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Combinations of polyphenols disaggregate Aβ1-42 by passing through in vitro blood brain barrier developed by endothelium, astrocyte, and differentiated SH-SY5Y cells

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Disaggregation of amyloid βeta (Aβ) is considered as one of the promising therapeutic strategies for Alzheimer's disease. Polyphenols are promising molecules for the disaggregation of Aβ. However, in order to find a potential therapeutic candidate, the *in vitro* analyses need to be performed on a model that mimics the blood-brain barrier (BBB) as much as possible. Therefore, we aimed to establish an *in vitro* BBB representative transwell system by using differentiated human neuroblastoma (SH-SY5Y), cerebral microvascular endothelial, and astrocyte cells to investigate transition and Aβ disaggregation capacity of punicalagin (PU), ellagic acid (EA), epigallocatechin gallate (EGCG), gastrodin, and their combinations on the established system. The efficiency of the established transwell systems was evaluated by measuring the transendothelial electrical resistance (TEER) and paracellular permeability coefficients (Pe) values. The transition and Aβ disaggregation capacities of the polyphenols were evaluated in the established tri-culture transwell system based on obtained TEER (50,07 Ω.cm^2) and Pe (65x10-6 cm/s) values. Our results revealed that all polyphenols can successfully pass across the BBB system and disaggregate Aβ. While Aβ disaggregation capacities of the polyphenols were in the range of 30.52–45.01%, the percentages of their combinations were higher (75% for EGCG-PU (Com 1) and 64% for EGCG-EA (Com 2)). Consequently, this study provides the first evidence that Com 1 and Com 2 are promising polyphenol combinations in terms of Aβ disaggregation. Besides, the developed tri-culture transwell system, containing differentiated SH-SY5Y cells, may provide a new tool that closely mimics the BBB for basic research and testing of candidate agents.

Key words: Aβ1-42, transwell systems, differentiated neuroblastoma cells, epigallocatechin gallate, punicalagin

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder that causes dementia in aging populations worldwide (Kumar et al., 2015). Several hypotheses have been propounded for the molecular basis of AD, such as Aβ cascade (Wong et al., 2010), cholinergic (Kumar and Baquer, 2016), tau protein hyperphosphorylation and accumulation (Jin et al., 2011), excitotoxicity and oxidative stress (Praticò, 2008). According to the Aβ cascade hypothesis, accumulation of Aβ peptide plays a significant role in the pathogenesis, leading to loss of the integrity of neuronal cells, epithelial cells (ECs), and blood-brain barrier (BBB) (Sharma et al., 2019). Currently, degradation or polymerization prevention of Aβ by polyphenols is considered as a promising approach to mitigate Aβ aggregation.

It has been stated in numerous studies that polyphenol compounds have a therapeutic potential in the modulation of the disease and disaggregation of Aβ (Ono et al., 2004; Yang et al., 2005; Phan et al., 2019). While some of the studies revealed that certain polyphenols destabilize Aβ fibrils in a dose-dependent manner, other studies suggested the combinations of

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polyphenols as a more promising approach (Giunta et al., 2010; Mori et al., 2019). Studies showed that Aβ polymerization prevention effects or disaggregation abilities of polyphenols are related to the rings found in their chemical structure which enable them to form covalent interactions with the hydrophobic amino-acid residues of Aβ (Yamin et al., 2009; Velander et al., 2017; Phan et al., 2019). Based on this knowledge and according to the chemical structures, punicalagin (PU), ellagic acid (EA), epigallocatechin gallate (EGCG), and gastrodin (GA) were selected as potential polyphenols in this study (Ehrnhoefer et al., 2008; Feng et al., 2009; Das et al., 2016; Liu et al., 2018).

However, in order to demonstrate Aβ disaggregation capacities of the polyphenols, the experiments need to be performed on an *in vitro* model that mimics the BBB as closely as possible. BBB is a complex and dynamic system that is composed of endothelial cells of cerebral capillaries and micro-vessels and its close contact with neighboring pericytes, astrocytes, and neurons. Maintaining the integrity of the barrier is important and highly related to communication among the cells (Abbott et al., 2006; Weiss et al., 2009).

In general, transwell systems are commonly used *in vitro* models that ensure high resemblance to the cellular architecture, resistance, and permeability of the *in vivo* BBB (Wilhelm and Krizbai, 2014; Ruck et al., 2015; Banerjee et al., 2016). The culturing method (co- or tri-culture) and cellular architecture (astrocytes, pericytes, endothelial and neuronal cells) are critical points for the quality of the *in vitro* transwell system and its similarity to the *in vivo* conditions as they hugely affect the levels of tight junction, integrity, and permeability of the *in vitro* system (Naik and Cucullo, 2012; Wilhelm and Krizbai, 2014). In particular, some of the researchers state that using differentiated human neuroblastoma cell line (SH-SY5Y) might be important in providing a more neuron-like phenotype (de Medeiros et al., 2019; Krishtal et al., 2019). Therefore, we decided to use differentiated SH-SY5Y cells whose differentiation has been optimized in a previous study of ours to establish the BBB model (Serdar et al., 2020).

The aims of this study were: to develop and select an *in vitro* BBB model using the differentiated SH-SY5Y cell line, to examine the transition and Aβ disaggregation capacity of the selected polyphenols (punicalagin, ellagic acid, EGCG, and gastrodin) and their combinations in the developed BBB model. For the first aim, co- and tri-culture *in vitro* transwell systems were structured with human astrocyte (SC-1810), human cerebral microvascular endothelial (hCMEC/D3), and the differentiated human neuroblastoma (SH-SY5Y) cell lines. After that, co- and tri-culture *in vitro* transwell systems were compared to each other and selected based on their transendothelial electrical resistance (TEER) values which is a widely accepted quantitative technique used to measure the integrity of tight junction dynamics and a strong indicator of the integrity of the cellular barriers before the evaluation of drugs or chemicals transition (Hatherell et al., 2011; Srinivasan et al., 2015). After the selection of the transwell system, cytotoxic effects of the polyphenols and combinations were determined using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) and Lactate dehydrogenase (LDH). Paracellular permeability coefficient (Pe) was measured using sodium fluorescein (NaF). Finally, Aβ disaggregation capacity was measured using Thioflavin T (ThT), Field Emission Scanning Electron Microscopy (FESEM) and aggregated Aβ Enzyme-linked Immunosorbent Assay (ELISA).

Our results proved that the tri-culture transwell system, consisting of brain endothelial, astrocyte, and differentiated SH-SY5Y cells, has higher barrier integrity values (TEER=50,07 $\Omega.cm^2$ and Pe=65x10-6 cm/s) compared to the other co-culture transwell systems. Transition experiments of the tri-culture transwell system also showed that all of the selected polyphenols can pass across the *in vitro* BBB system in the range of 61%-97%. However, polyphenol combinations showed much better Aβ disaggregation capacities than single polyphenol treatments (EGCG + PU (Com 1; 75%) and EGCG + EA (Com 2; 64%)).

This study is the first to provide comparative data on the tri- and co-culture transwell systems structured with differentiated SH-SY5Y, hCMEC/D3, and HA cells and also demonstrate the transition and Aβ disaggregation capacity of PU, EA, EGCG, and GA polyphenols and their combinations in the tri-culture transwell system. We suggest the tri-culture system, structured with differentiated SH-SY5Y cells, as a suitable model for *in vitro* BBB research and combinations of EGCG-PU or EGCG-EA as promising agents for Aβ disaggregation. The findings of this study may help in developing therapeutic strategies for AD with these polyphenols.

METHODS

Cell culture

In this study, human neuroblastoma SH-SY5Y (ATCC-CRL-2266), human cerebral microvascular endothelial (hCMEC/D3) (Cedarlane, Canadian), and human astrocyte (HA) (Cat no: 1800-1, ScienCell) cell lines were used. Human neuroblastoma SH-SY5Y cells were cultured in DMEM/Ham's F-12 Nutrient Mix (1:1) (Biochrom, Berlin, Germany) containing fetal bovine

serum (FBS, 10%), penicillin (1% (v/v)), streptomycin (1% (v/v)) in a humidified, 5% CO_2 incubator at 37°C. Differentiated and confirmed SH-SY5Y cells were used in this study. Differentiation and confirmation have been done with various chemical agents and neuronal markers respectively in our previous study. In this differentiation process, all cells were pre-treated with retinoic acid for 5 days followed by 5 days treatment of mixed medium consisting of Dibutyryl cyclic-AMP (dc-AMP, Sigma, Germany), potassium chloride (KCI, Thermo Fisher Scientific, USA), B27 (Thermo Fisher Scientific, USA), Glutamax I (Thermo Fisher Scientific, USA), Neurobasal (Thermo Fisher Scientific, USA) and brain-derived neurotrophic factor (Sigma, Germany) (Shipley et al., 2016; Serdar et al., 2020). Passages between 26 and 32 of hCMEC/D3 cells were used in the experiments for to provide more endothelial characteristics. All flasks were coated with rat tail type collagen (Gibco, USA) (150 µg/mL in PBS for one h) to allow better cell attachment. Cells were grown in EndoGRO-TM basal medium (Sigma, Germany, supplemented with EndoGRO-MV complete media kit), containing FBS (5%), EndoGRO-LS supplement (0,2%), rh EGF (5 ng/mL), heparin sulfate (0.75 U /mL), ascorbic acid (50 µg/mL), L-Glutamine (10 mM), hydrocortisone hemisuccinate (1,0 µg/mL), human bFGF (200 ng/mL) and antibiotics (1% (v/v) penicillin/streptomycin). For the human astrocyte cell line, passages between 1 and 10 were used in all experiments. The cells were seeded in poly-L-lysine (5 µg/cm^2, ScienCell, USA) pre-coated flasks and cultured in 5% FBS, 1% penicillin/streptomycin, 1% astrocyte growth supplement (AGS) in astrocyte medium. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 in an incubator.

