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Neuroprotective effects of coffee-derived exosome-like nanoparticles against Aβ-induced neurotoxicity

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Abstract. The present study aimed to provide experimental evidence that CDELNs (coffee-derived exosome-like nanoparticles) may be a candidate for the treatment or prevention of amyloid-β (Aβ)-induced Alzheimer's disease (AD). An *in vitro* Alzheimer's model was created with Aβ-induced toxicity in mouse hippocampal neuronal cells (HT-22). $A\beta_{(1\text{-}42)}$-exposed cells were treated with different concentrations of CDELNs (1–50 μg/ml) and the viability of cells was analyzed. The change in the mitochondrial membrane potential ($\Delta\Psi_m$) of cells was also determined. CDELNs treatment increased the viability of $A\beta_{(1\text{-}42\ )}$-toxicity-induced HT-22 cells significantly. The increase in the viability of $A\beta_{(1\text{-}42)}$-toxicity-induced cells was correlated with an improvement in $\Delta\Psi_m$. CDELNs treatment restored the dissipated $\Delta\Psi_m$. These results suggested that CDELNs protect neuronal cells against $A\beta_{(1\text{-}42)}$-induced neurotoxicity by repairing mitochondrial dysfunction. CDELNs might be a useful neuroprotective agent for the treatment or prevention of Aβ-induced AD. Further animal and clinical studies should be carried out to investigate the neuroprotective potential of CDELNs against Aβ-induced AD.

Key words: Alzheimer's disease — Amyloid-β — Coffee-derived exosome-like nanoparticles — HT-22 cell

Introduction

Alzheimer's disease (AD) is the most common type of dementia and is mostly seen in elderly people, predicted to affect over 100 million people globally by the year 2050 (Monteiro et al. 2023). AD is seen in different regions of the central nervous system manifested by loss of neurons, synapses, cognitive functions, and various neuropsychiatric and behavioral symptoms (Gilman 1997; Lleó et al. 2006). Despite the scientific developments, there is no effective neuroprotective treatment for AD (Burns and Iliffe 2009). AD is characterized by neuritic plaques and neurofibrillary tangles as a result of the accumulation of the amyloid-β (Aβ) peptide in the medial temporal lobe and the neocortical structures. $A\beta_{(1\text{-}42)}$ is the main component of amyloid plaques in AD brains. Autosomal dominant APP which is located very close to β- and y-secretase cleavage sites flank the Aβ formation sequence (Masters et al. 1985; Selkoe 2001). Although research continues on whether Aβ plaques are the cause or consequence of AD, the increased toxic effect due to overproduction and accumulation of Aβ peptides is thought to be the primary cause of neurodegeneration and cognitive decline. In light of the data obtained, it is known that the accumulation and density of Aβ in different regions directly affect the course of the disease and the clinical condition of the patient (Masters et al. 1985; Selkoe 2001; Rocchi et al. 2003). Mitochondrial dysfunction is an important contributing factor in the development and progression of Aβ-induced AD (Perez Ortiz and Swerdlow 2019; Wang et al. 2020). Neurocognitive changes and memory problems seen in Aβ-induced AD are mostly associated with mitochondrial dysfunction (altered mitochondrial dynamics, reduced

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mitochondrial membrane potential ($\Delta\Psi_m$), etc.) (Bhatia et al. 2022). Mitochondrial dysfunction results in some of the crucial functions in neuronal cells such as impaired energy production, increased oxidative stress, and neuronal cell death. The accumulation of Aβ on the mitochondrial membrane and the interaction of Aβ with the mitochondrial matrix also results in the degeneration of neuronal synapses (Pagani and Eckert 2011).

Aβ-induced *in vitro* AD model is a unique chemical-induced *in vitro* AD model to examine the *in vitro* neuroprotective effects of the agents against AD (Gouras et al. 2000; Liu et al. 2009; Brimson et al. 2012; Lee et al. 2016; Liu et al. 2017; Lu et al. 2018; Li et al. 2020; Cheng et al. 2021; Zhang et al. 2023). In addition, the HT-22 cell line derived from HT4 cells of the mouse hippocampus is used to establish in vitro models of neurodegenerative disorders and has been widely used for the development of treatments for neurodegenerative diseases including AD (Liu et al. 2017; Wang et al. 2019; Cheng et al. 2021; Prasansuklab et al. 2023).

