer and 100 nM $A\beta_{40}$ monomer (Fig. 2A and B). Thus, for permeation studies of inulin and Aβ, high concentrations that induced toxicity were excluded and only sub-toxic concentrations were used for cells treatment. Fig. 2C demonstrates absence of morphological changes on hCMEC/D3 cells after 24 h treatment with Aβ mixture consisting of 100 nM $A\beta_{40}$ monomers and 50 nM $A\beta_{42}$ oligomers when compared to control.

3.3. Treatment of hCMEC/D3 cells monolayer with Aβ mixture increases paracellular permeability

The integrity of the barrier formed by hCMEC/D3 cells grown on inserts after 24 h exposure to Aβ preparations was assessed by measuring permeation of ^{14}C-inulin across the cell monolayer. After 24 h treatment with 0, 50 and 100 nM $A\beta_{40}$ monomer in the presence or absence of 25 or 50 nM $A\beta_{42}$ oligomer, only Aβ mixture of 100 nM $A\beta_{40}$ monomer and 50 nM $A\beta_{42}$ oligomer disrupted the monolayer integrity and significantly increased apical to basolateral permeation of ^{14}C-inulin across hCMEC/D3 by 11–15% compared to control ($p < 0.05$; Fig. 3A and B). On the other hand, treatments with Aβ species separately, or mixtures at 50 nM each or 100 monomer and 25 oligomer failed to change inulin permeation (Fig. 3A and B).

3.4. Treatment with Aβ mixture decreases ^{125}I-$A\beta_{40}$ transport across hCMEC/D3 cell monolayer

Twenty-four hour treatment of hCMEC/D3 cells monolayer with 100 nM $A\beta_{40}$ monomer or 50 nM $A\beta_{42}$ oligomer added separately to