Development of the *in vitro* BBB model using co-and tri-culture transwell systems

All cell inserts were coated with appropriate extracellular matrix agents so as to obtain the transwell systems. Inserts were coated with rat-tail collagen for hCMEC/D3, poly-L-Lysine for HA cells, and poly-D-Lysine (10 µg/mL, Sigma, Germany) for differentiated SH-SY5Y cells. hCMEC/D3 cells were seeded on the inner side (Abluminal, blood side), and HA cells were seeded on the outer side of the transwell membrane inserts (0.4 µm, Greiner, Germany) in order to provide close contact between the cells (Fig. 1A). In the other co-culture system, SH-SY5Y cells were differentiated for 10 days with various agents in 12 well plates (1.13 cm^2/well) that had been coated with poly-D-lysin (Serdar et al., 2020). hCMEC/D3 cells were seeded on

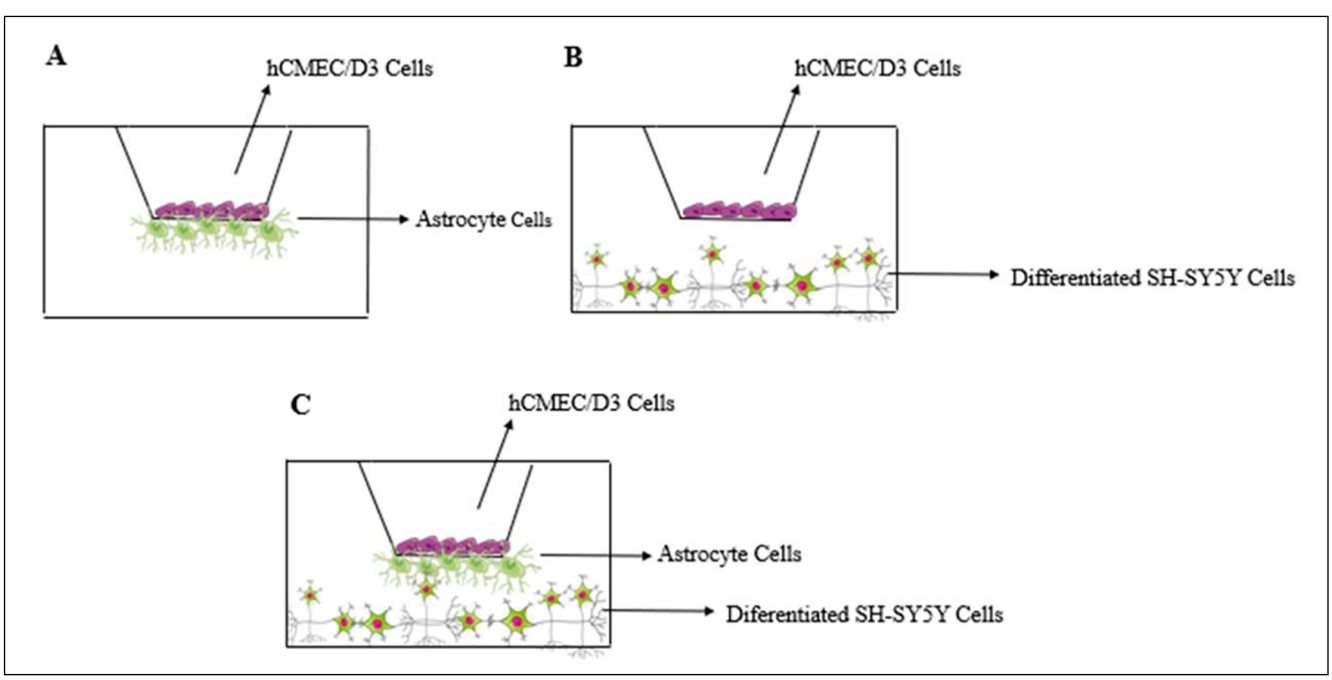


Fig. 1. Schematic representations of the *in vitro* transwell systems. (A) hCMEC/D3 cells seeded on the inner side (abluminal, blood side) and HA cells seeded on the outer side of the transwell membrane inserts. (B) hCMEC/D3 cells seeded on the inner side of the insert membrane and differentiated SH-SY5Y cells seeded on the bottom of the transwell system. (C) hCMEC/D3 cells seeded on the inner side of the insert membrane, HA cells seeded on the inner side of the transwell membrane inserts, and differentiated SH-SY5Y cells on the bottom of the transwell system.

the inner side of the insert membrane after the differentiation period. In this structure, hCMEC/D3 cells provide separation in the system between the apical layer and the basolateral compartment (luminal, brain side) without contact with the differentiated SH-SY5Y cells (Fig. 1B). Tri-culture transwell models were structured with hCMEC/D3, HA, and differentiated SH-SY5Y cell lines. In this model, HA cells were cultured on the outer side of the membrane insert (Nakagawa et al., 2009). The model provides contact between astrocytes and hCMEC/D3 cells but no contact with differentiated SH-SY5Y cells that are seeded on the bottom of the transwell (Fig. 1C).

Integrities of the co- and tri-culture transwell systems

TEER value provides an evaluation of the electrical resistance between luminal and abluminal transwell systems and it can be measured using a volt ohm meter (Srinivasan et al., 2015). TEER values are considered as a quantitative technique to measure the integrity of tight junction dynamics in cell culture models (Hatherell et al., 2011; Srinivasan et al., 2015). Generally, co- and tri-culture systems are characterized by relatively high TEER values and by low paracellular permeability coefficient (Pe) of marker compounds, such as small water-soluble tracer fluorescein (sodium fluorescein (NAF)). In this study, TEER and Pe values were used as quality parameters for the assessment of *in vitro* BBB transwell systems (Deli et al., 2005, Nakagawa et al., 2009, Hatherell et al., 2011, Eigenmann et al., 2013, De Laere et al., 2017). The integrities of the transwell systems were evaluated every two days (2^{nd}, 4^{th}, 6^{th}, and 10^{th}) with the TEER values ($\Omega.cm^2$) measured by an epithelial volt ohm meter (VOM 2, (World Precision Instrument, Sarasota, FL, USA) and STX2 electrodes (World Precision Instrument, Sarasota, FL, USA) and then calculated as described in Srinivasan et al.'s study (2015). Meanwhile, the mediums in the transwell systems were changed after each TEER reading. TEER values obtained from cell-free inserts, coated with rat-tail collagen, poly-D-Lysine, and poly-L-Lysine were used as blanks.

Paracellular permeability coefficients of the tri-culture transwell system

Paracellular permeabilities of the *in vitro* tri-culture transwell system were evaluated by measuring the Pe which indicates the transition of small water-soluble molecules, such as NaF (Deli et al., 2005) The fluorescence intensities of NaF were measured on the 4^{th}, 6^{th}, 8^{th}, and 10^{th} days after the constitution of the tri-culture transwell system. For this purpose, all inserts were transferred into a 12-well plate containing 1500 µL of pre- warmed Ringer HEPES buffer (150 mM NaCl, 2.2 mM CaCl2, 0.2 mM MgCl2, 5.2 mM KCl, 2.8 mM glucose, 5 mM HEPES, and 6 mM NaHCO3, pH 7.4) in the lower compartment. Mediums in the upper compartment of the inserts were then replaced with 500 µL of pre-warmed working solutions containing NaF at 10 µg/mL in Ringer HEPES buffer and collected at 0, 24, 48 and 72 h time points. The concentrations and transmittance of the NaF in the samples, obtained from the upper and lower compartments, were measured using fluorescence multiwell plate reader (excitation wavelength 485 nm, and emission wavelength 530 nm), and the Pe values were calculated as previously described (Deli et al., 2005; Eigenmann et al., 2013).

Determination of Aβ1-42 disaggregation efficacy of the polyphenols and combinations preparation of Aβ1-42 aggregates

Aβ1-42 aggregates were prepared in 1% ammonium hydroxide (NH_4OH) and diluted with 1X phosphate-buffered saline (PBS pH 7.4). After that, 10 µM Aβ1-42 were incubated at 37°C for 4, 24, 48, 72, and 96 h for the aggregation (Feng et al., 2009). Aggregation of Aβ was confirmed with FESEM and Aggregated Aβ ELISA assays (Thermo Fisher Scientific, USA) (Kang et al., 2011; Das et al., 2016). For FESEM analysis, Aβ1-42 samples or Aβ1-42 and polyphenols/combinations were deposited onto carbon- coated copper grids (100-mesh) and air-dried. After that, the samples were stained negatively using 2% (w/v in water) uranyl acetate. The stained samples were analyzed using a Zeiss Sigma 500 FESEM system with an accelerating voltage of 30 kV (Das et al., 2016).

Evaluation of cytotoxicity

The cytotoxic effect of aggregated Aβ1-42 on differentiated SH-SY5Y cells was assessed by performing WST-1 and lactate dehydrogenase (LDH) assays at 24, 48, and 72 h time points. The effects of the polyphenols on the viability of the differentiated SH-SY5Y cells were also determined at the 24^{th} and the 48^{th} h. Concentrations of the polyphenols that led to 90% viability of differentiated SH-SY5Y cells were accepted as non-toxic concentrations (EGCG (Sigma, E4143) 2.5 µM – punicalagin (Sigma, P0023) 7,5 µM – ellagic acid

(Sigma, E2250) 10 μM – gastrodin (Sigma, SMB00313) 25 μM). To examine simultaneous cytotoxic effects of Aβ1-42 and polyphenols, viabilities were assessed at 24, 36, and 48 h time points. Similarly, to examine simultaneous cytotoxic effects of Aβ1-42 and combinations, viabilities were also assessed at 12, 24, and 36 h time points. The treatment times that led to a minimum of 50% viability were accepted as appropriate treatment times for Aβ1-42 disaggregation efficacy experiments. To find maximum non-toxic polyphenol concentrations on hCMEC /D3 and HA cell viability, concentrations that are 2-10 times the determined concentrations of the polyphenols were also studied.

