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Human endometrial stem cell-derived small extracellular vesicles enhance neurite outgrowth and peripheral nerve regeneration through activating the PI3K/AKT signaling pathway

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Abstract

Nowadays, extracellular vesicles (EVs) such as exosomes participate in cell-cell communication and gain attention as a new approach for cell-free therapies. Recently, various studies have demonstrated the therapeutic ability of exosomes, while the biological effect of human endometrial stem cell (hEnSC)-derived small EVs such as exosomes is still unclear. Herein, we obtained small EVs from hEnSC and indicated that these small EVs activate the vital cell signaling pathway and progress neurite outgrowth in PC-12 cell lines. For this purpose, hEnSC-derived small EVs were extracted by ultracentrifuge and characterized by DLS, SEM, TEM, and western blot. Also, dil-staining of hEnSC-derived small EVs was done to determine the penetration of hEnSC-derived small EVs into PC12 cells. The MTT assay, scratch assay, and western blot assay were applied to PC12 cells that were exposed to different concentrations of small EVs (0, 50, 100, and 150 µg/ml). Our results demonstrated that small EVs significantly increased neurite outgrowth, proliferation, and migration in PC12 cells in a dose-dependent manner. Moreover, the analysis of western blots showed increased expression of the PI3k/AKT signaling pathway in PC12 cells exposed to hEnSC-derived small EVs in a dose-dependent manner. Also, the results of this study indicated that hEnSC-derived small EVs can enhance cell proliferation and migration and promote neural outgrowth by activating the PI3k/AKT signaling pathway. Accordingly, hEnSC-derived small EVs became an effective strategy for cell-free therapies. Altogether, these positive effects make hEnSC-derived small EVs a new efficient approach in regenerative medicine, especially for the cure of neural injury.

Keywords Exosomes, Nerve tissue regeneration, Human endometrial stem cells, PC12 cells, Small EVs, PI3k/AKT signaling pathway

Introduction

The nervous tissue, as a vital tissue for living, is capable of receiving and sending electrochemical signals that provide body information. Due to the limited capacity of nerve tissue and self-regeneration following traumatic nerve injuries or neurodegenerative diseases, alternative interference is required [1]. In nerve injury situations, clinical therapies still require improvement in tissue regeneration cases [2]. In this regard, the development

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of appropriate strategies for neurodegenerative diseases to enhance nerve regeneration post-injury, especially the strategy that accelerates axonal nerve regeneration, is precious for researchers [3]. Up until now, some strategies have been used to manage the symptoms, but none of these strategies are definitive treatments for neurodegenerative disorders [4, 5].

In several studies, researchers have demonstrated that mesenchymal stromal cells (MSCs) promote the regeneration of nerve injury and improvement of function [6], due to releasing paracrine factors with anti-inflammatory effects and modulating immune reactions [7–9]. MSCs were widely used in a variety of clinical trials, while concern about some side effects of systemic application, like poor cell survival, genetic alterations, and tumor growth, remains [10, 11]. Based on previous studies, researchers have illustrated that the main therapeutic roles of MSCs are divided into two categories: cell-mediated effects related to MSC differentiation and paracrine-mediated effects [12, 13] that the beneficial roles of them in tissue regeneration are mediated mostly by paracrine roles [14]. Paracrine secretion of MSCs includes growth factors, miRNAs, and extracellular vesicles (EVs) [13].

Small EVs such as exosomes are phospholipid bilayer vesicles with an approximately diameter of 30–150 nm that have bioactive substances like lipid, protein, and nucleic acids (DNA, microRNAs, circRNA, lncRNA, and other noncoding RNAs) that effect and mediate the signaling pathway of cells [15–17]. Exosomes can connect with the cell membrane of surrounding cells and selectively transfer bioactive substances into target cells [18]. Exosomal RNA as a primary messenger that reflects cell conditions is a combination of coding and non-coding [19]. Coding RNAs (mRNA) are functionally translated in recipient cells, and non-coding RNAs (microRNAs (miRNA)) mediate cell-cell communication by regulating gene expression [20, 21]. Therefore, RNA molecules within exosomes can modulate the microenvironment [22]. Previous studies have reported that the exosomes provided by MSC can enhance nerve regeneration and functional recovery post-nerve injury [23, 24]. Also, it was indicated that these exosomes have a wide variety of MSC effects [25, 26].

Indeed, exosomes extracted from MSCs have been demonstrated to have great potential for regenerating neurodegenerative diseases due to the neuroprotective and neuroregenerative properties of MSC-derived exosomes [27, 28]. Moreover, the long half-life of exosomes in body circulation, less immunogenicity, off-the-shelf employment, easy storage conditions, and their potency to cross the brain-blood barrier (BBB) make MSC-derived exosomes a precious substrate for therapeutic strategies [29] to replace MSCs as a safe cell-free clinical therapy approach compared to MSC-based therapies (Fig. 1) .

On the other hand, the source of extracted tissue and the microenvironmental surroundings of the donor cells affect the contents of the exosome [30]; therefore, a proper cell source should be applied for a particular clinical target. This study was designed to indicate the effects of human endometrial stem cell (hEnSC)-derived small EVs on neurite outgrowth, survival, proliferation and migration of cells, and cell signaling activation, and also find optimal concentrations of hEnSC-derived small EVs in neural therapy. Recalling that hEnSCs are a new source of adult MSCs that have been isolated from endometrium tissue, it should be noted that they have an immunomodulatory effect, possessing the ability for self-renewal and differentiation into osteoblasts, chondrocytes, adipocytes, and nerve cells [31]. Hence, due to these properties, hEnSC-derived small EVs are a novel approach for cell-free therapies as well as a proper choice for regenerative medicine.

Rat pheochromocytoma cell lines (PC12) are used as a classical model in in vitro situations for neuroscience investigation [32, 33]. In the current study, we aimed to extract small EVs from the supernatant of hEnSCs and indicate the dose effect of these small EVs on PC12 cells. Also, we wanted to investigate the role of hEnSC-derived small EVs on the survival, proliferation, and migration of cells by activating the PI3K/AKT signaling pathway.

Materials and methods

Isolation and characterization of hEnSCs

HEnSCs were isolated by our previous study method [34]. Briefly, human endometrium species tissue was obtained after hysterectomy at Imam Khomeini Hospital in Tehran, Iran. The specimens of endometrium tissue were maintained in a pre-warmed Hanks' balanced salt solution (HBSS; Invitrogen, Carlsbad, CA) with 1% (v/v) penicillin/streptomycin (P/S; Sigma-Aldrich) and amphotericin B (1 µg/mL), then the samples were placed in the cell culture dishes and washed with phosphate buffered saline (PBS) containing 1% penicillin/streptomycin. The specimens of tissue were dissolved by collagenase type I (2 mg/mL, Sigma-Aldrich) in an incubator at 37 °C and 5% CO_2 for at least 30 min. After that, Dulbecco's modified Eagle's medium F12 (DMEM/F12; Sigma) containing 5% fetal bovine serum (FBS) (Biosera) was added to the obtained suspension, and then the yield suspension was passed through cell strainers (70 µm). Finally, the obtained cell pellet was re-suspended in DMEM/F12 media with 20% serum and 1% P/S and incubated at 37 °C in an incubator. The cultural media were changed twice a week.