Exosomes are natural nanoparticles (NPs) of 30–150 nm in size, secreted by almost all cells. They have a particular lipid bilayer membrane and a natural targeting potential due to the proteins found in their membranes. Exosomes contain materials such as miRNA and proteins that carry the characteristics of the cell from which they originate. There are limitations and disadvantages in the use of exosomes derived from human cells as their genetic materials can change the phenotypic and genotypic characteristics of the targeted cell. Therefore, research on plant-derived exosome-like nanoparticles (PDELNs) has been intensified recently. PDELNs are small vesicles released by multivascular bodies mainly to communicate between cells and regulate immunity against pathogen attacks. Functional molecules of PDELNs are preserved in intracellular digestive vacuoles and mediated by unknown mechanisms to affect targeted cell's intracellular functions with EV carrier support (Bryniarski et al. 2015; Greenhill 2017). They are absorbed by intestinal macrophages and can be easily internalized by mammalian cells. PDELNs have many advantages such as low toxicity, efficient cellular uptake, high biocompatibility, stability, large-scale production, etc. They also have anti-inflammatory, immunomodulatory, and regenerative effects. Due to the many advantages of PDELNs, their potential use in medical applications arouses curiosity, and *in vitro* and *in vivo* studies have been carried out in this field (Zhang et al. 2016; Di Gioia et al. 2020; Kameli et al. 2021; Karamanidou and Tsouknidas 2021; Lian et al. 2022).

Despite the neuroprotective effects of coffee against AD being reported, there is no study that investigated the efficacy of coffee-derived exosome-like nanoparticles (CDELNs) for the treatment of AD in the literature so far. The present study investigated the possible neuroprotective effects of CDELNs on HT-22 cells against Aβ-induced AD.

Material and Methods

Isolation of CDELNs from coffee extracts

We used a low pH-based polyethylene glycol (PEG) precipitation method for isolation of CDELNs. The low pH-based PEG precipitation method for isolation of PDELNs has some advantages compared to other isolation methods. By using a low pH-based PEG precipitation method, PDELNs are isolated in high yields without compromising the stability and biochemical composition of PDELNs. Isolation of PDELNs in acidic conditions increases the amount of intact exosomal proteins and RNAs. Moreover, PDELNs are efficiently taken up by target cells when they are isolated by a low pH-based PEG precipitation method (Ban et al. 2015; Kalarikkal et al. 2020; Suresh et al. 2021). Briefly, 50 g coffee extracts (Indonesian Sumatra Mandheling DP Gr. 1) were homogenized in 500 ml distilled water using a grinder under no load condition for 3 min. The homogenized solutions were passed through a nylon mesh filter with a pore diameter of 200 μm to remove excess fiber and then transferred to falcon tubes. The solutions transferred to the tubes were subjected to differential centrifugation (2,000×*g* for 10 min, 6,000×*g* for 20 min, and 10,000×*g* for 40 min). After the differential centrifugation, the supernatants were taken, the pH values were fixed at 4.25 (using HCl or NaOH), and 10% (w/v) PEG-6000 (Sigma Aldrich) was added to each supernatant. Then, the supernatants were incubated overnight at 4°C. Following the incubation, the supernatants were centrifuged at 8,000×*g* for 30 min. The pellets obtained after centrifugation were dissolved in distilled water, and the obtained solution was then dialyzed against ultrapure water at 4°C using a dialysis membrane with a pore size of 10 kDa (MW cutoff) overnight. Then, the solution was passed through a 0.45 μm cellulose acetate filter. The quantity of exosomal protein was determined by a BCA (bicinchoninic acid) Protein Assay. Briefly, standards were prepared in eppendorf tubes according to the BCA Assay Kit procedure. 20 μl of prepared standards and samples were added to each well. Then 200 μl (Solution A + Solution B) mixture was added to each well and pipetted. The samples were incubated for 30 min at 37°C and then for 10 min at room temperature. The absorbance values of the standards and samples were measured at a wavelength of 570 nm using a microplate reader. A standard curve was created according to the absorbance values obtained from the standards, and the amount of CDELNs was determined for each sample.