Aβ1-42 disaggregation efficacy of the polyphenols and their combinations

For this purpose, Aβ1-42 and the polyphenols were incubated at 37°C for 12, 24, 36, and 48 h, and combinations of the polyphenols were also incubated at 37°C for 12, 24, 36 h. Disaggregation efficacy of the polyphenols or combinations were examined using Thioflavin T (ThT) (Sigma) and FESEM methods. ThT is a known fluorescent molecule that binds to aggregated Aβ1-42 *via* its benzyl groups. The decrease of fluorescence intensities indicates disaggregation or destabilization of the aggregated Aβ. In this study ThT fluorescence intensity was measured using a fluorescence plate reader (Ex: 450 nm, Em: 482 nm) (Ryan et al., 2013; Xue et al., 2017).

Experiments on the tri-culture transwell system Transition of the polyphenols

Transition of the polyphenols from the tri-culture transwell system was evaluated at the 6th h by Folin-Ciocalteu (FC) method, and transition percentages of the polyphenols were calculated as gallic acid equivalents. This method was performed as described in Sultana et al.'s (2014) study with modifications. The calibration was done using different concentrations of gallic acid (0.00, 5, 10, 20, 40, 60, 80 and 100 μg/mL). Briefly, 100 μL Folin-Ciocalteu reagent previously diluted with distilled water (1:10) was mixed with 0.02 μL of each sample. The solution was allowed to stand for 5 min at room temperature. Then 80 μL of 7.5% sodium carbonate solution was added to each tube and left for 1 h at room temperature. The absorbance was measured at λ=760 nm. Gallic acid equivalents were used to calculate a proportional constant for each polyphenol which would subsequently be utilized to calculate the concentrations of the polyphenols to be applied to the luminal side of the system in the following experiments.

Evaluation of the effects of polyphenols on the integrity of the tri-culture transwell system and disaggregation of Aβ1-42

The effects of the polyphenols, combinations, and Aβ1-42 on the integrities of the tri-culture transwell system were evaluated by measuring TEER values (Nakagawa et al., 2009; Srinivasan et al., 2015). Aβ1-42 was added from the abluminal side on the 4th day of the system construction. Subsequently, the polyphenols and their combinations were added from the luminal side. TEER value recording times were determined according to the ThT results in which disaggregation of Aβ1-42 had been expressed. Therefore, TEER values were recorded at the 24th h for Aβ1-42, 48th h for EGCG and EA, 36th h for PU and GA, and 24th h for Com 1 and Com 2. Aβ1-42 disaggregation capacity of the polyphenols and combinations were also analyzed by performing aggregated amyloid-beta ELISA assay.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA). The graphs represent data from at least three independent experiments, all performed in triplicate as means ± standard error of the mean (SEM). Changes were considered statistically significant at $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***). Statistical analyses were performed with GraphPad Prism 8. At least three replicates were measured for each experiment.

RESULTS

The integrity of the transwell systems

TEER values of co- and tri-culture transwell systems were measured on the 2nd, 4th, 6th, and 8th days and compared to each other. There were no significant differences among the TEER values of co-culture systems for the 2nd, 4th, 6th, and 8th days. ($p>0.05$) (Fig. 2A). However, TEER value of the tri-culture transwell system was significantly higher than those of the co-culture transwell systems on the 2nd day (12.47 ± 0.85 Ωcm^2 for hCMEC/D3-HA; $p<0.001$ and 16.38 ± 1.69 Ωcm^2 for hCMEC/D3-differentiated SH-SY5Y; $p<0.01$). Moreover, the TEER values on the 6th day of the tri-culture transwell system was found to be sig-

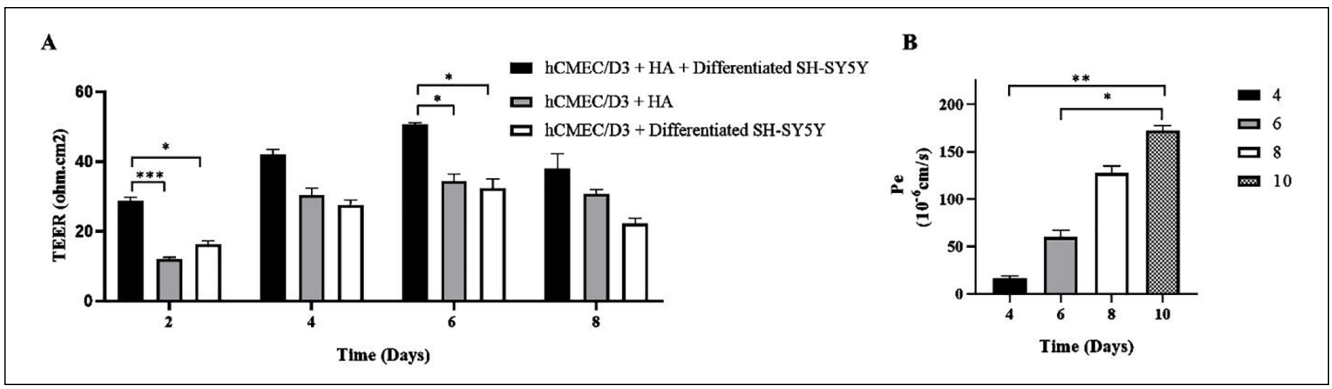


Fig. 2. Evaluation of TEER and Pe values of the transwell systems. (A) TEER values (Ω.cm²) of co- and tri-culture *in vitro* transwell systems. (B) Permeability coefficients (Pe) for sodium fluorescein of the tri-culture transwell system (Pe expressed in 10-6 cm/s). All data are presented as mean ± SEM of three independent experiments. ***p<0.001, **p<0.01, and *p<0.05 indicate statistically significant differences between the groups.

nificantly higher (50.76 ± 0.69 Ωcm²) than those of the co- culture transwell system (34.48±3.38 Ωcm² for hCMEC/D3-HA; and 32.48 ± 4.55 Ωcm² for hCMEC/D3-differentiated SH-SY5Y) ($p<0.05$). Since the obtained results clearly showed that the tri-culture transwell system provides higher TEER values than the co- culture system, we decided to continue our experiments with the tri-culture system. The TEER values were also compared with each other for all days. The high TEER values were obtained from the 4th and 6th days and no significant difference was found between these days (42.09 Ωcm² ± 3.02 and 50.76 Ωcm²±0.69 respectively) ($p>0.05$) (Fig. 2A). After that, the paracellular permeability feature of the membrane was assessed by measuring the transition of NaF, and the Pe coefficients of tri-culture transwell system were evaluated on the 4th, 6th, 8th, and 10th days (Fig. 2B). Data showed that Pe values tend to rise significantly from the 4th until the 10th day, especially on the 8th and the 10th days ($p<0.001$). Considering the highest TEER and the lowest Pe values, the 4th day was selected as the Aβ1-42 application day and the 6th day was determined as the model termination day.

Aggregation time of Aβ1-42

The suitable incubation time for the formation of Aβ1-42 aggregate was evaluated by performing FESEM and aggregated Aβ ELISA assays at the 4th, 24th, 48th, 72nd, and 96th h (Fig. 3A-E). Formations of Aβ aggregation were observed at all incubation times and no

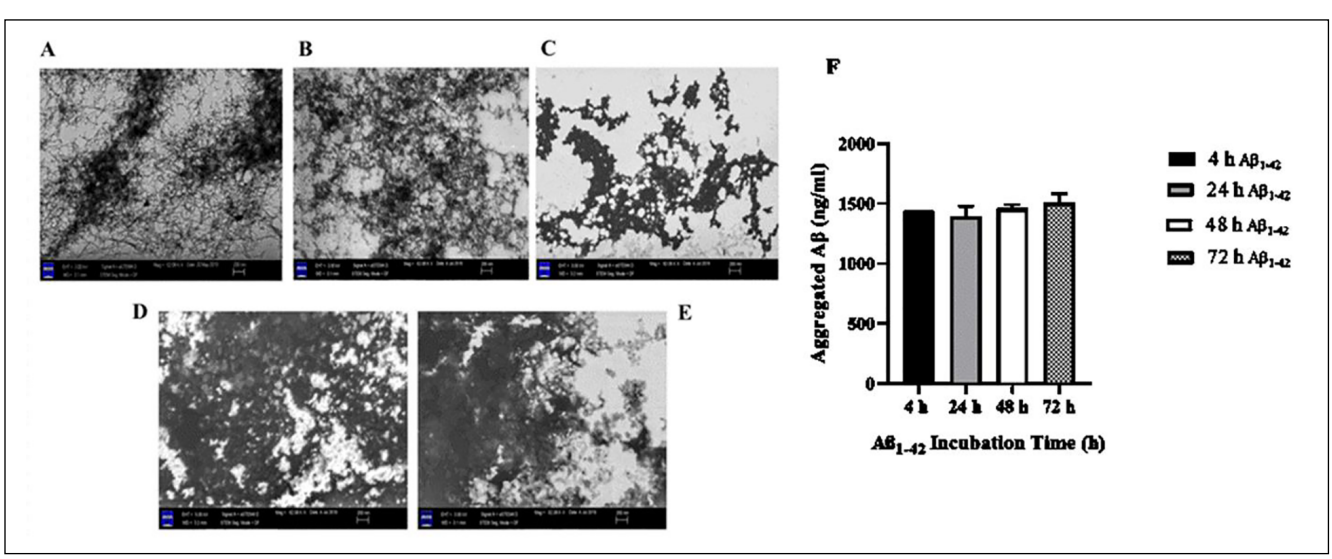


Fig. 3. FESEM images of Aβ1-42 aggregation at different time points (200 nm, 30 kV). (A) 4 h incubation of Aβ1-42, (B) 24 h incubation of Aβ1-42, (C) 48 h incubation of Aβ1-42, (D) 72 h incubation of Aβ1-42, (E) 96 h incubation of Aβ1-42. (F) Aggregated Aβ1-42 values at different incubation time points (4 h, 24 h, 48 h, 72 h). All data are presented as mean ± SEM of at least three independent measurements. *p<0.05 indicates statistically significant differences between different time points.

significant differences were found when aggregated Aβ ELISA assay was carried out as well (Fig. 3F). However, more globular cluster patterns were observed at the 48th, 72nd, and 96th h, which resemble the late stage of AD. Therefore, we decided to use the Aβ1-42 aggregate obtained at the 24th h for further experiments.