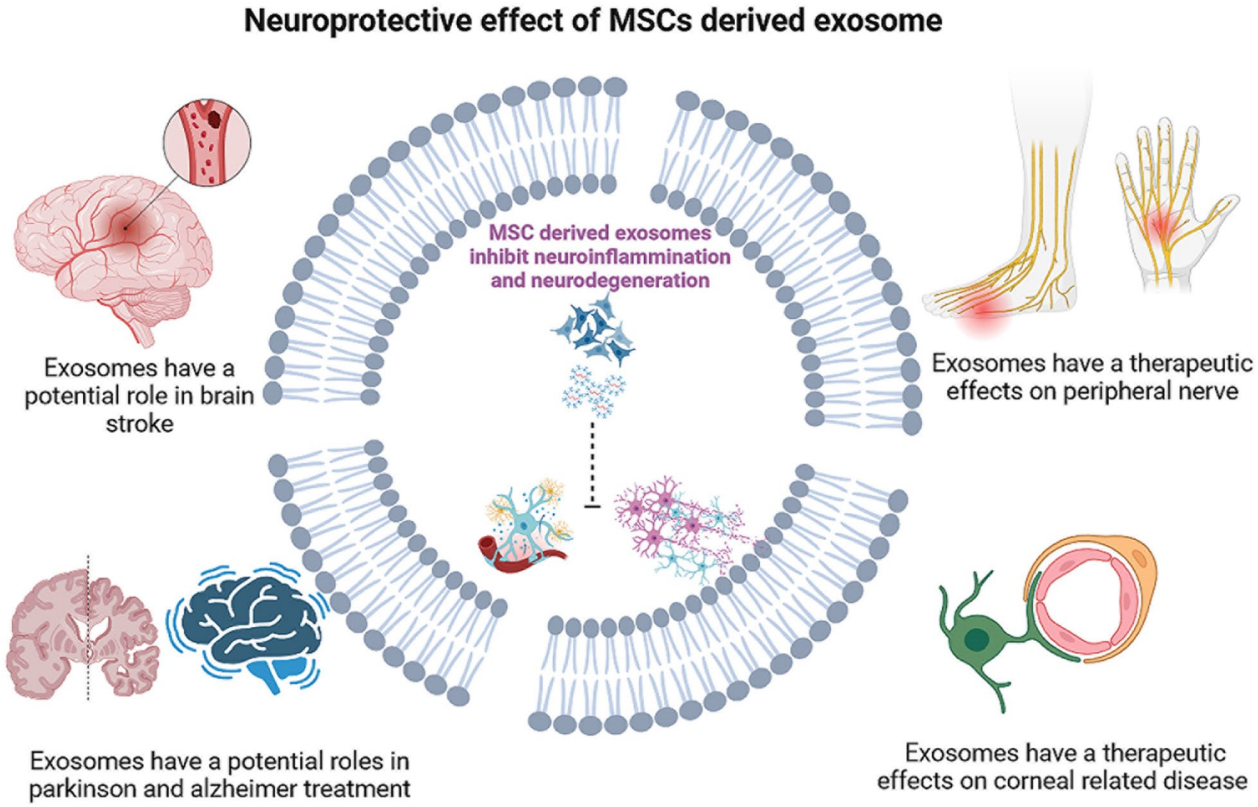

Fig. 1 Applications of MSc-derived exosomes for neuronal regenerative medicine, which have wide neuroprotective effects

Flow cytometry analysis was performed to verify the isolated hEnSCs with BD FACS Calibur (BD Biosciences, USA) and cells were expanded up to passage 3. The investigated surface markers were as follows: mesenchymal stem cell markers (CD90, CD105, and CD146), hematopoietic stem cell markers (CD34), and endothelial stem cell markers (CD31) [35]. The hEnSCs were washed several times with pre-warmed HBSS and 2% bovine serum albumin (BSA; Sigma, A7030), then incubated with a specific monoclonal antibody mentioned at specific recommended concentrations for 60 min. Flow cytometry result outcomes were analyzed by FlowJo (version 7.00) software.

Isolation of hEnSC-derived small EVs and concentration determination

HEnSCs were isolated from endometrium tissues as described by the previous method and cultured in complete DMEM/F12 media (supplemented with 10% FBS and 1% penicillin/streptomycin) and grown in 5% CO_2 at 37 °C [36]. When the confluence of cells was higher than 75% in a T175 culture flask, hEnSCs were washed with pre-warmed PBS and DMEM/F12 media supplemented with 5% exosome-free FBS were added. After 48 h, the hEnSCs culture supernatant was maintained and used for purification by differential centrifugation. To remove cells and debris, three accelerative centrifugations (Beckman Coulter X-14R centrifuge) were applied (500×g for 10 min at 4 °C, 2000×g for 20 min at 4 °C, and 10000×g for 45 min at 4 °C, respectively, and at each step the pellet was thrown away). The clear culture supernatant was placed in an ultracentrifuge device and centrifuged at 120,000×g for 60 min at 4 °C (Beckman Coulter XL90 ultracentrifuge). Subsequently, the obtained pellet was resuspended in filtered PBS and centrifuged again at 120,000×g for 60 min at 4 °C (Beckman Coulter XL90 ultracentrifuge) to omit protein contamination. At the end, the obtained hEnSC-derived small EVs were resuspended in cold PBS and maintained at −80 °C. Also in this study, the protein concentration of hEnSC-derived small EVs was identified by Bradford Protein Assay Kit (DNAbiotech, Iran).

Characterization of hEnSC-derived small EVs

Size distribution

Dynamic light scattering (DLS) analysis on DelsaMax Pro (Beckman, CA, USA) was done to determine the size distribution of isolated hEnSC-derived small EVs.

The extracted hEnSC-derived small EVs were diluted to 500 µg/ml using PBS and then transferred into a cuvette. After the laser and temperature equilibrium of the device (SZ-100 Dinamic Light Scatter, Horiba Jobin Jyovin) were stabilized, EVs samples were analyzed using the following parameters: Laser Wavelength (nm): 663.87, Refractive index: 1.37, Set temperature(C): 25, Peak Radius Low Cutoff(nm): 0.5, Peak Radius High Cutoff(nm): 5000, and Auto-attenuation Time Limit: 60s. The size of hEnSC-derived small EVs was measured through the % Intensity.

Electron microscopy

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were applied to investigate extracted exosome morphology and particle sizes. For SEM, 10-µl of extracted small EVs dilution was fixed by 4% glutaraldehyde and then dehydrated by an accelerating percent of ethanol. After dehydration and drying, a thin layer of gold was used to cover the exosome samples. Finally, the extracted small EVs were observed by SEM (QUANTA SEM system; FEI Company, Hillsboro, OR, USA).

To visualize extracts of small EVs, TEM was utilized. For this purpose, 20 µL of freshly extracted diluted small EVs was mixed with 4% glutaraldehyde; after that, 10 µL of it was loaded onto a carbon-coated grid, and then the sample was stained with 2% phosphotungstic. After staining and washing with PBS, the small EVs were observed with TEM (Zeiss, EM10C). In the current study, the approximate size of the extracted small EVs was estimated by Image J software.

Tracking uptake of hEnSC-derived small EVs

To verify the entry of the small EVs into cells, hEnSC-derived small EVs were labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Dil dye) and incubated for 12 h in an incubator (37 ℃ and 5% CO_2) with PC12 cells. To label hEnSC-derived small EVs, 1 µL of diluted Dil stock solution was dissolved in 1 mL of extracted hEnSC-derived small EVs and incubated for 45 min in a dark place at room temperature. Ultracentrifugation (100000 ×g for 60 min at 4 °C) was utilized to omit excess unincorporated Dil dye. Finally, four concentrations of Dil-labeled small EVs (0, 50, 100, and 150 µg per ml) were incubated with PC12 cells at 80% concentration in a 24-well plate for 12 h. In this study, PC12 cells were incubated with diluted Dil at a concentration of 0 µg/ml. Subsequently, the nuclei of cells were stained with DAPI, and then the cells were washed with PBS to eliminate extra dye. Internalization of Dil-labeled hEnSC-derived small EVs by PC12 cells was visualized by a fluorescence microscope (Olympus, BX51, Japan).

PC12 cell culture and hEnSC-derived small EVs

PC12 cell lines were cultured in complete RPMI media (supplemented with 10% FBS and 1% P/S). For small EVs treatment, PC12 cells were cultured in RPMI media with 5% FBS and 1% of P/S at a low density (2×10^3 cells/cm^2) on a tissue-culture plate and treated with four different concentrations of hEnSC-derived small EVs (0 as a control, 50, 100, and 150 µg per ml) that were added to the culture media for two consecutive days. In the current study, control cells were also cultured only in complete RPMI media under the same conditions.