Measurement of particle size by nanoparticle tracking analysis (NTA)

The particle size of NPs was measured by NTA, which is based on measuring the characteristic motion of NPs in solu-

tion based on Brownian motion. It detects information about particles in solution by capturing the after-light illumination scattered by a laser and uses the Stokes-Einstein equation and cell volume information to determine the concentrations and sizes of vesicles. CDELNs samples produced by the ultracentrifugation method were diluted using ddH_2O at a ratio of 1:100, 1:1000, and 1:10000 in 1.5 ml eppendorf tubes with a 0.1 μm-filter. The size of CDELNs in the diluted samples was analyzed with the NTA (Malvern Analytical, Nanosight NS300).

The determination of the distribution of particle size by scanning electron microscopy (SEM)

The distribution of diameters of CDELNs was analyzed by SEM (QUANTA 400F Field Emission). NPs were first washed with PBS solution and fixed with 2.5% glutaraldehyde solution. The samples were then washed with PBS and the glutaraldehyde was removed. Dehydration of the samples was achieved with increasing ethanol concentrations from 30% to 100%. Then, the samples were dried at room temperature and photographed with high resolution at different positions with appropriate magnification. The distribution of the diameters of CDELNs in obtained images was measured manually with the help of the ImageJ program (randomly selected on the SEM grid). The nanoparticle size and surface charge were analyzed by dynamic light scattering. The coffee-derived exosome samples were analyzed 3 times at 20°C.

Cytotoxicity assay

HT-22 cells acquired from the Health Sciences University Gulhane Stem Cell Research Laboratory were cultured in 75-cm^2 culture flasks in DMEM (Dulbecco's Modified Eagle's medium) with 10% fetal bovine serum (Invitrogen), 2 mM glutamine, 100 units/ml penicillin and 100 mg/ml streptomycin in a medium using 95% humidity and 5% CO2 at 37°C (pH: 7.2–7.4). Cell viability and number were determined with 0.4% trypan blue dye using an automated cell counter (RWD C100). Non-viable cells absorb the trypan blue dye and appear blue-stained under the light microscope. Thus, non-living cells could be counted by distinguishing them from living cells. In all experiments, before starting the experiments, the rate of viable cells was determined as (Living cell rate (%) = the number of cells that did not receive dye/total number of cells ×100) and the experiments were started at the values where the cell viability was 95%. HT-22 cells were incubated with CDELNs at concentrations of 1–50 μg/ml for 24 hours. After 24 hours of incubation, the cell viability of HT-22 cells was analyzed by WST-1 assay. Before performing the WST-1 viability test, the WST-1 stock solution (ABP Biosciences) was prepared and 100 μl of the stock solution was applied to each well. The cells were then incubated for 2 hours. After 2 hours of incubation, absorbance values were measured with a microplate reader at 450 nm wavelength.

Establishment of an in vitro AD model with $A\beta_{(1\text{-}42)}$ toxicity

1 mM stock solution was prepared by dissolving lyophilized $A\beta_{(1\text{-}42)}$ peptides in distilled water and DMSO. The prepared stock solution was diluted with serial dilutions to prepare $A\beta_{(1\text{-}42)}$ solutions in different concentrations (2.5, 5, 10, 25 and 50 μM). HT-22 cells were seeded in 96-well plates at 10^4 cells/ml in each well. Cells were incubated with $A\beta_{(1\text{-}42)}$ peptides at different concentrations (5, 10, 25 and 50 μM) for 24 hours. After incubation, a WST-1 cell viability test was performed to determine the cell viability, and the effective $A\beta_{(1\text{-}42)}$ dose value was determined. In the *in vitro* AD model, which we created with $A\beta_{(1\text{-}42)}$ toxicity at the effective dose in HT-22 cells, the cells were incubated with CDELNs at concentrations of 5–50 μg/ml for 24 hours and cell viability was determined after 24 hours of incubation.