Cytotoxic effects of Aβ1-42 and polyphenols on differentiated SH-SY5Y cells

The effects of aggregated Aβ1-42 on the viability of differentiated SH-SY5Y cells were examined with WST-1 and LDH analysis at the 24th, 48th, and 72nd h. Our results showed that 10 μM of Aβ1-42 decreased differentiated SH-SY5Y cell viability to 82% at the 24th, 54% at the 48th and 52% at the 72nd h (Fig. 4A). The cell cytotoxicity results were 21% at the 24th, 52% at the 48th and 70% at the 72nd h (Fig. 4B).

Cytotoxic effects of polyphenols on the cells were also assessed at a wide range of concentrations at the 24th and 48th h (EGCG (1-20 μM), PU (2-10 μM), EA (10-100 μM), and GA (10-100 μM)). For the subsequent experiments, the polyphenol treatment time that provides 90% cell viability was chosen. Determined concentrations were 2.5 μM for EGCG, 7.5 μM for PU, 10 μM for EA, and 25 μM for GA (supplement data 1). After that, differentiated SH-SY5Y cells were treated with 10 μM Aβ1-42 and the determined polyphenol concentrations simultaneously. The results showed that the differentiated SH-SY5Y cell viability values did not decrease below 50% until 48 h. Therefore, disaggregation effects of the polyphenols were investigated until 48 h (Fig. 5A). Based on the viability results of Aβ1-42 and the polyphenols, combinations were composed as follows: 2.5 μM EGCG + 7.5 μM PU (Com 1), 2.5 μM EGCG + 10 μM EA (Com 2), 2.5 μM EGCG + 25 μM GA (Com 3), 7.5 μM PU + 10 μM EA (Com 4), 7.5 μM PU + 25 μM GA (Com 5), and 25 μM GA + 10 μM EA (Com 6). Viability assays were performed for all combinations in the presence of Aβ1-42. The results showed that there was no significant toxicity on differentiated SH-SY5Y cells for Aβ1-42 + Com 1 and Aβ1-42 + Com 2 groups at 12 h and 24 h (81%, 70% for 12 h and 71%, 65% for 24 h, respectively) (Fig. 5B). To investigate the effects of the polyphenols on hCMEC/D3 and HA cell viability, WST-1 assays were also performed at the 24th h. The same concentrations of the polyphenols that had been used for differentiated SH-SY5Y cells were assessed (EGCG 2,5 μM, punicalagin 7,5 μM, ellagic acid 10 μM, and gastrodin 25 μM). However, in order to determine the nontoxic concentration ranges of the polyphenols on the cells, the cells were also treated with higher polyphenol concentrations, ranging from 2 to 10 folds. Results revealed that the selected polyphenols and their combinations did not show toxic effects on hCMEC/D3 or HA cells at those concentrations (Fig. 6A, B, C).

Aβ1-42 disaggregation effects of the polyphenols and polyphenol combinations

Disaggregation effects of the polyphenols and polyphenol combinations on aggregated Aβ1-42 were assessed with ThT, FESEM, and aggregated Aβ ELISA assays. The results clearly showed that the presence of polyphenols and polyphenol combinations increased the disaggregation of Aβ1-42. We observed significant decreases in ThT values at 24 h, 36 h, and 48 h, which indicate the disaggregation of Aβ1-42 ($p<0.001$). Maximum disaggregation occurred at the

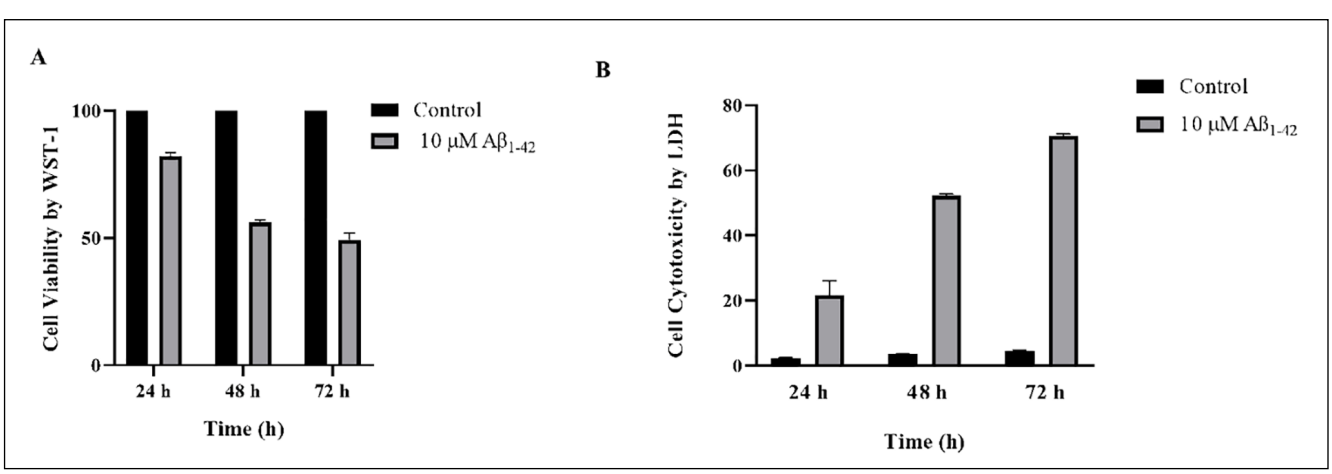


Fig. 4. The viability of differentiated SH-SY5Y cells treated with Aβ1-42 for 24 h, 48 h and 72 h, measured by WST-1 (A) and LDH (B) assays. All data are presented as mean ± SEM of at least three independent measurements. Cell viability is presented as percentage relative to control.

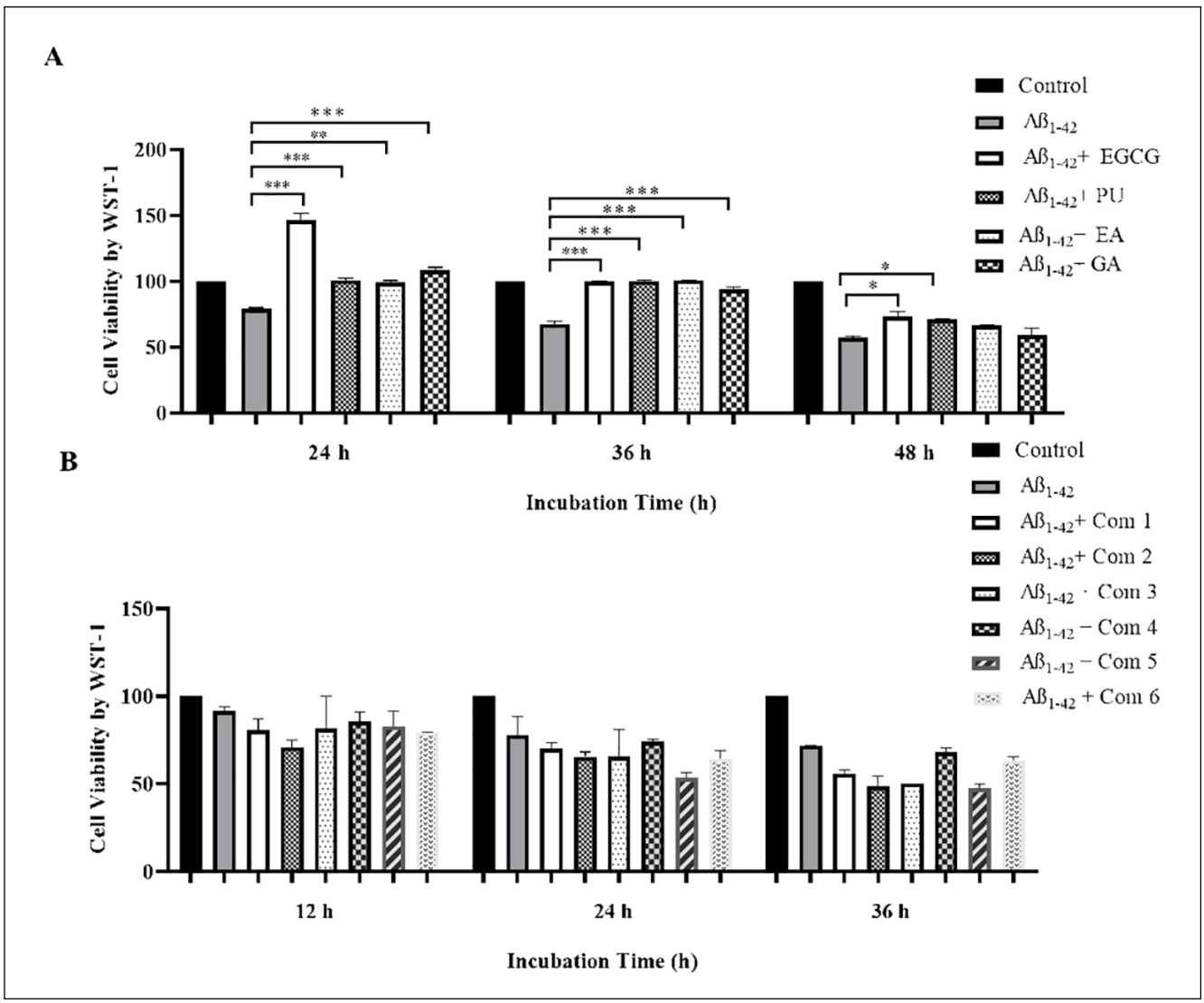


Fig. 5. The viability of differentiated SH-SY5Y cells treated with Aβ1-42 and polyphenols (A). The viability of differentiated SH-SY5Y cells treated with Aβ1-42 and polyphenol combinations (B). Data are presented as mean ± SEM of at least three independent measurements. ***p<0.001, **p<0.01, and *p<0.05 indicate statistically significant differences between the groups.