Neurite outgrowth assay

After 24 h and 48 h post-treatment of PC12 cells with four different concentrations of hEnSC-derived small EVs (0, 50, 100, and 150 µg per ml), PC12 cells were fixed by 4% glutaraldehyde. In this experiment, each concentration was divided into one separate group, and each group was investigated in triplicate. The percentage of neurite outgrowth in each group was quantified, and the average neurite length of each neuron was estimated by Image J software. In this assay, a total of about 50 neurons were considered from each group.

Cell proliferation assay

To determine the effect of different doses of hEnSC-derived small EVs on PC12 cell proliferation, a MTT assay was performed. As a brief, 1×10^4 cells per well were seeded in a 48-well culture plate with complete RPMI media. After 24 h, PC12 cells were treated in triplicate with 0, 50, 100, and 150 µg/mL of hEnSC-derived small EVs to indicate the viability and proliferation of PC12 cells. MTT assays were applied after 1-, 3-, and 5-days post-exosome treatment. To do this, media was thrown out, and then PC12 cells were thoroughly washed with PBS and exposed to 5 mg/mL of 3-(4,5-dimethylthiazolyl2)−2,5-diphenyltetrazolium bromide (MTT, Sigma, USA). After 4 h of incubation in an incubator, DMSO solution was added to dissolve purple MTT formazan crystals and incubated for 15 min. The Eliza reader at 570 nm was used to read the absorbance of the plate.

Cell migration assay

The cell migration assay was investigated by scratch testing. As a brief, 1×10^5 cells per well density were cultured in complete RPMI media (supplemented with 10% of FBS and 1% of P/S) in a 12-well plate. After 48 h, a deep and straight scratch was created in each well of the culture plate by using a sterile pipette tip. Subsequently, the PC12 cells were washed several times with PBS and treated in triplicate with 0, 50, 100, and 150 µg/mL

hEnSC-derived small EVs for two consecutive days. In this step, PC12 cells were cultured in RPMI media without FBS for inhibition of cell proliferation. The surrounding area of PC12 cell migration was imaged post-24 and 48 h by an inverted microscope (Olympus IX 71, 100x magnification, Olympus, Japan). Also, the nucleus of cells was stained by DAPI staining, and PC12 cell migration was determined by a fluorescent microscope.

Western blot analysis

Western blot analyses were done to characterize hEnSCs-derived small EVs and these small EVs' effect on activation of the PI3k/AKT signaling pathway. Lysis of cells and hEnSCs-derived small EVs were done in RIPA buffer. Equal amounts of protein (25 µg) were taken by using a BCA kit (DNA Biotech, Iran) and denatured at 100 °C for 15 min. Electrophoresis on a 12% SDS-polyacrylamide gel was done to separate proteins. After that, the proteins were transferred onto a PVDF membrane, and 5% blocking buffer containing BSA was used (Sigma, Germany). Then, blots were incubated with diluted (1:1000) primary antibodies against CD9 (sc-13118, Santa Cruz Biotechnologies, USA), CD63 (sc-5275, Santa Cruz Biotechnologies, USA), CD81 (sc-166029, Santa Cruz Biotechnologies, USA), PI3 (sc-67306, Santa Cruz Biotechnologies, USA), AKT (E-AB-30471, Elabscience®, USA), and β-Actin (sc-47778, Santa Cruz Biotechnologies, USA) at 4 °C overnight, followed by several washes in 1X Tris-buffered saline with 0.1% Tween-20 (TBST) buffer, and then incubated with horseradish peroxidase (HRP)-conjugated diluted (1:1000) secondary antibodies (sc-516102, Santa Cruz Biotechnologies, USA) (sc-2357,Santa Cruz Biotechnologies, USA). The unbound antibodies were omitted by several washings with 1X TBST buffer (3X-10 min). Finally, the expression of proteins was illustrated by a chemiluminescence gel imaging analysis device (Tanon, China).

Statistical analysis

A one-way analysis of variance (ANOVA) was applied with a significance threshold of $P < 0.05$ to assess the statistical differences between the groups under an unequal variance assumption. For all statistical analyses, GraphPad Prism Version 9.0.0 (121) was applied.

Results

Characterization of isolated hEnSCs

The spindle-shaped hEnSCs were positive for MSC markers CD90 (98.5%), CD105 (98%), and CD146 (89.2%). hEnSCs were also negative for hematopoietic CD34 (1.2%) and endothelial CD31 (0.01%) markers, as shown in Fig. 2.

Characterization of hEnSC-derived small EVs

The hEnSC-derived small EVs were assessed by Bradford analysis, DLS, western blot, SEM, and TEM. Obtained images from SEM and TEM indicated that the isolated small EVs were round and 60–120 nm in diameter (Fig. 3a and b). The Bradford assay illustrated that the concentration yield of extracted hEnSC-derived small EVs was nearly 1000 µg/ml (Fig. 3c). Also, DLS illustrated that the size distribution of these derived small EVs almost ranged from 60 to 120 nm (Fig. 3d) and verified the SEM and TEM outcomes. Finally, western blot analysis demonstrated positive small EVs-specific surface marker expression of CD81, CD63, and CD9 (Fig. 3e).

The hEnSC-derived small EVs uptake by PC12

Researchers have illustrated that MSCs-derived exosomes can be taken up by target cells, thereby regulating their biological behavior [29]. As such, we indicated the penetration of hEnSC-derived small EVs into PC12 cells. In our tracking experiment, different concentrations of Dil-labeled small EVs were incubated with PC12 cells for 12 h, and fluorescence microscopy was used for detection of the cellular uptake of hEnSC-derived small EVs (Fig. 4a). Also, the intensity of the Dil stain was measured (Fig. 4b). As shown in this Figure, hEnSC-derived small EVs can enter the cytoplasm of PC12 cells, mainly localizing to the perinuclear region, and small EVs uptake is also increased with increasing small EVs concentration. Furthermore, no significant difference of intensity was detected between 100 and 150 µg/ml concentrations of small EVs.

hEnSC-derived small EVs induce axonal growth in PC12 cells

In this study, the PC12 cell line has been selected as an in vitro model for studying the effects of hEnSC-derived small EVs on inducing axonal outgrowth in neurons. The influence of 0, 50, 100, and 150 µg/ml small EVs on the PC12 cell morphology was investigated after 24 and 48 h post-treatment (Fig. 5a). Exposure of PC12 cells to various concentrations of hEnSC-derived small EVs resulted in concentration-dependent increases in neurite length. As shown in Fig. 5b, in the attendance of 50, 100, and 150 µg/ml concentrations of small EVs, axonal outgrowth significantly increased by 136%, 274% and 308% in axons, compared to control, respectively. After 48 h, the neurite length in the group with 100 and 150 µg/ml small EVs was significantly increased, which demonstrated the greatest neurite