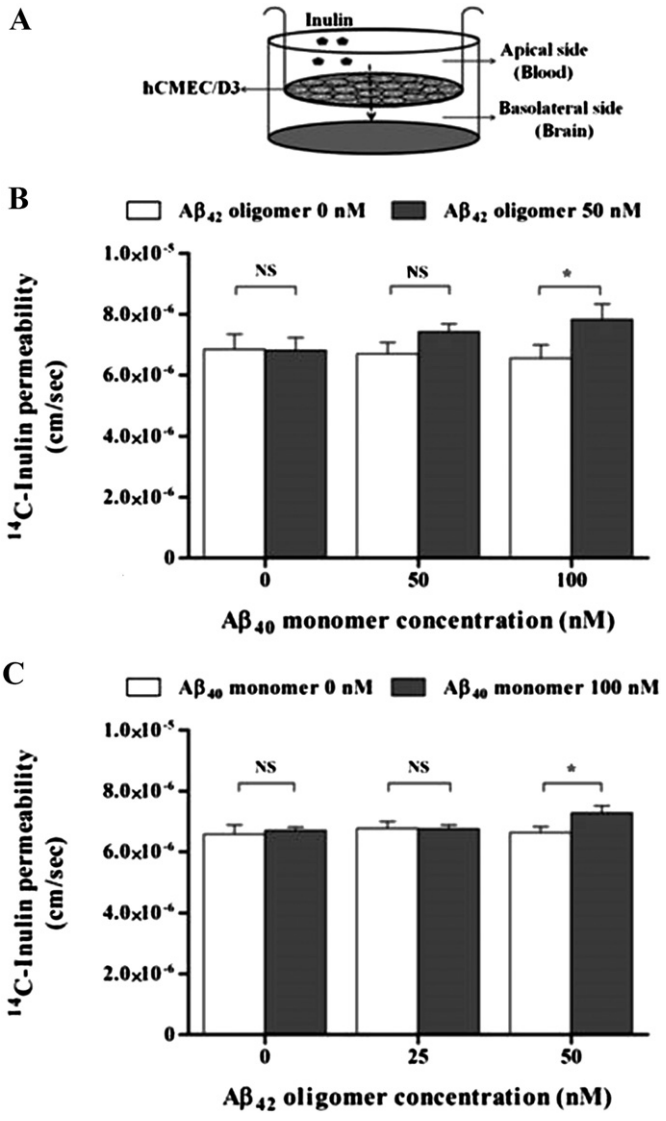

Fig. 3. Effect of Aβ preparations on the integrity of an hCMEC/D3 monolayer model of the BBB endothelium. Apical to basolateral ^{14}C-inulin permeation across the hCMEC/D3 cell monolayer was monitored for 2 h after 24 h treatment with different Aβ preparations at sub-toxic nanomolar concentrations. (A) Schematic presentation of hCMEC/D3 monolayer shows the direction of inulin permeation when added to the apical side. (B) Concentration dependent studies on the effect of $A\beta_{40}$ monomer with or without 50 nM $A\beta_{42}$ oligomer on inulin permeation, and (C) Concentration dependent studies on the effect of $A\beta_{42}$ oligomer with or without 100 nM $A\beta_{40}$ monomer on inulin permeation. ^{14}C-inulin permeaion in the apical to basolateral direction was significantly enhanced after treatment with Aβ mixture of 100 nM $A\beta_{40}$ monomer and 50 nM $A\beta_{40}$ oligomer indicating the disruptive effect of this Aβ mixture on the integrity of the hCMEC/D3 cells. Data represent mean ± SEM from three independent experiments, * $P < 0.05$.

the cells failed to alter the transport of ^{125}I-Aβ_{40} from basolateral to apical side (representative for brain to blood clearance). However, significant reduction in ^{125}I-Aβ_{40} transport from basolateral to apical side ($p < 0.05$) was only observed following exposure of hCMEC/D3 cells to a mixture of 100 nM Aβ_{40} monomer and 50 nM Aβ_{42} oligomer (Fig. 4). In particular, this reduction in Aβ_{40} transport quotient was a result of decreased transport of Aβ_{40} across the hCMEC/D3 cells (Supplementary Fig. 1). Transport studies on monomeric ^{125}I-Aβ_{40} for 6 h across hCMEC/D3 cells monolayer showed a significant reduction in ^{125}I-Aβ_{40} transport quotients ($CQ_{B \rightarrow A}$) by 20–25% across cells treated with 100 nM Aβ_{40} monomer and 50 nM Aβ_{42} oligomer mixture; while a reduction trend in ^{125}I-Aβ_{40} $CQ_{B \rightarrow A}$ was observed with other mixtures investigated at different combined concentrations, this reduction was not statistically significant when compared to control treatment (Fig. 4). Besides, treatment with all Aβ preparations for 24 h had no effect on ^{125}I-Aβ_{40} degradation as measured by TCA assay. The percent degradation of ^{125}I-Aβ_{40} in the media of hCMEC/D3 were similar for all treatments with degradation % values in the range of 15.7–17% (Fig. 5).