48th h in EGCG and EA applied groups (30.52% and 45.01%, respectively; p <0.001) and at the 36th h in PU and GA applied groups (41.84% and 36.68% respectively; p<0.001) (Fig. 7A). Afterward, ThT values of Com 1 and Com 2 groups showed a remarkable decrease in aggregated Aβ1-42 , especially at the 24th h (75% and 64%, respectively (p<0.001)) (Fig. 7B). Based on these data, Com 1 and Com 2 were chosen for further experiments. Disaggregation effects of the polyphenols and combinations were also evaluated by performing aggregated Aβ ELISA and FESEM assays for the times in which maximum disaggregation results had been obtained by ThT assay. Aβ ELISA results confirmed that each polyphenol, Com 1, and Com 2 combinations had a powerful and statistically significant disaggregation effect on Aβ1-42 (p<0.001) (Fig. 7C). FESEM images also showed that polyphenols and combinations disaggregated Aβ1-42 in a different pattern. For instance, we observed that groups treated with EGCG and groups treated with GA tend to alter Aβ1–42 aggregate appearance to a fibrillar-like one, PU-treated group tends to reduce aggregation density leading to an amorphous appearance, and EA-treated group reduces the foliated structure of Aβ1-42 aggregate (Fig. 7D, H). The appearance of Com 1-treated Aβ1-42 was fibrillar and fragmental, similar to EGCG and PU groups, and Com 2-treated Aβ1–42 was similar to EA-treated group (Fig. 7I, J).

Transition percentages of the polyphenols through the tri-culture transwell system Transition

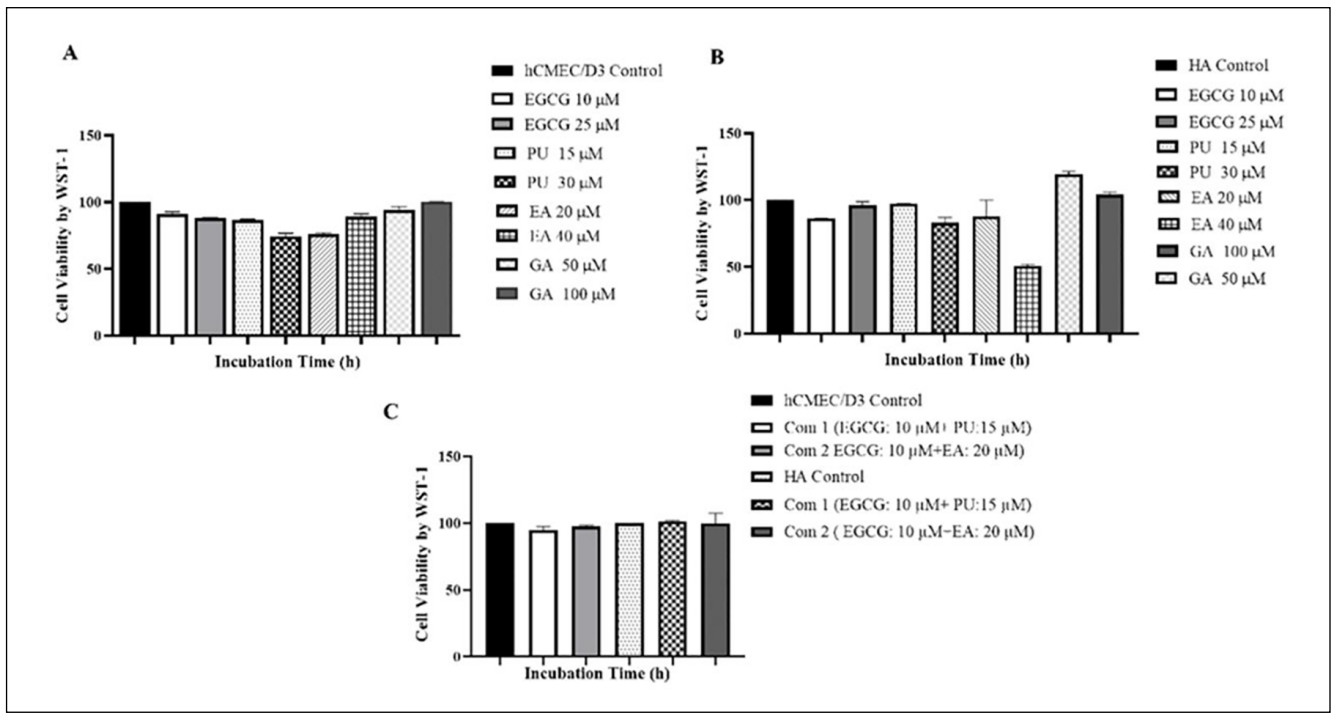


Fig. 6. The effect of different concentrations of polyphenols and combinations on hCMEC/D3 and HA cell viabilities. The effect of polyphenols on hCMEC/D3 cells (A), HA cells (B) and polyphenol combinations on hCMEC/D3 and HA cells (C). Data are presented as mean ± SEM obtained from at least three independent measurements. Cell viability is presented as percentage relative to control.

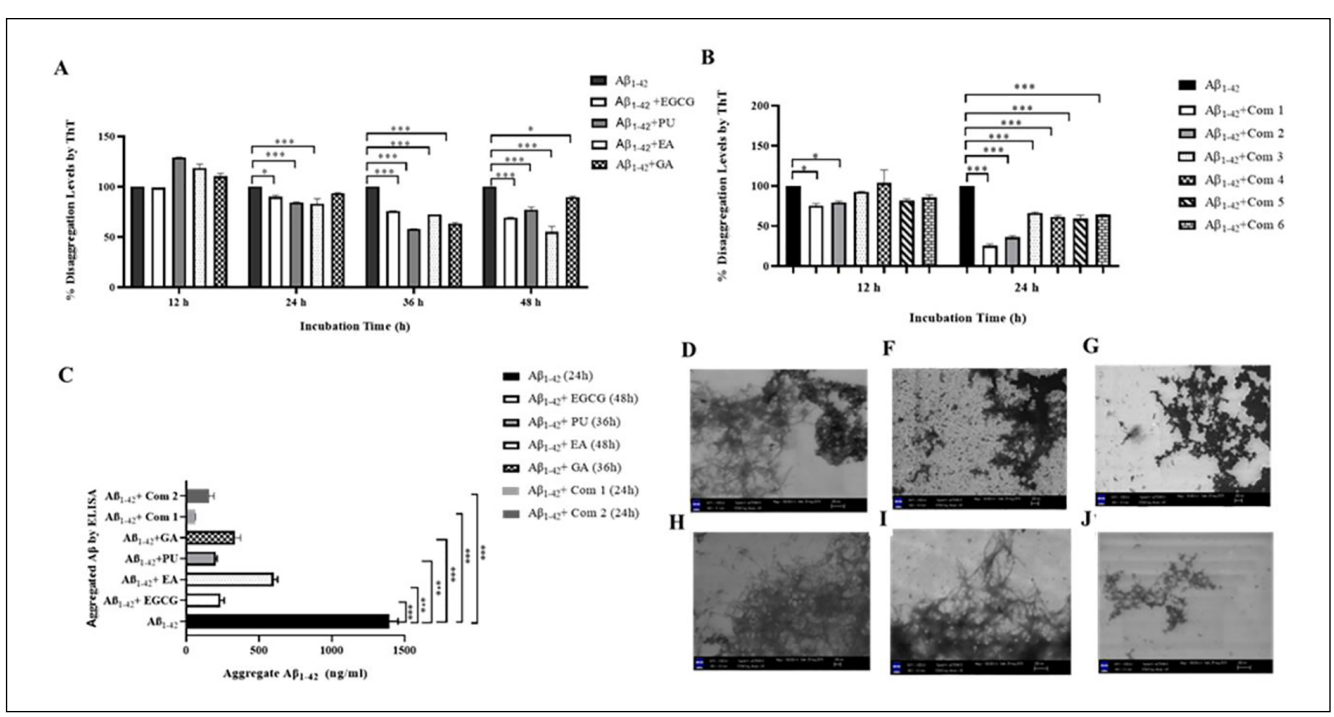


Fig. 7. The effects of polyphenols (A) and polyphenol combinations (B) on Aβ1-42 aggregation by ThT assay. Aggregated Aβ ELISA kit (C) and FESEM images (FESEM, 200 nm, 30kV) (D-J), (D) EGCG (48h), (F) PU (36 h), (G) EA (48 h), (H) GA (36 h), (I) Com 1 (24 h), (J) Com 2 (24 h). Data are presented as mean ± SEM obtained from at least three independent measurements. ***p<0.001, *p<0.05 indicate statistically significant differences between the groups.

percentages of the polyphenols through the tri-culture transwell system were evaluated at the 6th h after the treatments. The factors were calculated from the transition percentages of the polyphenols between luminal and abluminal sides at the 6th h. Polyphenol concentrations to be applied to the luminal side of the system were found by multiplication of the proportional transmittance constants with the concentration of the polyphenols (Table 1). Transition percentages of EGCG, PU, EA and GA were found at the 6th h as 87.82%, 61.19%, 97.70%, and 70.18%, respectively.

Integrity of the tri-culture transwell system and Aβ1-42 disaggregation efficiency of polyphenols and combinations

The effects of Aβ1-42, the polyphenols, and combinations on the integrity of the tri-culture transwell system were examined by measuring TEER values. There were significant differences in the TEER values between the tri-culture transwell systems treated with Aβ1-42 and those that weren't treated with Aβ1-42 ($p<0.01$). Besides, some polyphenols and combinations enhanced TEER values of the system significantly when compared with the Aβ1-42 treated group ($p<0.01$ for Aβ1-42-EA, $p<0.01$ for Aβ1-42-GA, $p<0.05$ for Com 1, and $p<0.05$ for Com 2) (Fig. 8A). Disaggregation efficiencies of polyphenols and combinations were also confirmed as samples were taken from the basolateral side of the tri-culture transwell system and aggregated Aβ ELISA test was performed on them. All the groups showed significant differences, but the more efficient groups were Com 1 and Com 2 as they disaggregated Aβ1-42 at 75% and 64% percentages respectively ($p<0.001$) (Fig. 8B).