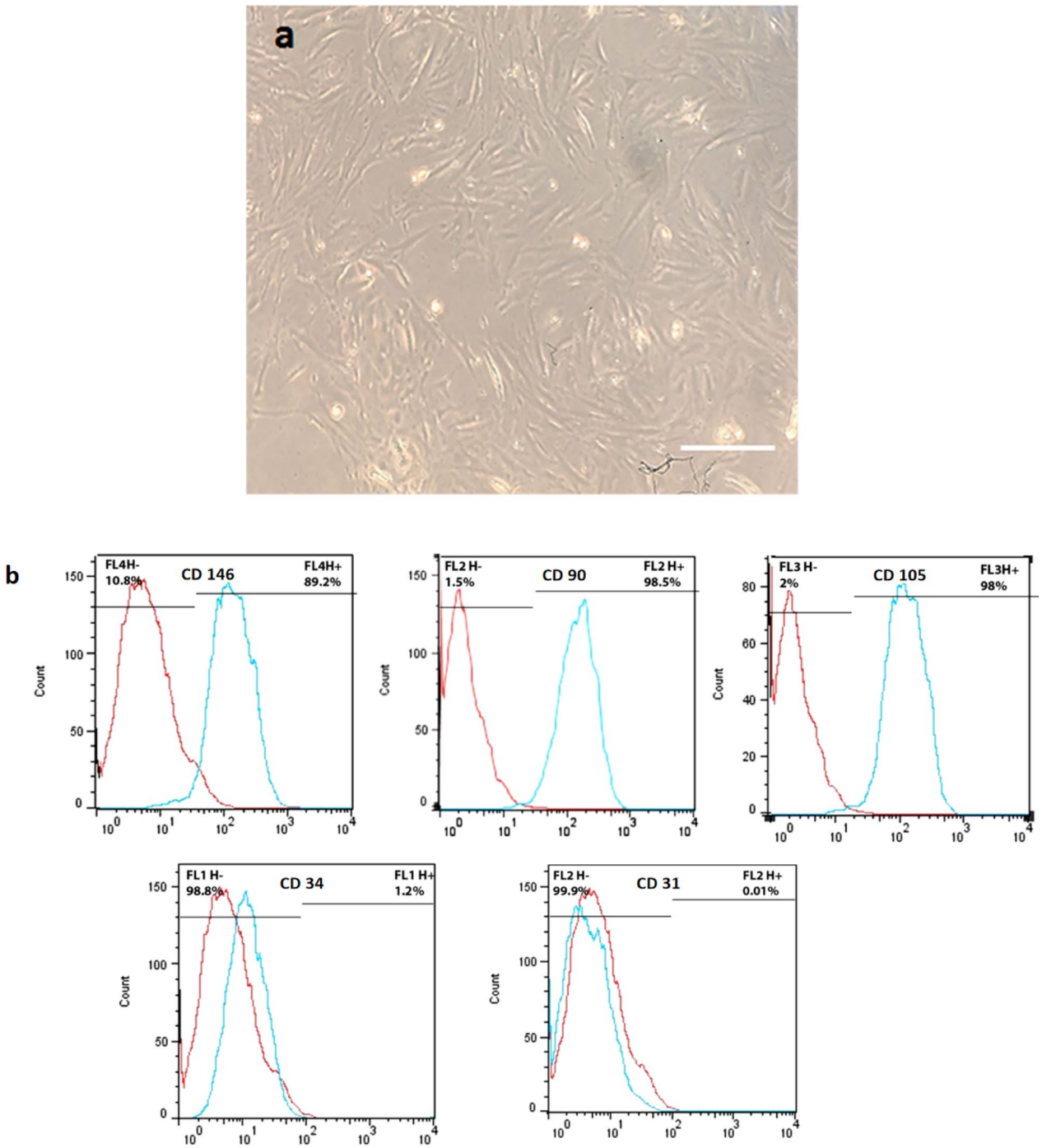

Fig. 2 Characterization of hEnSCs. **a** Morphological characteristics of hEnSCs at passage 3 (scale bar = 100 µm); **b** Flow cytometry analysis of hEnSCs surface markers demonstrated positive expression of CD90, CD44, and CD105 (mesenchymal stromal cell markers) and negative expression of CD31 (endothelial cell marker) and CD34 (hematopoietic cell markers) in hEnSCs

length (Fig. 5). As a result, the differentiation and neurite outgrowth of PC12 cells are induced by the presence

of hEnSC-derived small EVs and the extended neurites can connect to each other to create synapse structures.

The hEnSC-derived small EVs promote PC12 cell proliferation

To investigate the effect of hEnSC-derived small EVs on PC12 cell viability and proliferation, PC12 cells were exposed to various concentrations of hEnSC-derived small EVs, and MTT assays were applied after 1-, 3-, and 5-days post-treatment (Fig. 6a). In this study, increasing concentrations of small EVs can lead to increased PC12 cell viability and proliferation. Furthermore, no cytotoxic symptoms of hEnSC-derived small EVs, were determined. Also, a significant increase in proliferation of the cells treated with 100 and 150 µg/mL compared to the untreated cell groups 5 days later was observed (Fig. 6b and c).

hEnSC-derived small EVs enhance cellular migration

Next, the migratory and proliferative effects of hEnSC-derived small EVs on PC12 cells were evaluated. The result of the scratch assay is depicted in Fig. 7a and b. As shown in these Figures, the cell-covered area at 0, 50, 100, and 150 µg/mL of hEnSC-derived small EVs was depicted and analyzed after 24 and 48 h post-wounding. Scratch closure assay results demonstrated that the migration of treated PC12 cells was obviously increased in the presence of hEnSC-derived small EVs as compared to untreated cells (Fig. 7a and b). After 48 h, the scratch area was obviously filled with cells that were treated with 100 and 150 µg/mL of these small EVs, and there was no significant difference between these groups. But untreated cells as well as cells treated with 50 µg/mL small EVs were not completely filled after 48 h of scratching (Fig. 7c). Hence, it can be concluded that 100 and 150 µg/mL of hEnSC-derived small EVs greatly enhance cell migration and there was no significant difference was detected between them (Fig. 7c).

hEnSC-derived small EVs activate the PI3K/Akt signaling pathway in PC12 cells

The PI3K/Akt pathway is one of the substantial intracellular signaling pathways that plays a key role in promoting proliferation and inhibiting the apoptosis process [37]. In this study, western blot experiments were applied to identify the downstream signaling pathway involved in hEnSC-derived small EVs induced in PC12 cells. The results of this section revealed that PC12 cells treated with hEnSC-derived small EVs obviously increased protein levels of PI3K and AKT in PC12 cells in a dose-dependent manner compared to control cells (Fig. 8). Furthermore, the highest expression level of PI3k and AKT proteins was observed in PC12 cells treated with 150 µg/mL small EVs (Fig. 8). Accordingly, it can be concluded that the promoter effect of hEnSC-derived small EVs on cell survival, proliferation, migration, and anti-apoptosis happens through the PI3K/AKT signaling pathway [38].

Discussions

Nerve injury is one of the main challenges researchers face due to the limited self-repair capacity of nerves. Accordingly, the achievement of a perfect functional recovery from a nerve injury is not easy. Cells typically communicate together by interaction directly and/or by releasing growth factors, cytokines, and exosomes [39]. In recent years, small EVs, such as exosomes, have been known as an extracellular vehicle of intercellular communication that is released by all kinds of cells and can be isolated from conditioned media. All secreted small EVs not only share a similar evolutionary conserved protein but also share cell type-specific proteins that relate to their cellular origin [40].

Recently, MSCs have been the subject of a wide variety of clinical trial studies due to their anti-inflammatory, anti-apoptosis, immune-regulating, and vascular regeneration properties [41, 42]. Also, several studies have revealed that MSCs have significant therapeutic effects through their secreted small EVs such as exosomes. Currently, the use of MSC-derived small EVs such as exosomes to regenerate nerve injury is in its early stages, and only a few documents have demonstrated the effect of exosomes on nerve cells in detail. Also, in previous studies, it was not specifically investigated the hEnSC-derived exosomes effect on nerve cells. Due to that, we decided to evaluate the effect of hEnSC-derived small EVs on nerve cells and determine the optimal dose of these small EVs for nerve repair. According to our last research on hEnSC-derived exosomes, encapsulating small EVs in polymeric nerve guide conduit promoted

(See figure on next page.)