Determination of relative mitochondrial membrane potential ($\Delta\Psi_m$)

Relative $\Delta\Psi_m$ was measured by fluorescence microscopy using JC-1 dye. JC-1 is a cationic dye that selectively accumulates into the mitochondria under the effect of an electrochemical gradient and gives a red color when the mitochondrial membrane is polarized (Esmekaya et al. 2017). First, 1 μl of Component B was mixed with 500 μl of PBS. Then, 100X JC-1 stock solution (ABP Biosciences) was diluted 1:100 with PBS to obtain 1X JC-1 solution. 100 μl of Component B mixture was added into wells and cells were incubated at 37°C for 10 minutes in a 5% CO_2 incubator. Then, 1X JC-1 solution was added into wells and cells were incubated at 37°C for 15 minutes in a 5% CO_2 incubator. After incubation, 1X JC-1 solution was withdrawn and wells were washed with 100 μl of warm PBS. The samples were taken on a slide and viewed with a fluorescent microscope (Olympus).

Data analysis

All experiments were repeated five times and the data were presented as mean ± standard deviation (SD). The normality distribution of the data was determined by the Kolmogorov-Smirnov test. The data were analyzed by one-way ANOVA, followed by the Tukey HSD multiple comparison test. $p < 0.05$ was considered to be statistically significant.

Results

Characterization of CDELNs

CDELNs were isolated from homogenized coffee extracts using the low pH-based PEG method. The size distribution of CDELNs were determined using NTA. The shape and size distribution of CDELNs was also confirmed and visualized by SEM. SEM images of coffee CDELNs showed that CDELNs had a homogeneous population and were mostly spherical in shape (Fig. 1A). The median +/− interquartile range diameter of CDELNs population was 48.89 ± 19.75 nm (Fig. 1B). Zeta potential analysis indicated that CDELNs had a negative zeta potential value of −23.8 ± 1.42 mV (Fig. 2). Zeta potential analysis, which evaluates the surface potential of colloidal particles, is widely used to estimate the colloidal stability of isolated plant-derived exosome-like nanoparticles. These results demonstrated that isolated exosome from coffee has high purity and high stability.

Cytotoxicity results

In vitro Alzheimer's model was created with Aβ-induced toxicity in HT-22 cells. HT-22 cells were incubated with $A\beta_{(1\text{-}42)}$ peptides at different concentrations (5–50 mM) for 24 hours and cytotoxicity of cells with Aβ-induced toxicity was measured with WST-1. $A\beta_{(1\text{-}42)}$ peptides showed a dose-dependent cytotoxic effect on cells (Fig. 3). We chose a concentration of 25 mM $A\beta_{(1\text{-}42)}$ to create an *in vitro* Alzheimer's model. HT-22 cells were treated with 25 mM $A\beta_{(1\text{-}42)}$ and 25 mM $A\beta_{(1\text{-}42)}$ + different concentrations of CDELNs for 24 hours and then, the effects of CDELNs on $A\beta_{(1\text{-}42)}$-induced toxicity were analyzed. The viability of cells treated with 25 mM $A\beta_{(1\text{-}42)}$ + different concentrations of CDELNs was significantly higher ($p < 0.05$) than the viability of cells treated only with 25 mM $A\beta_{(1\text{-}42)}$. The increase in viability was highest for the lowest dose of CDELNs. The viability of cells treated with 25 mM $A\beta_{(1\text{-}42)}$ + 5 µg/ml CDELNs was significantly higher than the viability of 25 mM $A\beta_{(1\text{-}42)}$ + 10, 25 and 50 µg/ml CDELNs-treated cells (Fig. 4).