DISCUSSION

This study is the first to establish and compare a novel co- and tri-culture BBB system consisting of human brain endothelial, astrocyte, and differentiated SH-SY5Y cells with the aim of constructing an *in vitro* tool that mimics the *in vivo* BBB environment. Our results revealed that the tri-culture transwell system has higher barrier properties than the co-culture systems. Our study also showed for the first time that combinations of the polyphenols produce a synergistic effect on the Aβ1-42 disaggregation, and that Com 1 and Com 2, consisting of EGCG-PU and EGCG-EA, were more effective than the other combinations.

Currently, studies emphasize that in order to reach closer results to *in vivo*, cells used in an *in vitro* model need to be characteristically resemblant to the *in vivo* environment. Some researchers have also emphasized that using SH-SY5Y cells without differentiation is not appropriate for *in vitro* studies (Shipley et al., 2016; de Medeiros et al., 2019;, Krishtal et al., 2019). The primary difference mentioned in the literature is the polarization difference between undifferentiated and differentiated SH-SY5Y cells which plays a significant role in acquiring the neuron-like characteristics (Goldie et al., 2014; de Medeiros et al., 2019; Chandrasekaran et al., 2000). Krishtal et al. (2019) also advocated that the *in vivo* reflection level of an *in vitro* system can facilitate the adaptation of the obtained results to *in vivo*. On the other hand, they demonstrated that longer neurites and larger surface areas of differentiated SH-SY5Y cells might cause sensitivity to Aβ1-42 toxicity. For this reason, to examine the Aβ disaggregation effects of the polyphenols, we intended to develop an *in vitro* transwell system using differentiated SH-SY5Y cells. Immortalized brain endothelial, astrocyte, pericyte, neuron, or glial cell

Table 1. Transition percentages of the polyphenols through the tri-culture BBB are presented as gallic acid equivalent. Data are presented as mean ± SEM obtained from at least three repeated measurements. *Proportional transmittance constants were calculated from the transition percentages of the polyphenols at the 6th h and the constants were used to calculate the concentrations of the polyphenols to be applied to the luminal side of the transwell systems for the subsequent experiments.

Applied polyphenol concentrations (luminal side)	Gallic acid equivalents of the applied polyphenols (luminal side) (μg/ml)	Gallic acid equivalents of polyphenols (6th abluminal side) (μg/ml)	Transition percentages of the polyphenols at 6th (%)	*Proportinal constants
EGCG (25 μM)	85.78	75.33	87.82	1.14
PU (15 μM)	49.61	30.36	61.19	1.63
EA (20 μM)	50.12	48.97	97.70	1.02
GA (100 μM)	31.62	22.19	70.18	1.42

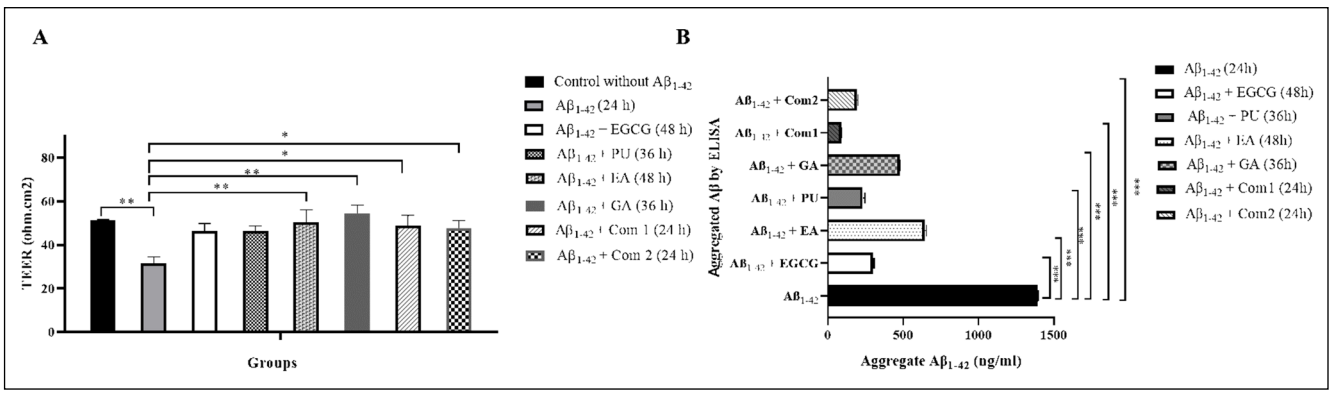


Fig. 8. (A) TEER values of the tri-culture BBB model treated with Aβ1-42 and the polyphenols. (B) Disaggregation effect of polyphenols/polyphenol combinations on Aβ1-42 in tri-culture transwell system measured by aggregated Aβ ELISA kit. Data are presented as mean± SEM obtained from at least three independent measurements. ***p<0.001 indicates statistically significant differences between the groups.

lines, which have been developed from rat, mouse, and human tissues, are frequently used for *in vitro* BBB systems. These cell lines offer the benefit of their ease of use and show expression of tight junction proteins and other BBB specific proteins. However, they generally develop TEER<100 Ω*cm^2 (Weksler et al., 2005; Helms et al., 2015). It is emphasized in the literature that establishing co- or tri-culture transwell systems with these cells contributes to tight junction (TJ) formation and higher TEER values, which reflect the functionality and integrity of the BBB (Nakagawa et al., 2007; 2009; Hatherell et al., 2011). Besides, there is evidence that a close contact between astrocytes and neuron cells regulates important features for BBB function (Banerjee et al., 2016). Therefore, we used differentiated neuronal cells and astrocytes in close contact in the BBB system.

Commonly used methods for the verification of an *in vitro* BBB system and formation of TJs are the evaluation of TEER values, paracellular permeability assessment by using various chemicals such as NaF, and determination of protein expressions. TEER values are considered as a quantitative technique to measure the integrity of tight junction dynamics in cell culture models (Srinivasan et al., 2015). However, when *in vivo* TEER values have been considered, they may exceed 1000 Ωcm^2. On the other hand, TEER values obtained from *in vitro* studies are in the range of 20 to 200 Ωcm^2, which is considerably lower (Reichel et al., 2003; Förster et al., 2008). Eigenmann et al. (2013) express that despite this limitation, *in vitro* models with human immortalized cell lines possess several advantages and may be favorable tools for obtaining first mechanistic insights into BBB permeability of drugs. Since there are no exact TEER and Pe values for *in vitro* BBB systems, various TEER values can be seen in the literature for mono, co- and tri-culture transwell systems established with different cell lines (Hatherell et al., 2011; Rahman et al., 2016).

There is also discrepancy in the literature about the effect of co- and tri-culture systems on TEER values. While some studies advocate that co- and tri-culture systems support the formation of TJ connection and rising of TEER values, several studies indicate the ineffectiveness of co- and tri-culture transwell systems on TEER values (Nakagawa et al., 2009; Hatherell et al., 2011, Eigenmann et al., 2013). For instance, Hatherell et al. (2011) constructed a tri-culture model with hCMEC/D3, pericytes, and astrocyte cells. The model has been confirmed with only TEER values. TEER values of their tri-culture models were found to be significantly higher than the mono-culture values (44±0.9 Ωcm^2 and 39 ± 1.4 Ωcm^2 respectively). However, the research group also advocated that a tri-cultured transwell system with endothelial, pericyte, and astrocyte cells did not improve tight junction formation or TEER values of the system when compared to the co-cultivation of astrocytes with endothelial cells. De Laere et al. (2017) also advocate that co-culture system with hCMEC/D3 endothelial cells and human primary astrocytes provides the highest TEER values on the 10th day (30.63 ± 1.11 Ωcm^2). However, Nagakawa et al. (2009) emphasized that although all co-culture models showed higher TEER than the brain endothelial cell monolayers, triple culture models showed higher TEER values (354 ± 15 Ωcm^2 on the 4th day) when compared to co-culture models. In addition, Xue et. al. (2013) found that the TEER value of the tri-culture model established with neurons, astrocytes, and rat microvascular endothelial cells (BMECs) was 268.67 Ω×cm^2.

Generally, in the literature, BBB models which are established with primary cell cultures show higher TEER values than BBB models structured with immortalized cell lines. Therefore, the higher TEER value

of Nagakawa et al's (2009) study might be related to the used primary cell lines (primary cultures of rat brain capillary endothelial cells (RBEC), pericytes, and astrocytes). Overall, as can be seen in the literature, there are many TEER values and higher TEER values are obtained with primary cell culture compared to immortalized cells. Therefore, we thought that it would be more appropriate to evaluate each study within itself. Comparison of our TEER values for co- and tri-culture transwell systems demonstrated that the highest TEER value belonged to the tri-culture-BBB system, established with astrocyte and differentiated SH-SY5Y cells ($p<0.05$). Therefore, we decided that the tri-culture transwell system has a more reflective property of a neurovascular unit, including brain endothelial cells, astrocytes, differentiated SH-SY5Y cells (neuron). In many studies, evaluation of *in vitro* systems has been performed only by TEER measurement and without Pe assessment (Hatherell et al., 2011; Eigenmann et al., 2013; Maherally et al., 2018). We thought that verifying an *in vitro* transwell system by TEER and Pe values at the same time may provide information on the integrity, paracellular permeability, and efficient usage period of the system. Our TEER and Pe results revealed that the tri-culture transwell system did not lose its features for up to 10 days.