Fig. 3 Characterization of hEnSC-derived small EVs. **a** Illustration of size distribution of the isolated hEnSC-derived small EVs by SEM (scale bar 1 µm); **b** TEM photomicrographs of hEnSC-derived small EVs (scale bar = 100 nm); **c** Size distribution of hEnSC-derived small EVs by DLS; **d** Determination of small EVs concentration based on the mass concentration of BSA (small EVs concentration and 1000 µg/ml concentration of BSA was no significant difference). **e** The expression of small EVs marker proteins CD9, CD63, CD81 and cytochrome C (as a negative marker) by western blot assay. Each experiment was investigated in triplicate ($n = 3$)

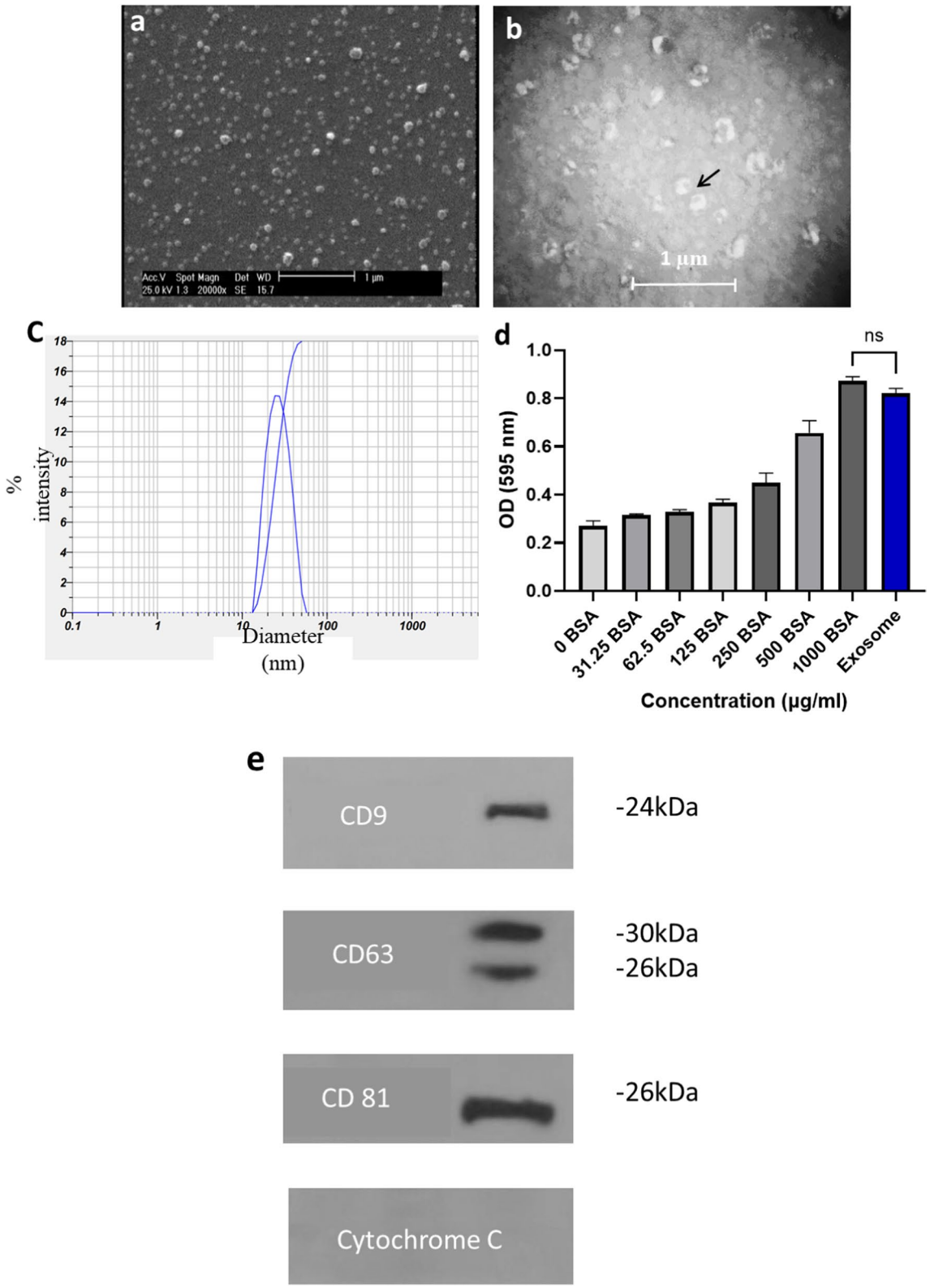

Fig. 3 (See legend on previous page.)

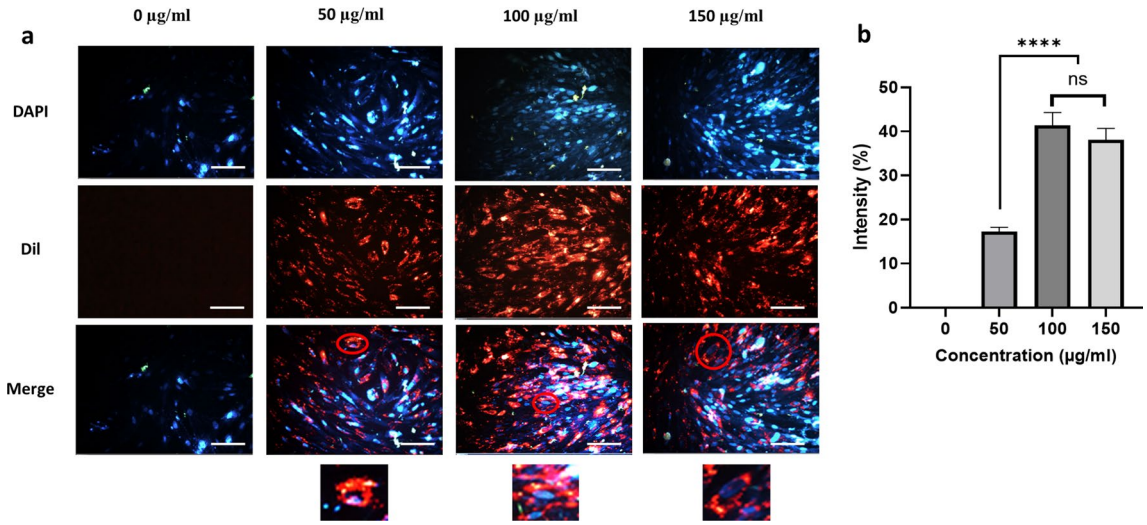

Fig. 4 Cellular internalization of hEnSC-derived small EVs by PC12 cells. **a** PC12 cells were incubated with different dil dye labeled small EVs concentration (0, 50, 100, and 150 µg/ml) for 12 h, and cells nuclei were stained with DAPI. Increased dye labeled small EVs were observed inside the cells by increasing small EVs concentration. Also as shown in figure stained small EVs (red spots) was completely penetrate in cells and concentrated around the nuclei (blue core) (scale bar = 100 µm); **b** Also, the intensity of Dil stain was measured by image j. *ns* not significant/****$P < 0.0001$. Each experiment was investigated in triplicate ($n = 3$)

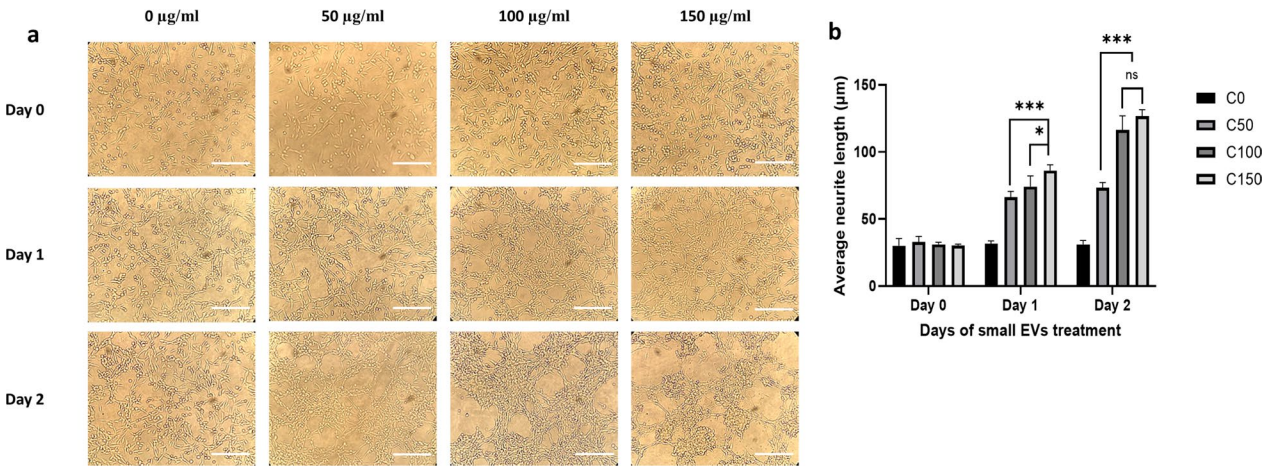

Fig. 5 Neurite length of PC12 cells induced with different concentrations of hEnSC-derived small EVs (scale bar = 100 µm); **a** The effects of 0, 50, 100, and 150 µg/ml small EVs on the PC12 cell morphology was observed in the four groups on day 0, 1, and 2 (magnification, x400). **b** Comparison of the neurite length of PC12 cells between the four groups on day 0, 1, and 2. Data are presented as mean ± standard error of the mean. * $P < 0.05$ / ***$P < 0.001$. Each experiment was investigated in triplicate ($n = 3$)

the repair of sciatic nerve injury by influencing Schwann cells in the sciatic nerve injury of rat model [24].