$\Delta\Psi_m$ results

Control and CDELNs-treated cells were stained with JC-1 to evaluate $\Delta\Psi_m$ by fluorescence microscopy. The representative fluorescence microscopy images are presented (Fig. 5A). The red fluorescence (JC-1 aggregates) represents intact mitochondria, the green fluorescence (JC-1 monomers) represents a decrease in the $\Delta\Psi_m$ and the yellow fluorescence (merged) represents damaged mitochondria. As seen in Figure 5A, treatment of HT-22 cells with different doses of CDELNs increased red fluorescence signals compared to control cells. CDELNs treatment caused an increase in $\Delta\Psi_m$. So, the mitochondria of CDELNs-treated cells became more polarized compared to the mitochondria of untreated control cells. These results demonstrated that CDELNs treatment restored the dissipated $\Delta\Psi_m$ and improved mitochondria in $A\beta_{(1\text{-}42)}$-neurotoxicity-induced HT-22 cells.

Discussion

The disproportion between the production, accumulation, and clearance of Aβ peptides is one of the most important

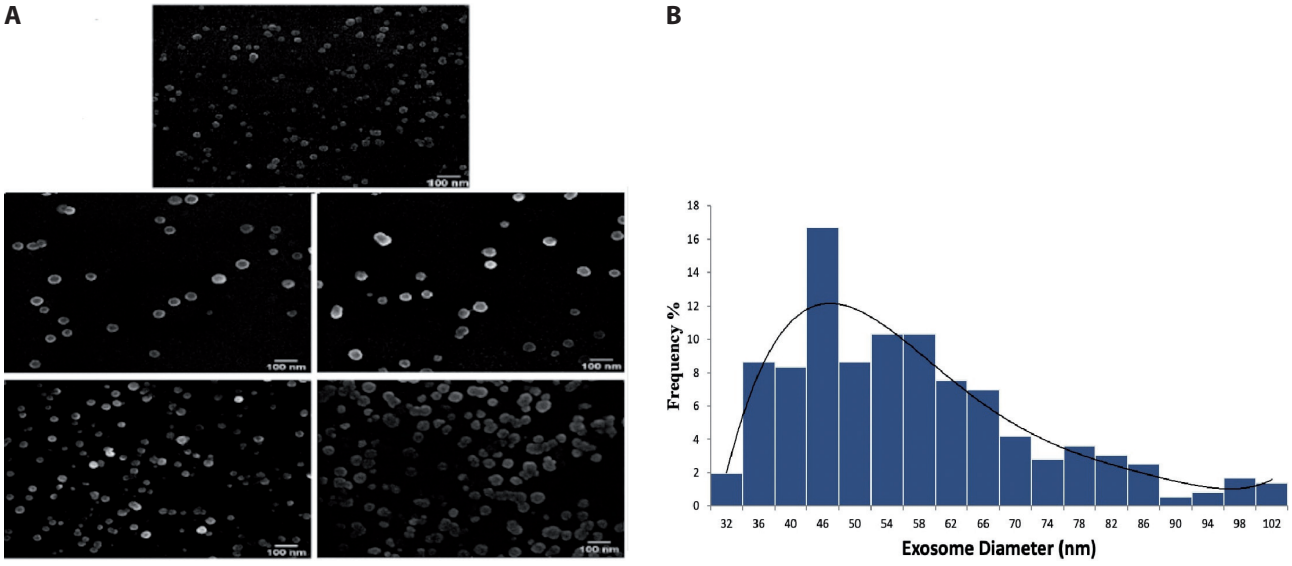

Figure 1. A. Exosome-like nanoparticles isolated from coffee extracts were imaged by scanning electron microscopy (SEM; scale bar = 100 nm). **B.** The size distribution derived from images randomly selected on the SEM grid.

initial factors in the neuropathogenesis of AD (Reddy and Oliver 2019; Hampel et al. 2021; Knopman et al. 2021; Ono and Watanabe-Nakayama 2021; Sehar et al. 2022; Shi et al. 2022). The neuronal and synaptic losses and neurotransmitter deficiencies that are observed due to the accumulation of Aβ peptides are thought to lead to dementia by impairing cognitive functions (Shen and Kelleher 2007; Xia et al. 2015; Selkoe and Hardy 2016). In the present study, we investigated the possible neuroprotective effects of CDELNs against Aβ-induced AD. HT-22 cells were exposed to $A\beta_{(1\text{-}42)}$ and treated with different concentrations of CDELNs (1–50 μg/ml). The effects of CDELNs on the viability of $A\beta_{(1\text{-}42)}$-exposed cells were analyzed by WST-1 assay. The neuronal cell death induced by $A\beta_{(1\text{-}42)}$ was decreased due to CDELNs treatment. The viability of cells treated with 25 mM $A\beta_{(1\text{-}42)}$ + different concentrations of CDELNs was significantly higher ($p < 0.05$) than the viability of cells treated only with 25 mM $A\beta_{(1\text{-}42)}$. The treatment with the lowest dose of CDELNs (5 μg/ml) had the highest effect on inhibition of $A\beta_{(1\text{-}42)}$-induced toxicity. These results demonstrat that treatment of CDELNs improves the viability of neuronal cells and protects them against $A\beta_{(1\text{-}42)}$-induced neurotoxicity.