Recently, the identification of compounds that destabilize Aβ aggregate is seen as a therapeutic approach for the amyloidogenic pathology in AD (Kim et al., 2015; Phan et al., 2019). Phan et al. (2019) also emphasized that the chemical structure of the polyphenols is crucial in the solubilization of Aβ1-42 aggregates. Studies showed that polyphenols can prevent or disentangle the aggregation of Aβ by their hydrophobic forces, electrostatic and aromatic interactions with the target protein's side chain residues (Porat et al., 2004; Hills and Brooks, 2007; Bieschke et al., 2010; Das et al., 2016; Velander et al., 2017). Based on this information, EGCG, PU, EA, and gastrodin polyphenols – representing flavonoid, tannin, phenolic acid, and phenolic glycoside groups respectively-were selected for our study. Since these polyphenols contain different hydrophobic and hydrophilic groups, we thought they could be more interactive with the hydrophobic and hydrophilic amino acids in the Aβ structure and provide effective dissolution. Our FESEM, ThT, and aggregated Aβ results confirmed that EGCG (2.5 μM) and EA (10 μM) effectively dissolved and disrupted the plaque structure of Aβ at 48 h ($p<0.001$). Ehrnboefer et al.'s work (2008) supports this finding as they showed that EGCG effectively bound to the Aβ aggregate by forming stable hydrogen bonds that inhibit fibrillogenesis of Aβ1-42. Biesche et al. (2010) also showed that 50 μM EGCG dissolved 15 μM Aβ aggregate into small non-toxic amorphous protein structures. Our EA-related results were also consistent with the only *in vitro* findings of Feng et al. (2009), but the concentration of EA used in that study is ten times higher than ours. The reason for their high EA concentration may be due to the experiments being performed in cell-free tubes. Although many *in vitro* and *in vivo* results show that gastrodin has a therapeutic capacity for the treatment of AD, no *in vitro* study has revealed the dissolving performance of gastrodin on Aβ1-42 plaques. Our results showed that 25 μM gastrodin effectively dissolved 10 μM Aβ plaques at 36 h ($p<0.001$). This data represent a contribution to the literature. Punicalagin is another polyphenol with which we have difficulties in comparing our results to the previous studies. In one study, Aβ1-42 anti-aggregation properties and neuronal toxicity effects of punicalagin were investigated in PC12 cells *via* in silico interactive modeling (Das et al., 2016). They showed that 100 μM punicalagin bound to 10 μM Aβ1-42 through stearic and hydrogen bond interactions and inhibited neurotoxicity at 48 h. They also demonstrated that it bound to Aβ1-42 from Leu17den Val39 residues due to the large molecular structure of punicalagin. In our study, 10 μM of Aβ effectively dissolved at 36 h with a much lower concentration of PU (7.5 μM) ($p<0.001$).

Recent studies also emphasize that combinations of polyphenolic compounds can create synergistic effects and thus provide more effective results on Aβ disaggregation (Giunta et al., 2010; Choi et al., 2013; Zheng et al., 2019; Mori et al., 2019). In our study, after the evaluation of Aβ1-42 dissolving capacity of the polyphenols individually, we found that all combination groups (6 groups, each formed with two different polyphenols) can dissolve Aβ1-42 plaques in various ratios. High level of Aβ disaggregation were seen in the Com 1- and Com 2-treated groups ($p <0.001$). Our study is the first in the literature to reveal that the synergistic combinations of these polyphenols effectively dissolve Aβ1- 42 aggregation in a short time.

In the literature, studies related to the Aβ disaggregation capacity of polyphenols were generally performed in a tube and without examining their transition ability through the BBB system (Ehrnhoefer et al., 2008; Feng et al., 2009; Das et al., 2016). Since the effectiveness of polyphenols directly depends on their passing feature through the BBB, examination of their transition is critical. For this reason, we evaluated the transition ability of the polyphenols in our developed tri-culture transwell BBB system, which has never been researched before. Our results showed that all polyphenols can pass through the *in vitro* tri-culture transwell BBB system with a high percentage. Afterwards, we evaluated the disaggregation capacity of

the polyphenols in the Aβ-containing tri-culture transwell system. Moreover, the effects of polyphenols on the integrity of the Aβ1-42- containing transwell system were analyzed using TEER values. Our data clearly showed that the addition of Aβ1-42 to the tri-culture transwell system led to a decrease in TEER values. These results may possibly be related to Aβ1-42 toxicity and damage to TJ connections. However, our TEER data may also suggest that the addition of polyphenols/combinations enhanced TJ connections and reduced BBB leakage induced by Aβ1–42 aggregates.

CONCLUSION

In vitro BBB models are important tools for the studies on Aβ1-42 disaggregation. Therefore, we propose the tri-culture system, developed by human endothelium, astrocyte, and differentiated SH-SY5Y cells, as a suitable model. Besides, our findings provide the first basis for further studies on the therapeutic potentials of the Com 1 and Com 2 combinations, consisting of EGCG-PU and EGCG-EA, on Aβ1-42 disaggregation in AD.

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REFERENCES

Abbott NJ, Rönnbäck L, Hansson E (2006) Astrocyte-endothelial interactions at the blood-brain barrier. Nat Rev Neurosci 7: 41–53.

Banerjee J, Shi Y, Azevedo HS (2016) In vitro blood–brain barrier models for drug research: state-of-the-art and new perspectives on reconstituting these models on artificial basement membrane platforms. Drug Discov Today 21: 1367–1386.

Bieschke J, Russ J, Friedrich RP, Ehrnhoefer DE, Wobst H, Neugebauer K, Wanker EE (2010) EGCG remodels mature α-synuclein and amyloid-β fibrils and reduces cellular toxicity. PNAS 107: 7710–7715.

Chandrasekaran V, Zhai Y, Wagner M, Kaplan PL, Napoli JL, Higgins D (2000) Retinoic acid regulates the morphological development of sympathetic neurons. J Neurobiol 42: 383–393.

Choi RCY, Zhu JTT, Yung AWY, Lee PSC, Xu SL, Guo AJY, Tsim KWK (2013) Synergistic action of flavonoids, baicalein, and daidzein in estrogenic and neuroprotective effects: A development of potential health products and therapeutic drugs against Alzheimer's disease. Evid Based Complement Alternat Med 2013.

Das S, Stark L, Musgrave IF, Pukala T, Smid SD (2016) Bioactive polyphenol interactions with β amyloid: a comparison of binding modelling, effects on fibril and aggregate formation and neuroprotective capacity. Food Funct 7: 1138-1146.

De Laere M, Sousa C, Meena M, Buckinx R, Timmermans JP, Berneman Z, Cools N (2017) Increased transendothelial transport of CCL3 is insufficient to drive immune cell transmigration through the blood-brain barrier under inflammatory conditions in vitro. Mediat Inflamm 2017: 6752756.

de Medeiros LM, De Bastiani MA, Rico EP, Schonhofen P, Pfaffenseller B, Wollenhaupt-Aguiar B, Klamt F (2019) Cholinergic differentiation of human neuroblastoma SH-SY5Y cell line and its potential use as an in vitro model for Alzheimer's disease studies. Mol Neurobiol 56: 7355–7367.

Deli Máiria A, Abrahám CS, Kataoka Y, Niwa M (2005) Permeability studies on in vitro blood-brain barrier models: physiology, pathology, and pharmacology. Cell Mol Neurobiol 25: 59–127.

Ehrnhoefer DE, Bieschke J, Boeddrich A, Herbst M, Masino L, Lurz R, Wanker EE (2008) EGCG redirects amyloidogenic polypeptides into unstructured, off-pathway oligomers. Nat Struct Mol Biol 15: 558–566.

Eigenmann DE, Xue G, Kim KS, Moses AV, Hamburger M, Oufir M (2013) Comparative study of four immortalized human brain capillary endothelial cell lines, hCMEC/D3, hBMEC, TY10, and BB19, and optimization of culture conditions, for an in vitro blood-brain barrier model for drug permeability studies. Fluids Barriers CNS 10: 33.

Feng Y, Yang SG, Du XT, Zhang X, Sun XX, Zhao M, Sun GY, Liu RT (2009) Ellagic acid promotes Aβ42 fibrillization and inhibits Aβ42-induced neurotoxicity. Biochem Biophys Res Commun 390: 1250–1254.

Förster C, Burek M, Romero IA, Weksler B, Couraud PO, Drenckhahn D (2008) Differential effects of hydrocortisone and TNFα on tight junction proteins in an in vitro model of the human blood-brain barrier. J Physiol 586: 1937–1949.

Giunta B, Hou H, Zhu Y, Salemi J, Ruscin A, Shytle RD, Tan J (2010) Fish oil enhances anti-amyloidogenic properties of green tea EGCG in Tg2576 mice. Neurosci Lett 471: 134–138.

Goldie BJ, Barnett MM, Cairns MJ (2014) BDNF and the maturation of post-transcriptional regulatory networks in human SH-SY5Y neuroblast differentiation. Front Cell Neurosci 8: 325.

Hatherell K, Couraud PO, Romero IA, Weksler B, Pilkington GJ (2011) Development of a three-dimension all-human in vitro model of the blood-brain barrier using mono-, co-, and tri-cultivation transwell models. J Neurosci Methods 199: 223–229.

Helms HC, Abbott NJ, Burek M, Cecchelli R, Couraud PO, Deli MA, Brodin B (2015) In vitro models of the blood-brain barrier: An overview of commonly used brain endothelial cell culture models and guidelines for their use. J Cereb Blood Flow Metab 36: 862–890.

Hills RD, Brooks CL (2007) Hydrophobic cooperativity as a mechanism for amyloid nucleation. J Mol Biol 368: 894–901.

Jin M, Shepardson N, Yang T, Chen G, Walsh D, Selkoe DJ (2011) Soluble amyloid β-protein dimers isolated from Alzheimer cortex directly induce Tau hyperphosphorylation and neuritic degeneration. PNAS 108: 5819–5824.

Kang IJ, Jeon YE, Yin XF, Nam JS, You SG, Hong MS, Kim MJ (2011) Butanol extract of Ecklonia cava prevents production and aggregation of beta-amyloid, and reduces beta-amyloid mediated neuronal death. Food Chemical Toxicol 49: 2252–2259.

Kim HY, Kim HV, Jo S, Lee CJ, Choi SY, Kim DJ, Kim Y (2015) EPPS rescues hippocampus-dependent cognitive deficits in APP/PS1 mice by disaggregation of amyloid-β oligomers and plaques. Nat Commun 6: 8997.

Krishtal J, Metsla K, Bragina O, Tõugu V, Palumaa P (2019) Toxicity of amyloid-β peptides varies depending on differentiation route of SH-SY5Y cells. J Alzheimers Dis 71: 879–887.

Kumar A, Singh A, Ekavali (2015) A review on Alzheimer's disease pathophysiology and its management: an update. Pharmacol Rep 67: 195–203.