Similar to our research, Xie et al. [43] demonstrated that adipose mesenchymal stromal cells (ADSCs)-derived exosomes promoted proliferation and migration of PC12. Also, they investigated different concentrations of ADSCs-derived exosomes on PC12 cell function and concluded that 100 µg/ml is an optimal concentration to use therapeutically for nerve damage. Authors investigated the proliferation, migration and apoptosis of PC12 cells when exposed to different concentration of ADSCs-derived exosomes (0, 10, 50 and 100 µg/ml) [43].

In comparison to their study, we also indicated the effect of various concentrations of hEnSC-derived small EVs (0, 50, 100, and 150 µg/ml) on viability, proliferation, and migration on PC12 cell lines. However, we investigated neurite outgrowth and also stain hEnSC-derived small EVs to demonstrate small EVs

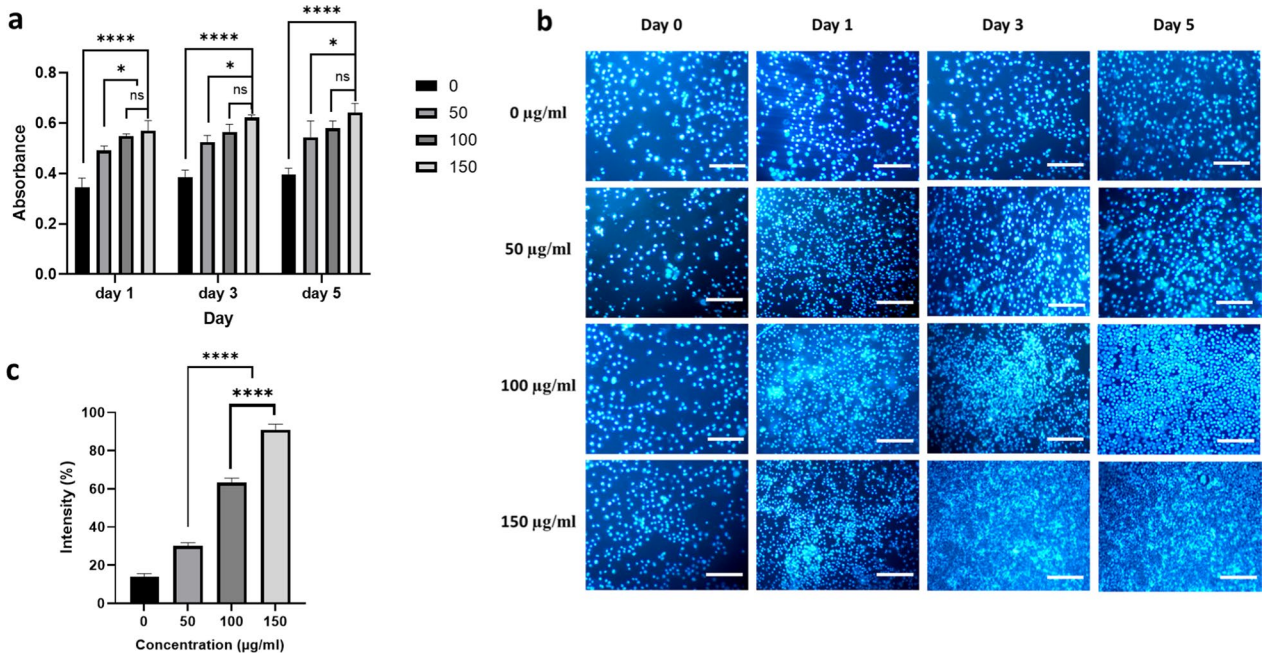

Fig. 6 hEnSC-derived small EVs enhance PC12 cell proliferation. **a** PC12 cells were treated with different concentration of small EVs and MTT assay was carried out after 1, 3, and 5 days after treatment. Absorbance was measured at 570 nm. MTT data demonstrated increased proliferation of treated cells by increasing small EVs concentration; **b** DAPI staining of PC12 cells were treated by different concentration of small EVs (0, 50, 100, and 150 µg/ml) (scale bar = 100 µm) **c** Also, post 5 days the intensity of DAPI staining was calculated by image J. The 100 and 150 µg/ml concentration of small EVs have the most effect in cell proliferation. [ns. not significant / $*P < 0.05$ / $****P < 0.0001$]. Each experiment was investigated in triplicate ($n = 3$)

penetration in PC12 cells. Furthermore, we assessed a higher concentration of 100 µg/ml small EVs to demonstrate a more precisely optimal concentration.

Also, in another study, Shariati et al. [44]. demonstrated that human ADSCs-derived exosomes have a promotive effect on cell viability, neuronal differentiation, and neural marker expression in PC12 cells [44]. Although their study investigated only one concentration (40 µg/ml) effect of human ADSCs-derived exosomes in PC12 cells, they also compared the effect of human ADSCs-derived exosomes and nerve growth factor (NGF) on PC12 cells.

In another study, Hsu et al. [23]. demonstrated the neurotrophic and neuroprotective effects of human umbilical cord MSC (UCMSC)-derived exosomes. They used locally UCMSC-derived exosomes with appropriate scaffolds to reduce nerve injury-induced pain in rat models. They showed that implantation of UCMSC-derived exosomes with scaffolds promoted IL-10 and the expression of myelin basic protein (MBP) after 21 days. Hence, they reported that UCMSC-derived exosomes could be a promising therapeutic for nerve injury-induced pain. However, they did not investigate various concentrations of the UCMSC-derived exosomes effect in in vitro conditions [23].

In the current study, the effects of hEnSC-derived small EVs on nerve regeneration were explored in PC12 cells as an in vitro model. Here, hEnSC-derived small EVs were extracted from hEnSCs supernatant and explained and characterized. The total yield of small EVs extracted from the ultracentrifuge method was nearly 1000 µg/ml. Also, as depicted in Fig. 3a, b, and **c**, hEnSC-derived small EVs were homogeneous in size distribution, with an approximately 60 nm diameter.

Western blot results revealed that the extracted small EVs were positive for CD81, CD63, and CD9 as an exosomal surface marker protein (Fig. 3e). Then the effects of hEnSC-derived small Vs on cell viability and proliferation in PC12 cells were investigated. As shown in Fig. 4, hEnSC-derived small EVs could be incorporated into the cytoplasm of PC12 cells, causing neurite outgrowth, proliferation, and migration, as well as promoting the proliferative signaling pathway of PC12 cells (Figs. 5 and 6, and 7).