The results of this study showed that CDELNs treatment improved mitochondria by restoring the dissipated $\Delta\Psi_m$ in $A\beta_{(1\text{-}42)}$-exposed cells. Mitochondria of CDELNs-treated $A\beta_{(1\text{-}42)}$-exposed HT-22 cells showed a lower green/red fluorescence ratio than mitochondria of untreated control cells. The lower green/red fluorescence indicates higher $\Delta\Psi_m$ and polarized mitochondria. Mitochondrial dysfunction and bioenergetic deficits caused by Aβ aggregation may play a central role in the pathogenesis of AD (Chen et al. 2006; Spuch et al. 2012; Chaturvedi et al. 2013; Swerdlow 2018; Flannery and Trushina 2019; Li et al. 2020; Sharma et al. 2021). However, the underlying mechanism of mitochondrial dysfunction in AD has not been clarified yet (Castellani et al. 2002; Sheng et al. 2012). Some authors suggested that mitochondrial dysfunction related to reductions in the activities of mitochondrial electron transport chain (ETC) enzymes (especially complex I) may play an important role in the pathogenesis of AD (Fosslien 2001; Manczak et al. 2004; Sharma et al. 2009; Breuer et al. 2012; Jhonson et al. 2020; Kilbride et al. 2020, 2021). Complex I has been shown to be impaired and mitochondrial genes encoding complex I has been reported to be downregulated in AD (Fosslien 2001; Manczak et al. 2004; Breuer et al. 2012). Because the entry point for most electrons into the respiratory chain is complex I, it plays a key role in energy metabolism (Sharma et al. 2009).

$\Delta\Psi_m$ is more susceptible to reductions in complex I activity than reductions in the other ETC complex activities (Kilbride et al. 2021). The inhibition of complex I activity reduces the proton gradient and leads to loss of $\Delta\Psi_m$ which is crucial for maintaining the viability of a neuron (Martínez et al. 2016). Dissipation of $\Delta\Psi_m$ results in the opening of the mitochondrial transition pore and the release of pro-apoptotic molecules into the cytosol which is an early event of apoptotic cell death (Nicholls and Budd 2000; Qi et al. 2003; Onyango et al. 2016). The increase in viability of CDELNs-treated $A\beta_{(1\text{-}42)}$-exposed neuronal cells may be attributable to the ability of CDELNs to improve dissipated $\Delta\Psi_m$ *via* restoring complex I activity by reducing the binding of Aß to mitochondria in the present study.

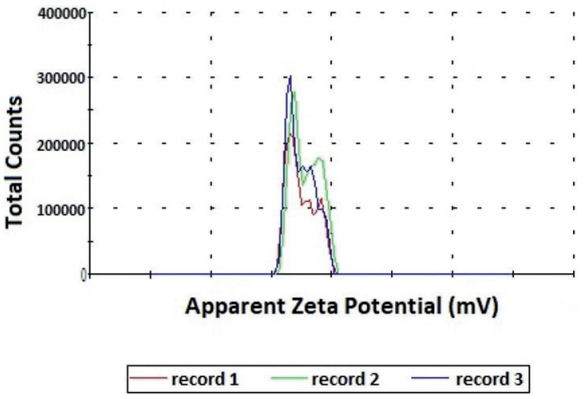

Figure 2. Zeta potential distribution of CDELNs (coffee-derived exosome-like nanoparticles).