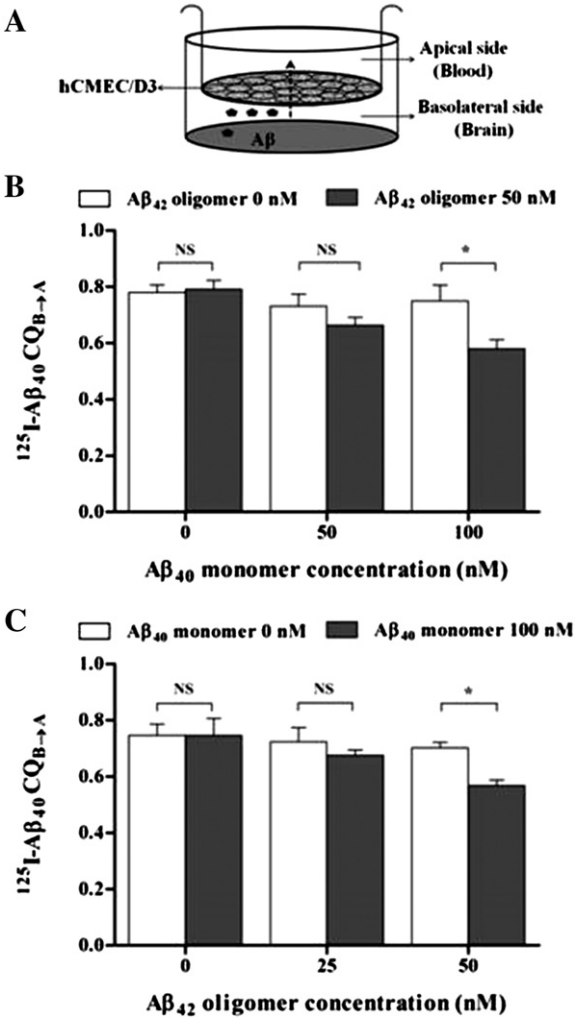

Fig. 4. Transport of ^{125}I-Aβ_{40} across hCMEC/D3 cell monolayer after exposure to Aβ preparations. (A) Schematic presentation of hCMEC/D3 monolayer shows the direction of transport of ^{125}I-Aβ_{40} added to the basolateral side. (B) Transport quotient $CQ_{B \rightarrow A}$ of ^{125}I-Aβ_{40} across hCMEC/D3 monolayer treated for 24 h with increasing concentrations of Aβ_{40} monomer with or without 50 nM Aβ_{42} oligomers, and (C) Transport quotient $CQ_{B \rightarrow A}$ of ^{125}I-Aβ_{40} across hCMEC/D3 monolayer treated for 24 h with increasing concentrations of Aβ_{42} oligomers with or without 100 nM Aβ_{40} monomer. While Aβ mixture of 100 nM Aβ_{40} monomer and 50 nM Aβ_{42} oligomer significantly reduced $CQ_{B \rightarrow A}$ of ^{125}I-Aβ_{40}, other Aβ preparations did not affect ^{125}I-Aβ_{40} transport. Data represent mean ± SEM from three independent experiments; * $P < 0.05$.

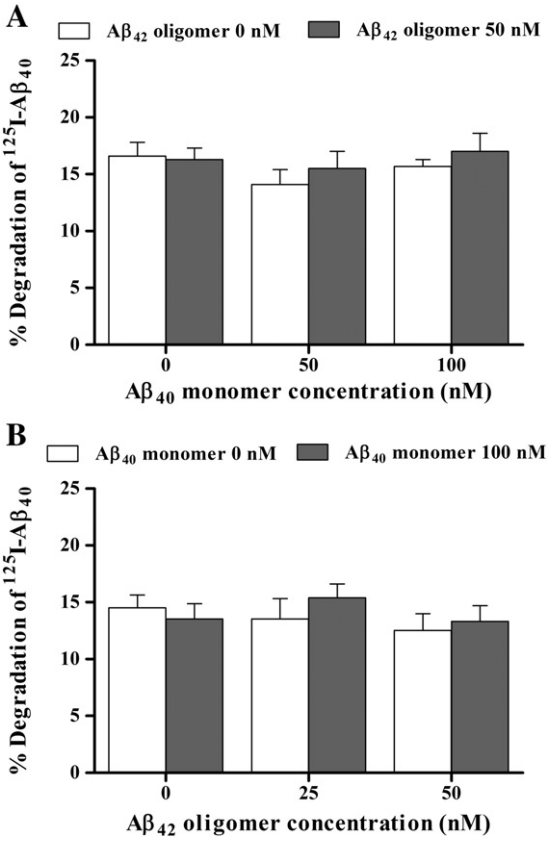

Fig. 5. Degradation of ^{125}I-Aβ_{40} by hCMEC/D3 cell monolayer after exposure to Aβ preparations. (A) % degradation of ^{125}I-Aβ_{40} by hCMEC/D3 monolayer treated for 24 h with increasing concentrations of Aβ_{40} monomer with or without 50 nM Aβ_{42} oligomers, and (B) % degradation of ^{125}I-Aβ_{40} by hCMEC/D3 monolayer treated for 24 h with increasing concentrations of Aβ_{42} with or without 100 nM Aβ_{40} monomer. None of Aβ preparations altered % degradation of ^{125}I-Aβ_{40} after 24 h treatment. Data represent mean ± SEM from three independent experiments; * $P < 0.05$.

3.5. Aβ mixture differentially affects the expression of LRP1 and RAGE in hCMEC/D3 cells but not P-gp and Aβ degrading enzymes