Kumar P, Baquer N (2016) P1-110: Neuroprotective role of 17B estradiol against amyloid beta neurotoxicity in synaptosomes of aging female rats. Alzheimer's Dement 12: P444.

Maherally Z, Fillmore H L, Tan SL, Tan SF, Jassam SA, Quack FI, Pilkington GJ (2018) Real-time acquisition of transendothelial electrical resistance in an all-human, in vitro, 3-dimension blood-brain barrier model exemplifies tight-junction integrity. FASEB J 32: 168–182.

Mori T, Koyama N, Tan J, Segawa T, Maeda M, Town T (2019) Combined treatment with the phenolics (-)-epigallocatechin-3-gallate and ferulic acid improves cognition and reduces Alzheimer-like pathology in mice. J Biol Chem 294: 2714–2731.

Naik P, Cucullo L (2012) In vitro blood-brain barrier models: current and perspective technologies. J Pharmaceutical Sci 101: 1337–1354.

Nakagawa S, Deli MA, Kawaguchi H, Shimizudani T, Shimono T, Kittel Á, Niwa M (2009) A new blood-brain barrier model using primary rat brain endothelial cells, pericytes and astrocytes. Neurochem Int 54: 253–263.

Nakagawa S, Deli MA, Nakao S, Honda M, Hayashi K, Nakaoke R, Niwa M (2007) Pericytes from brain microvessels strengthen the barrier integrity in primary cultures of rat brain endothelial cells. Cell Mol Neurobiol 27: 687–694.

Ono K, Hasegawa K, Naiki H, Yamada M (2004) Anti-amyloidogenic activity of tannic acid and its activity to destabilize Alzheimer's β-amyloid fibrils in vitro. Biochim Biophys Acta 1690: 193–202.

Phan HTT, Samarat K, Takamur Y, Azo-Oussou AF, Nakazono Y, Vestergaard MC (2019) Polyphenols modulate Alzheimer's amyloid beta aggregation in a structure-dependent manner. Nutrients 11: 756.

Porat Y, Mazor Y, Efrat S, Gazit E (2004) Inhibition of islet amyloid polypeptide fibril formation: a potential role for heteroaromatic interactions. Biochemistry 43: 14454–14462.

Praticò D (2008) Evidence of oxidative stress in Alzheimer's disease brain and antioxidant therapy: Lights and shadows. Ann N Y Acad Sci 1147: 70–78.

Rahman NA, Rasil ANHM, Meyding-Lamade U, Craemer EM, Diah S, Tuah AA, Muharram SH (2016) Immortalized endothelial cell lines for in vitro blood-brain barrier models: a systematic review. Brain Res 1642: 532–545.

Reichel A, Begley DJ, Abbott NJ (2003) An overview of in vitro techniques for blood-brain barrier studies. Methods Mol Med 89: 307–324.

Ruck T, Bittner S, Meuth SG (2015) Blood-brain barrier modeling: Challenges and perspectives. Neural Regeneration Res 10: 889–891.

Ryan TM, Caine J, Mertens HDT, Kirby N, Nigro J, Breheney K, Roberts BR (2013) Ammonium hydroxide treatment of Aβ produces an aggregate free solution suitable for biophysical and cell culture characterization. PeerJ 1: e73.

Serdar BS, Erkmen T, Ergür BU, Akan P, Koçtürk S (2020) Comparison of medium supplements in terms of the effects on the differentiation of SH-SY5Y human neuroblastoma cell line. Neurol Sci Neurophysiol 37: 82–88.

Sharma P, Srivastava P, Seth A, Tripathi PN, Banerjee AG, Shrivastava SK (2019) Comprehensive review of mechanisms of pathogenesis involved in Alzheimer's disease and potential therapeutic strategies. Prog Neurobiol 174: 53–89.

Shipley MM, Mangold CA, Szpara M L (2016) Differentiation of the SH-SY5Y human neuroblastoma cell line. J Vis Exp 108: 53193.

Srinivasan B, Kolli AR, Esch MB, Abaci HE, Shuler ML, Hickman JJ (2015) TEER measurement techniques for in vitro barrier model systems. J Lab Autom 20: 107–126.

Sultana B, Fatima B, Mushtaq M (2014) In vitro synergism of antimutagenic and antioxidant activities of Phoenix dactylifera fruit. Food Sci Biotechnol 23: 881–887.

Velander P, Wu L, Henderson F, Zhang S, Bevan DR, Xu B (2017) Natural product-based amyloid inhibitors. Biochem Pharmacol 139: 40–55.

Weiss N, Miller F, Cazaubon S, Couraud PO (2009) The blood-brain barrier in brain homeostasis and neurological diseases. Biochim Biophys Acta 1788: 842–857.

Weksler BB, Subileau EA, Perrière N, Charneau P, Holloway K, Leveque M, Couraud PO (2005) Blood-brain barrier-specific properties of a human adult brain endothelial cell line. FASEB J 19: 1872–1874.

Wilhelm I, Krizbai IA (2014) In vitro models of the blood-brain barrier for the study of drug delivery to the brain. Mol Pharm 11: 1949–1963.

Wong DF, Rosenberg PB, Zhou Y, Kumar A, Raymont V, Ravert HT, Pontecorvo MJ (2010) In vivo imaging of amyloid deposition in Alzheimer disease using the radioligand 18F-AV-45 (flobetapir F 18). J Nucl Med 51: 913–920.

Xue C, Lin TY, Chang D, Guo Z (2017) Thioflavin T as an amyloid dye: fibril quantification, optimal concentration and effect on aggregation. R Soc Open Sci 4: 160696.

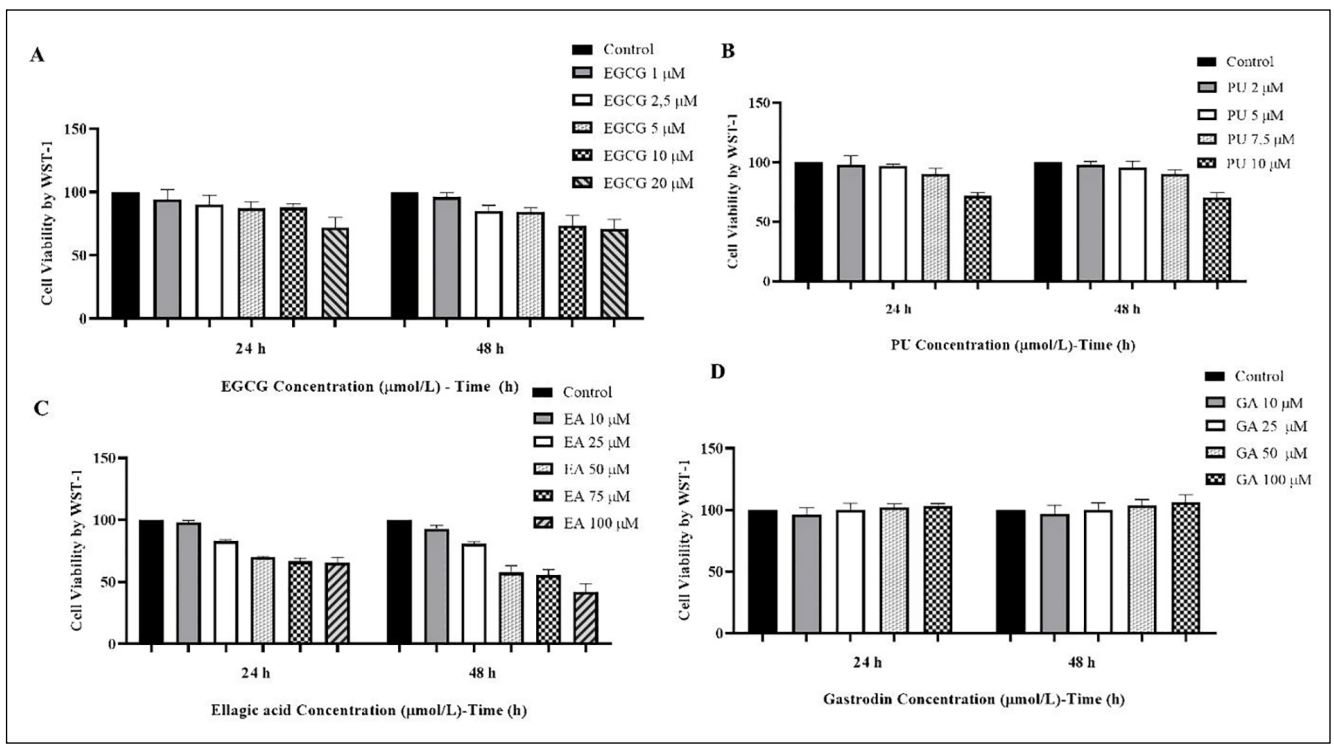
Xue Q, Liu Y, Qi H, Ma Q, Xu L et al. (2013) A novel brain neurovascular unit model with neurons, astrocytes and microvascular endothelial cells of rat. Int J Biol Sci 9: 174–189.

Yamin G, Ruchala P, Teplow DB (2009) A peptide hairpin inhibitor of amyloid β-protein oligomerization and fibrillogenesis. Biochemistry 48: 11329–11331.

Yang F, Lim GP, Begum AN, Ubeda OJ, Simmons MR, Ambegaokar SS, Chen PP, Kayed R, Glabe CG, Frautschy SA, Cole GM (2005) Curcumin inhibits formation of amyloid beta oligomers and fibrils, binds plaques, and reduces amyloid in vivo. J Biol Chem 280: 5892–5901.

Zheng Q, Kebede MT, Kemeh MM, Islam S, Lee B, Bleck SD, Lazo ND (2019) Inhibition of the self-assembly of Aβ and of tau by polyphenols: mechanistic studies. Molecules 24: 2316.

SUPPLEMENTAL MATERIALS



Supplement data 1. The viability of differentiated SH-SY5Y cells treated with the polyphenols for 24 and 48 h measured by WST-1 (A) EGCG (1-20 μM), (B) PU (2-10 μM), (C) EA (10-100 μM), (D) GA (10-100 μM). Data are presented as mean± SEM obtained from at least three independent experiments. Cell viability is presented as percentage relative to control.