The neuroprotective effects of coffee against AD have been reported in the literature so far. Experimental and clinical studies have shown that coffee consumption may reduce the risk of the development and the progression of AD (Barranco et al. 2007; Dostal et al. 2010; Kolahdouzan and Hamadeh 2017; Bae 2020; Colombo and Papetti 2020). Lindsay et al's study (2022) showed that coffee consumption was associated with a 31% lower risk of AD in the Canadian population. The study of Maia and Mendonca (2002) reported reduced risk of AD by 60% due to caffeine consumption. $A\beta_{1\text{-}40}$

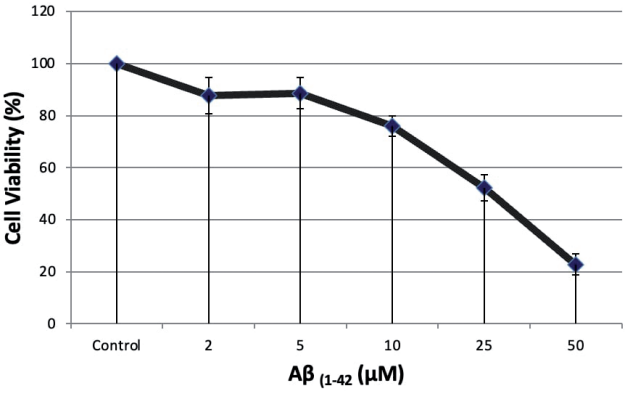

Figure 3. Effect of 2.5, 5, 10, 25 and 50 μM amyloid-β $A\beta_{(1\text{-}42)}$ on cell viability of HT22 cells.

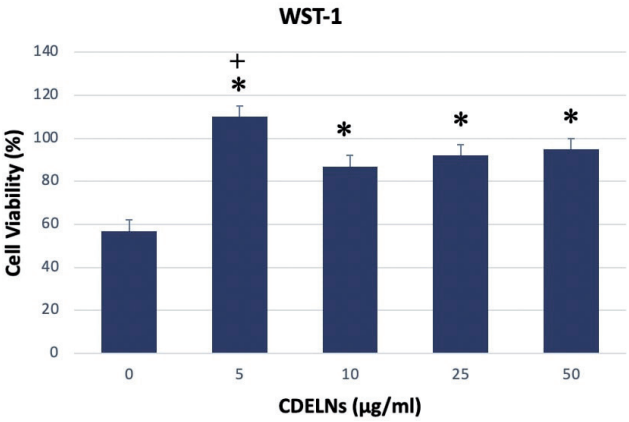

Figure 4. Effect of CDELNs (coffee-derived exosome-like nanoparticles) on the viability of $A\beta_{(1\text{-}42)}$-induced toxicity (25 mM) in HT22 cells. * $p < 0.05$ *vs.* control group; $^{+}$ $p < 0.05$ *vs.* other treatment groups.

and $A\beta_{1\text{-}42}$ levels were shown to be reduced in the cortex and hippocampus regions due to coffee administration (Arendash et al. 2009). A single dose of encapsulated coffee concentrate increased peripheral serum brain-derived neurotrophic factor (BDNF) by 91% at 60 minutes and by 66% at 120 minutes compared to baseline levels (Nieber 2017). The study of Sukumaran and Shree (2021) also reported that coffee extracts can alleviate the symptoms of AD.

Despite the neuroprotective role of coffee extracts against AD have been studied, there is no study that investigated the efficacy of CDELNs for the treatment of AD in the literature so far. We first time investigated the neuroprotective effects of CDELNs against Aβ-induced AD. Our results showed that CDELNs might be a useful neuroprotective agent for the treatment of Aβ-induced AD by improving $\Delta\Psi_m$ and decreasing neuronal cell death. Penetration of coffee extracts through the blood-brain barrier (BBB) is low due to their large sizes. This limits the use of coffee extracts in AD treatment. On the other hand, CDELNs have small sizes and greater ability to penetrate BBB. They can cross the BBB by transcytosis likewise by immune cells and infectious agents (Liu et al. 2020). Due to their small sizes and great ability to penetrate through BBB, CDELNs may be much more effective than large-sized coffee extracts in the treatment of AD.