Given the important role of P-gp and LRP1 in the transport of Aβ across the BBB, we determined the effect of Aβ mixture (100 nM Aβ_{40} monomers and 50 nM Aβ_{42} oligomers) on the expression of these proteins in hCMEC/D3 cells in order to explain the reduced transport of Aβ_{40} across the monolayer. The results of western blot analysis following 24 h treatment demonstrated that the expression of P-gp in hCMEC/D3 was not altered by Aβ_{40} monomers, Aβ_{42} oligomers, or Aβ mixture treatments (Fig. 6A and B). However, treatment with Aβ mixture significantly decreased the expression of LRP1 ($P < 0.01$). This reduction was not observed with Aβ_{40} monomers or Aβ_{42} oligomers (Fig. 6A). Densitometry analysis of western blot bands showed a significant 20% reduction in the expression of LRP1 after treatment with Aβ mixture (Fig. 6B). On the other hand, RAGE showed a significant 23% increase in protein level after exposure to Aβ mixture for 24 h, yet cells treated with Aβ_{40} monomers or Aβ_{42} oligomers did not show any significant alterations in RAGE protein expression (Fig. 6A and B). Consistent with the results of TCA degradation assay, there were no significant changes observed in the expression of the Aβ degrading enzymes IDE and NEP following 24 h exposure to Aβ_{40} monomers, Aβ_{42} oligomers and Aβ mixture (Fig. 7A and B).

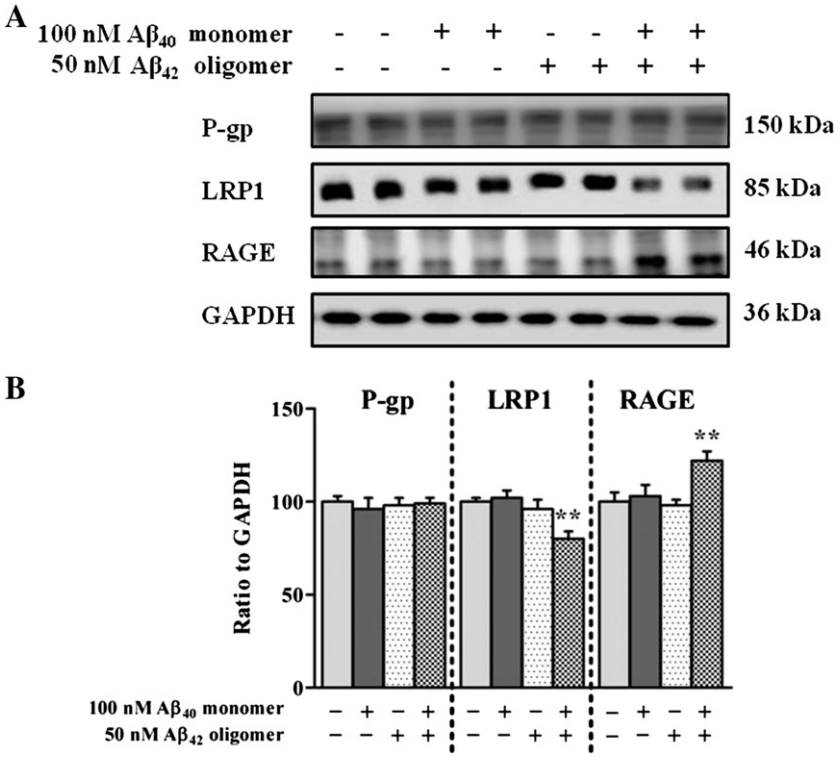

Fig. 6. Expression of P-gp, LRP1 and RAGE in hCMEC/D3 cells. (A) Western blot analysis of P-gp, LRP1, and RAGE protein expressions in hCMEC/D3 cells after 24 h exposure to control media, 100 nM monomeric $A\beta_{40}$, 50 nM $A\beta_{42}$ oligomers, or Aβ mixture of both. (B) Densitometry analyses showed similar expression level of P-gp in all treatment groups, significantly higher RAGE expression and lower LRP1 expression in hCMEC/D3 cells treated with Aβ mixture. Data represent mean ± SEM from three independent experiments; ** $P < 0.01$.

4. Discussion

In AD patients, Aβ peptides coexist in the brain as a heterogeneous mixture of different Aβ species with different sizes, solubility and conformational structures [7]. Although qualitative as well as quantitative changes in Aβ species have a central role in the pathogenesis of AD, specific key changes that contribute significantly to the development and progression of AD are still a matter of debate [5,39]. Available