The florescent microscopic image analysis of small EVs uptake illustrated that almost most of the small EVs were seen near the nucleus of PC12 cells by increasing the concentration of hEnSC-derived small EVs. On the other hand, it was demonstrated that the highest cellular uptake of hEnSC-derived small EVs led to most cell

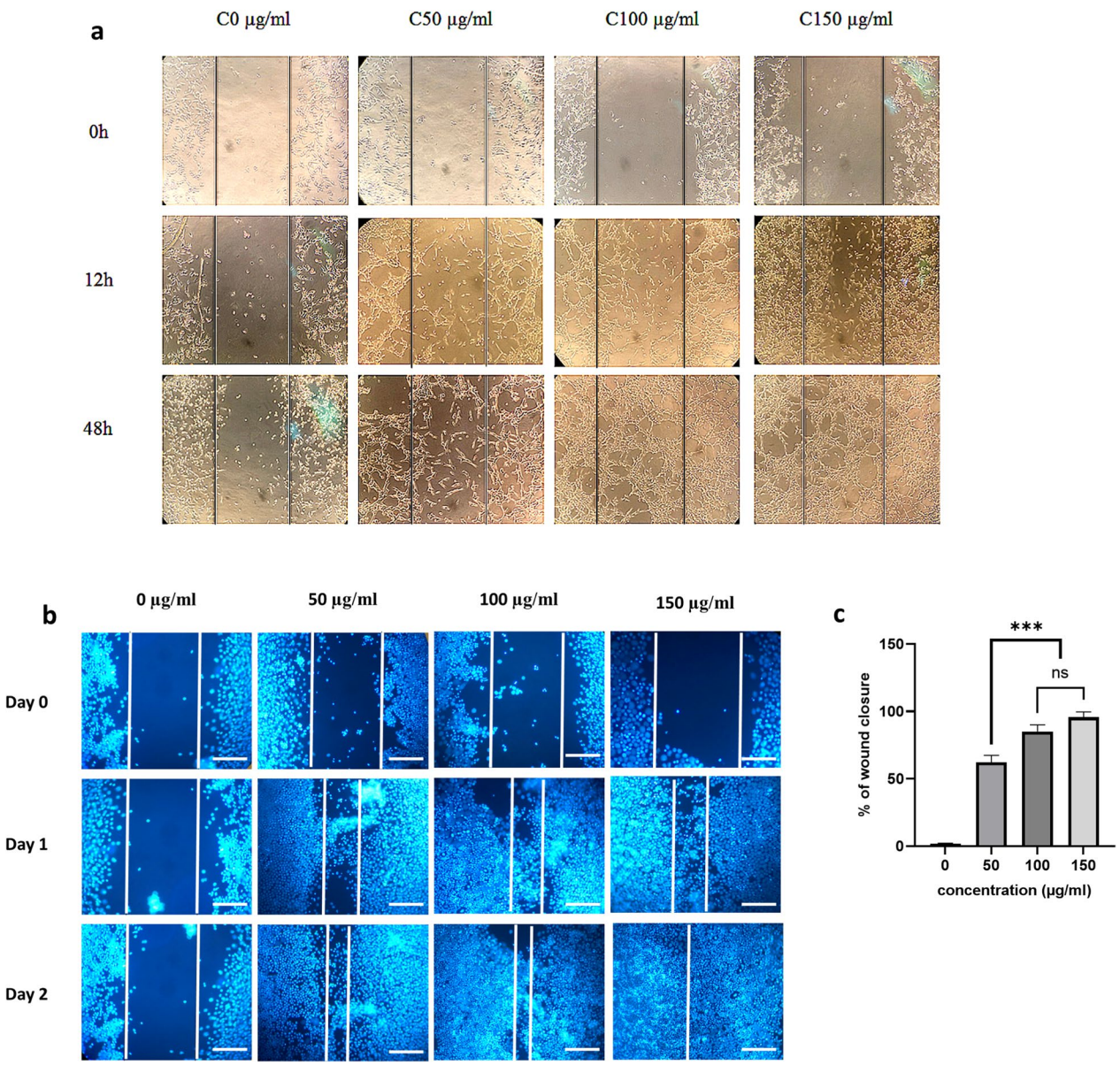

Fig. 7 hEnSC-derived small EVs enhance PC12 cell migration. **a** Migration rates of PC12 cells following co-culture with 0, 50, 100, and 150 µg/mL hEnSC-derived small EVs for 24 h and 48 h. Scratches incubated with 0 and 50 µg/mL exosomes were not filled 48 h post scratching; **b** DAPI staining of PC12 cells following co-culture with 0, 50, 100, and 150 µg/mL hEnSC-derived small EVs after scratching test (scale bar = 100 µm); **c** Also, the percentage of wound closure after 48 h was drawn in graph. [ns. not significant / ***$P < 0.001$]. Each experiment was investigated in triplicate ($n = 3$)

proliferation and also higher upregulation of PI3k/AKT pathways. The results of this section demonstrated that PC12 cells treated with 100 and 150 µg/mL hEnSC-derived small EVs revealed approximately equal neurite outgrowth and cell proliferation and also upregulated PI3k/AKT pathways because of high cell confluence and contact inhibition of PC12 cells.

Furthermore, the MTT assay and scratch wound healing results revealed that 100 and 150 µg/mL of small EVs increased PC12 cell survival, proliferation, and migration much more than 50 µg/mL, and the created scratch areas were totally filled after 48 h of treatment by 100 and 150 µg/mL concentrations of small EVs exposure (Figs. 6 and 7). The expression levels of proliferative signaling pathways (PI3k/AKT) were promoted in PC12 cells that were treated with hEnSC-derived small EVs compared to untreated PC12 cells (Fig. 8). Also, with the increased concentration of small

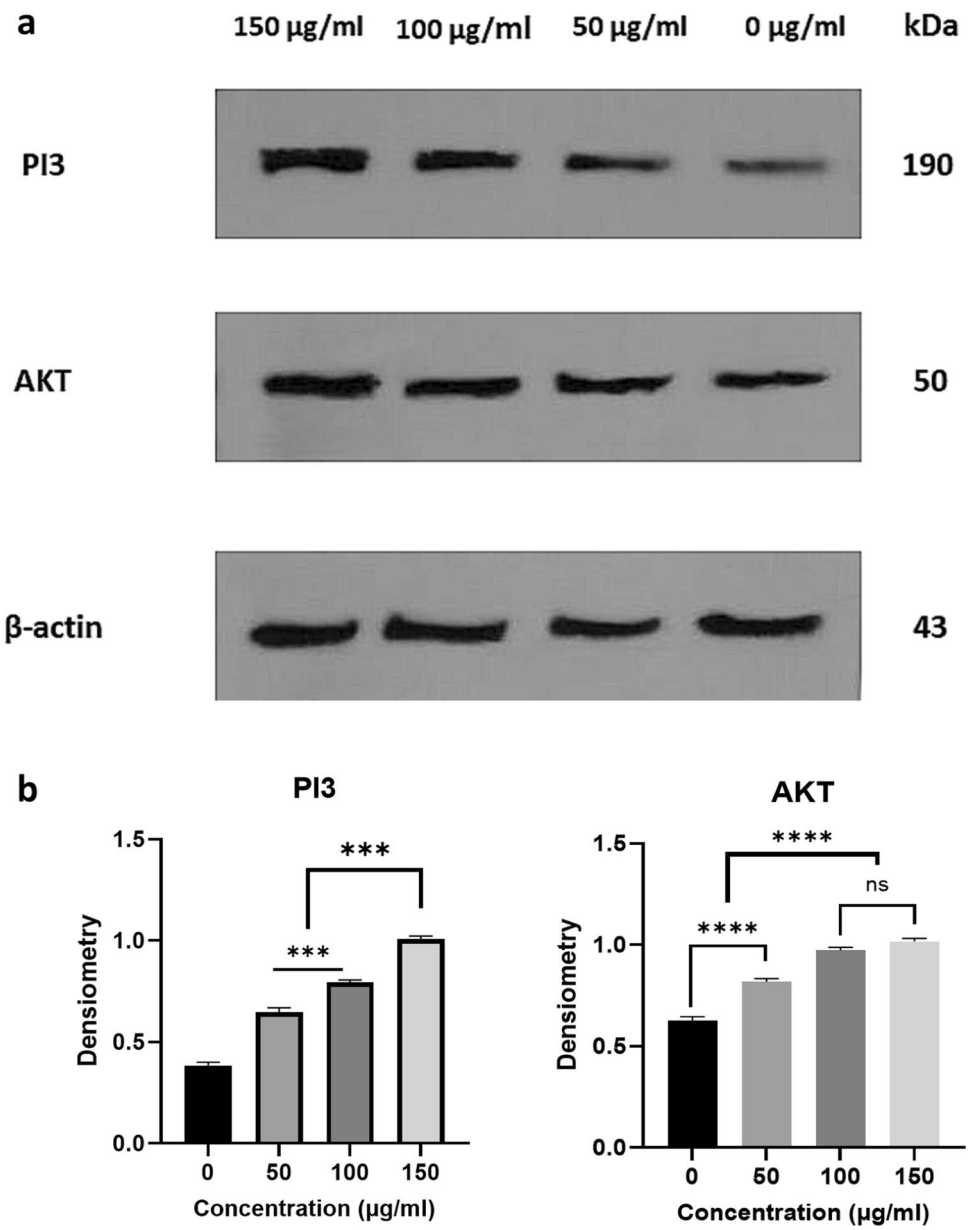

Fig. 8 hEnSC-derived small EVs activates the PI3k/Akt signaling pathway in PC12 cells. **a** Western blot data demonstrated increased expression of PI3k/Akt in treated PC12 cells by increasing small EVs concentration; **b** Bar graph of western blot densitometry. Analysis was done by image J. ns. not significant/***$P < 0.001$/****$P < 0.0001$. Each experiment was investigated in triplicate ($n = 3$)

EVs, the expression levels of these proteins in PC12 cells are increased.