In the present study, the HT-22 cell line was chosen as a cellular model to investigate the potential neuroprotective effect of CDELNs against Aβ-induced AD. The results of the study showed that CDELNs may be used as a neuroprotective agent for the treatment or prevention of Aβ-induced AD. CDELNs markedly improved the viability of $A\beta_{(1\text{-}42)}$-exposed neuronal cells. The protection provided by CDELNs against $A\beta_{(1\text{-}42)}$-induced neurotoxicity was associated with the reversal of mitochondrial depolarization. CDELNs significantly restored the dissipated $\Delta\Psi_m$. CDELNs may bring an innovative perspective to the solution of AD apart from conventional methods. Further animal and clinical studies

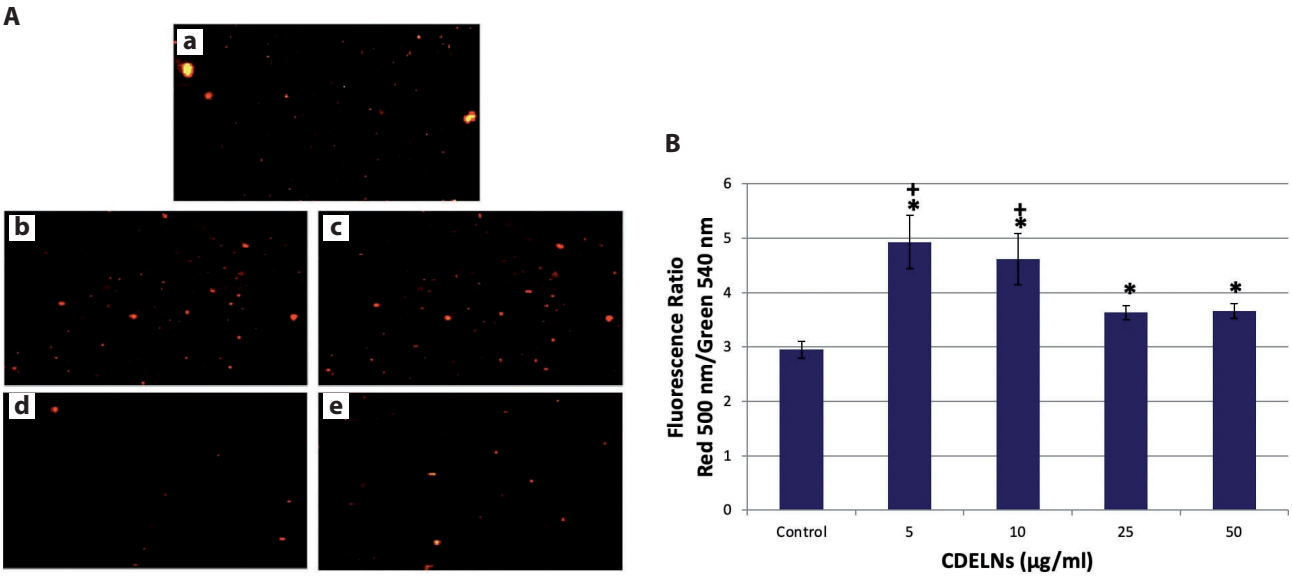

Figure 5. A. Fluorescence photographs of merged green and red channels of JC-1 staining in control (**a**) and 5 µg/ml (**b**), 10 µg/ml (**c**), 25 µg/ml (**d**) and 50 µg/ml (**e**) CDELNs (coffee-derived exosome-like nanoparticles)-treated groups. **B.** Red/green fluorescence ratio of $\Delta\Psi_m$ measurement. Data for each group was expressed as mean ± SD of five independent experiments. The experiments were performed in each group and samples were measured and analyzed for each experiment. * $p < 0.05$ *vs.* control group; $^{+}$ $p < 0.05$ *vs.* other CDELNs-treated groups. (See online version for color figure.)

should be carried out to investigate the treatment potential of CDELNs against Aβ-induced AD.

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Conflict of interest. The authors declare no conflict of interest.

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