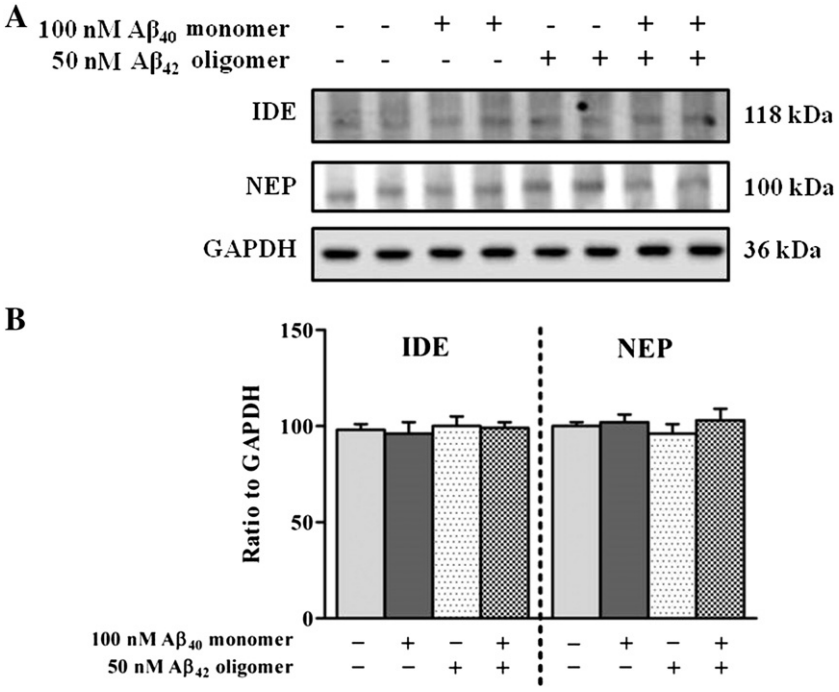

Fig. 7. (A) Western blot analysis of IDE and NEP protein expression in hCMEC/D3 cells after 24 h exposure to control media, 100 nM monomeric $A\beta_{40}$, 50 nM $A\beta_{42}$ oligomers or Aβ mixture of both. (B) Corresponding densitometry analysis showed that none of the Aβ preparations altered the expression of IDE or NEP. Data represent mean ± SEM from three independent experiments.

studies indicate that soluble Aβ pool has high toxicity against neurons and is highly correlated with the severity of AD [8,11]. Some studies have identified specific individual species in the Aβ soluble pool that exerts significant neuronal toxicity, however, the significance of these results remains unclear. It is difficult to attribute Aβ toxicity to a single species because: 1) it is difficult to prepare highly pure and stable specific Aβ assemblies (in vitro or in vivo) due to the unusual behavior of Aβ peptides in the analytical procedures [40], 2) there is a lack of standardization of concentrations or purification methods, and 3) the more important issue is that in the brains of AD patients multiple species of Aβ exist and are expected to change during aging and the course of disease [41–43]. In addition, physiological, or at most pathological, concentrations of specific Aβ species are likely to be the most relevant to the biological situation. Otherwise, it is difficult to compare the intrinsic toxicity of Aβ mixtures to that of specific species. Instead, studies investigating the effect of a mixture of Aβ species (at least the known, abundant and pathogenic ones) at physiological and pathological level are more likely to be informative of the in vivo condition [5].

The intent of this study was to in vitro investigate the effect of different preparations of Aβ on BBB endothelial cells integrity and $A\beta_{40}$ clearance using hCMEC/D3 cell monolayer as a representative model for the BBB. Brain endothelial cells, astrocytes and pericytes are involved in normal function of BBB [44]. While the astrocytes, pericytes and basement membrane play important role in regulating BBB function, only the capillary endothelium forms the physical barrier separating brain from blood and control barrier functions of the BBB [44]. Although, this model is an in vitro model, it expresses many BBB-specific properties including stringent restriction of paracellular diffusion of large molecules [35,45]. Moreover, permeability coefficients of hCMEC/D3 cell monolayer were well correlated with in vivo permeability coefficients making this cell line a useful model to study BBB barrier functions [35].

Total $A\beta_{40}$ has been detected in the brains of AD patients at a level that is 10 times higher than the level of $A\beta_{42}$. However, the pathological levels of $A\beta_{40}$ monomer and $A\beta_{42}$ oligomers in Aβ soluble pool were 100 nM and in the range of 10 to 100 nM for $A\beta_{40}$ monomer and $A\beta_{42}$ oligomers, respectively [33,46]. Therefore, for the purpose of our study, we used $A\beta_{40}$ monomer and $A\beta_{42}$ oligomers at previously identified pathologically relevant concentrations at 100 nM and in the range of 10 to 100 nM for $A\beta_{40}$ monomer and $A\beta_{42}$ oligomers, respectively.