As mentioned, the small EV components and their effects on recipient cells depend on the source of tissue extracted and the environmental context of the surrounding cells. On the other hand, small EVs, such as exosomes, carry various agents and exert their biological effects by transporting them to recipient cells. Previous studies also confirmed that neural progenitor cell migration and neurogenesis could be impressed by various factors, including channels and signaling pathways [45]. Also, recalling the progressive trend in numerous engineering [46], biomaterials [47], and biotechnology fields [48], utilizing the small EVs in treating multiple disorders and diseases in the future works is anticipated [49]. Accordingly, based on our results, we can conclude that hEnSC-derived small EVs significantly enhance cell survival, proliferation, and migration and also decrease cell

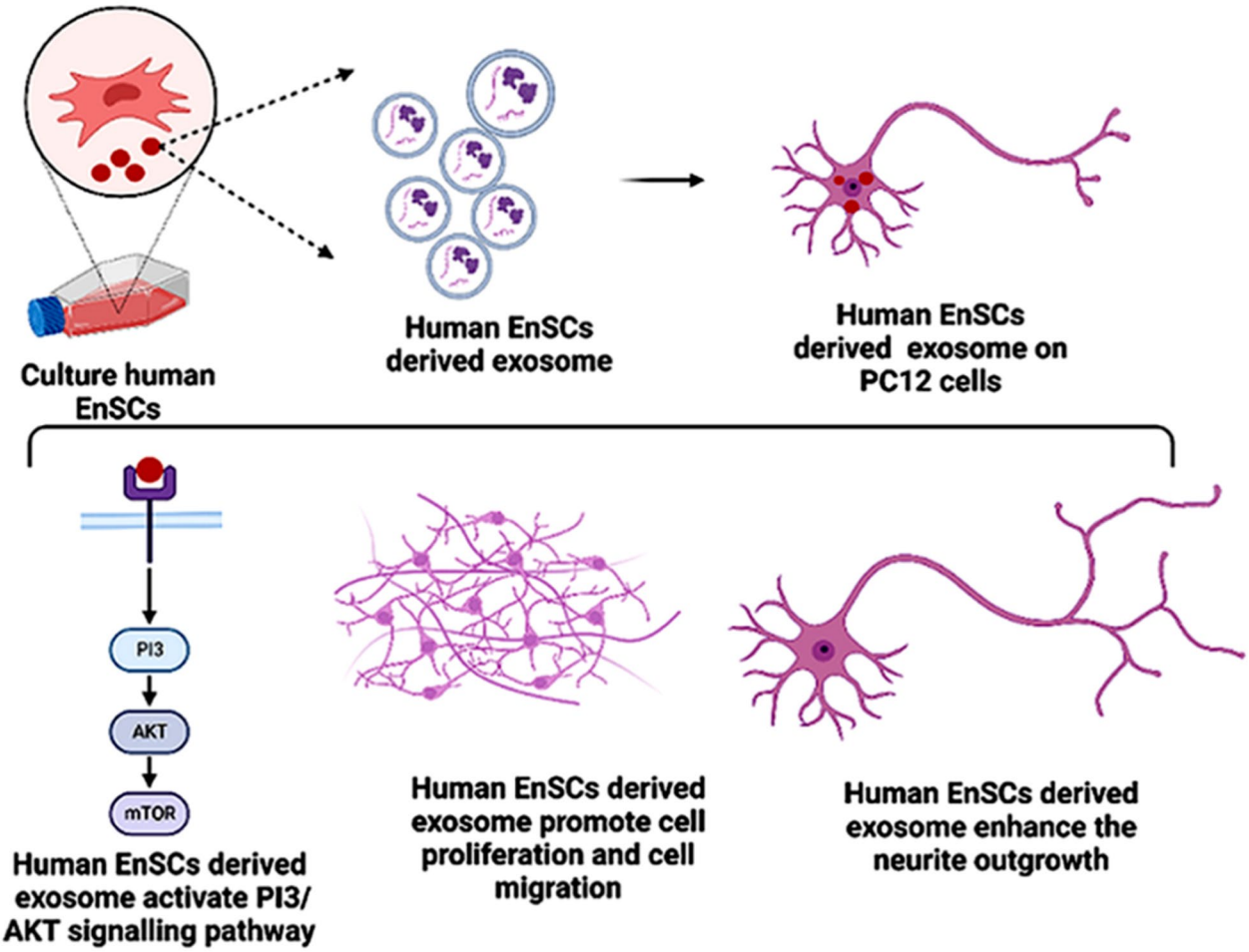

Fig. 9 hEnSC-derived exosomes promote neurite outgrowth, cell proliferation, and migration due to activating the PI3k/AKT signaling pathway

apoptosis in PC12 cells through PI3K/Akt signaling pathway activation (Fig. 9).

All of the outcomes of the current study illustrate new insights into the therapeutic effect of hEnSC-derived small EVs on nerve regeneration. Indeed, our outcomes demonstrated the enhancement role of hEnSC-derived small EVs through activation of the PI3K/Akt signaling pathway in the regeneration of nerve injury, while this signaling pathway play pivotal role in other human tissue functions [50]. However, this study has some limitations, such as investigating downstream molecular data like real-time PCR to indicate the PI3k/AKT signaling pathway activation. Also, future studies require investigating the duration of hEnSC-derived small EVs effects to provide a more comprehensive understanding of their therapeutic window. In addition, further studies should be designed in association with the PI3K/Akt signaling pathway, hEnSC-derived small EVs, and regeneration of nerve tissue injury in an in vivo model.

Conclusions

Due to the limited capacity of nerve tissue regeneration post-injury and the lack of appropriate strategies, MSC-derived small EVs could be a suitable candidate for the cure of nervous tissue injuries without the cell therapy side effects. The MTT assay and scratch wound healing results revealed that 100 and 150 µg/mL of small EVs increased PC12 cell survival, proliferation, and migration much more than 50 µg/mL, and the created scratch areas were totally filled after 48 h of treatment by 100 and 150 µg/mL concentrations of small EVs exposure. The expression levels of proliferative signaling pathways (PI3k/AKT) were promoted in PC12 cells that were treated with hEnSC-derived small EVs compared to untreated PC12 cells. Also, with the increased concentration of small EVs, the expression levels of these proteins in PC12 cells are increased. Hence, the outcomes of the current study illustrated that hEnSC-derived small EVs penetrate the PC12 cells and enhance viability, proliferation, and cell growth in a dose-dependent manner by

activating PI3k/AKT signaling pathways. Accordingly, hEnSC-derived small EVs can be a promising strategy for nervous tissue regeneration.

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Author contributions
MS did so and collected all the data. SE designed and supervised the study. NB wrote the draft and revised it. JA verified the research protocol and obtained results. All the authors read the submitted and revised versions and approved them.

Data availability
Not applicable.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent of publication
Not applicable.

Competing interests
The authors declare that they have no competing interests with respect to the research, authorship, and/or publication of this article.

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