MTT toxicity studies were initially performed to exclude toxic concentrations of Aβ species when added separately or as mixtures. This is important in order to evaluate the effect of non-toxic concentrations on hCMEC/D3 monolayer integrity as a cell-based BBB model. Thus, all subsequent permeability and active transport studies of $A\beta_{40}$ were conducted at concentrations $\leq$100 nM $A\beta_{40}$ monomer and/or $\leq$50 nM $A\beta_{42}$ oligomers.

Our results demonstrated an effect of the soluble Aβ pool, at nanomolar levels, on the integrity of the hCMEC/D3 model of the BBB assessed by inulin permeation. While several studies have described the disruptive effect of $A\beta_{40}$ oligomers or Aβ monomers (40 or 42) on BBB permeability [24,29,30,47], the single species of Aβ required high micromolar concentrations to exert such toxic effect. Unlike these studies, in the current investigation only Aβ mixture that contains both $A\beta_{40}$ monomers and $A\beta_{42}$ oligomers, both in nanomolar concentrations, was able to disrupt the hCMEC/D3 monolayer integrity following 24 h treatment, while at the examined concentrations of individual $A\beta_{40}$ monomers or $A\beta_{42}$ oligomers had no effect on the monolayer integrity. This finding suggested that Aβ mixture disrupts the function of tight junction proteins, thereby enhancing paracellular permeation across the hCMEC/D3-BBB model. Previous studies showed that treatment of brain endothelial cells with $A\beta_{40}$ monomers, $A\beta_{42}$ monomers or $A\beta_{40}$ oligomers required high micromolar concentrations to decrease expression of tight

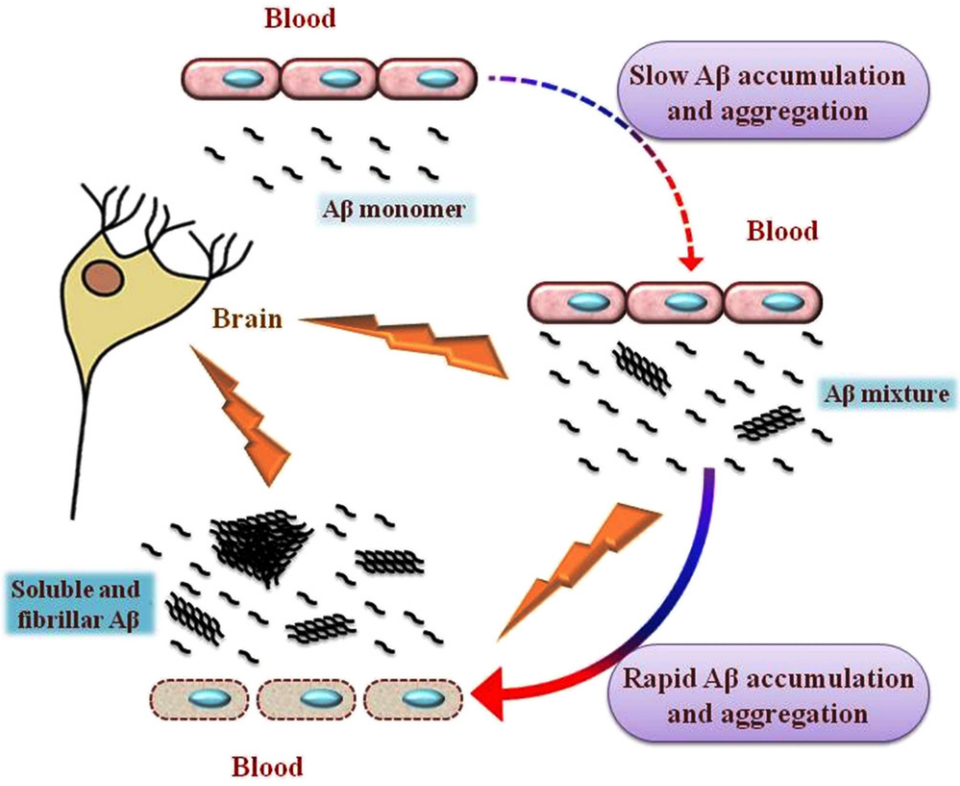

Fig. 8. Schematic presentation for a model describing toxic effect of soluble Aβ pool against BBB endothelial cells. Faulty clearance of monomeric Aβ results in its brain accumulation. Accumulated Aβ initiates a cascade of Aβ aggregation to form soluble aggregates of different sizes and types. In addition to their intrinsic neurotoxicity, soluble Aβ aggregates act synergistically with monomeric $A\beta_{40}$ in the form of Aβ mixture to disrupt BBB endothelial cells and enhance more Aβ accumulation by halting its clearance across BBB. This accelerates the formation of a wide range of soluble and insoluble Aβ assemblies enhancing the development of CAA and AD.

junction proteins, re-localize tight junction proteins from the plasma membrane, and enhance paracellular permeation of the culture monolayer [29,30]. The mix of $A\beta_{40}$ monomers and $A\beta_{42}$ oligomers at nanomolar concentrations may provoke a more pronounced effect on tight junction protein expression and localization than the individual forms, resulting in a leaky BBB that is unable to provide maximal protection of the brain against circulating neurotoxins.

In spite of leaky hCMEC/D3 monolayer as a result of Aβ mixture treatment, the transport of ^{125}I-$A\beta_{40}$ was restricted and significantly decreased following the treatment, suggesting Aβ to disrupt its own clearance machinery. This finding confirms Aβ clearance as an active process mediated by transport proteins, and is consistent with studies reporting association between the brain load of Aβ and its disposition [48]. This reduction in the transport of ^{125}I-$A\beta_{40}$ following Aβ mixture treatment was accompanied by differential effects on the expression of P-gp, IDE and NEP, LRP1, and RAGE in hCMEC/D3 cells. While cells treatment with Aβ mixture for 24 h had no effect on P-gp, IDE and NEP expressions, LRP1 and RAGE expressions were significantly decreased and increased, respectively. P-gp has been reported to efflux Aβ across BBB toward the blood [14]. Several in vivo and in vitro studies [23–25] have shown reduction in P-gp expression following treatment with Aβ monomer or oligomers; however, in the current study this effect was not observed. This discrepancy could be related to differences in experimental protocols used including Aβ concentrations (nanomolar vs micromolar levels) and/or treatment times (24 h vs longer treatment time up to 96 h).

LRP1, a member of the LDL family receptor, is highly expressed at the abluminal side of the brain endothelial cells. It mediates transport of Aβ from the brain to the blood and its expression has been reported to decrease with aging and in patients with AD [38]. Findings from the current study demonstrated that treatment with Aβ mixture down-regulated LRP1 expression in hCMEC/D3 cells, which is consistent with previously reported results following in vivo administration of 100 μg $A\beta_{42}$ monomers over 26 h that caused a significant reduction in LRP1 expression in brain tissue of FVB wild type mice [25], however with more realistic and relevant concentrations provided by our study. In contrast to LRP1, Aβ mixture up-regulated the cellular expression of RAGE, which is consistent with a pattern observed in AD patients' brains [17]. While the effect of Aβ mixture on LRP1 and RAGE was obvious 24 h following treatment, the effect of the individual Aβ preparations on LRP1 and RAGE, or on P-gp, IDE, and NEP would be expected to occur following a longer exposure time. Our results suggest that LRP1 and RAGE are more sensitive than P-gp, IDE, and NEP to Aβ changes in the brain. Given the role of LRP1 and RAGE in efflux and influx, respectively, of Aβ across BBB endothelium, changes in their expression would be expected to alter Aβ transport across the BBB.

Collectively, our in vitro findings, in addition to previous in vitro and in vivo studies, suggest that faulty clearance of Aβ may start as a slow cascade of monomeric Aβ accumulation and aggregation that result in the formation of a soluble Aβ pool (Aβ mixture). Aβ mixture possesses greater disruption effect on the integrity and functionality of the BBB endothelium compared to either species alone, and enhances rapid accumulation of Aβ in the brain, which in turn could accelerate the pathogenesis of CAA and AD (Fig. 8).

5. Conclusion

Study of a soluble Aβ pool containing a mixture of monomeric and oligomeric Aβ peptide provides a physiologically relevant way to probe the pathogenesis of AD. While previous in vitro and in vivo studies investigated the toxicity of specific Aβ species on the BBB, here, we provide for the first time evidence that a mixture of Aβ possess a greater disruptive effect over and above that of individual Aβ species on the integrity and function of hCMEC/D3 cells as an in vitro model of BBB. This concept may also apply to other biological effects of Aβ.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2014.06.029.

Conflict of interest

The authors declare no competing financial interest.

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