

Human Adipose Derived Stem Cell Exosomes Enhance the Neural Differentiation of PC12 Cells

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Abstract

Background: Human adipose stem cells (hADSCs) are proper cell sources for tissue regeneration. They mainly mediate their therapeutic effects through paracrine factors as exosomes. The exosomes contents are protein, lipid and RNA. Exosomes are effective in restoring the function of neurons and astrocytes in neurodegenerative diseases, and improve the therapeutic outcomes. We investigated the effect of hADSCs derived exosomes on survival and neural differentiation of PC12 cells *in vitro*.

Methods and Results: The isolated hADSCs, were characterized by flow cytometry. Exosomes were separated from hADSC-condition medium using Exo-spin™ kit and characterized by DLS and TEM. Then acridine orange staining was performed to confirm entrance of exosomes into PC12 cells. PC12 cells were treated with culture medium containing NGF and exosome. Cell viability was assessed by MTT assay, and neural differentiation by ICC technique and qRT-PCR.

TEM and DLS data confirmed the isolation of exosomes according to their size (30-100nm) and acridine orange staining indicated entrance of exosomes to target cells. MTT assay showed that cell viability was significantly increased in exosome treated group. ICC technique revealed that the expression of Map2 was superior in the exosome treated group. Based on qRT-PCR data, *Map2* and *β-tub III* gene expression was increased in the exosome treated group. Significant expression of *Gfap* was seen in the NGF and NGF/EXO treated groups.

Conclusions: Present study indicated that hADSCs derived exosomes might enhance cell viability and promote neuronal differentiation and expression of mature neural marker in PC12 cells.

Introduction

The nerve tissue has a limited ability to spontaneously regenerate following neurodegenerative diseases or traumatic injuries, requiring innovative approaches for tissue regeneration. In this regard, researchers are always looking for an appropriate treatment of neurodegenerative diseases that characterized by degeneration and loss of neurons and glia. Up to now, some drugs and surgeries have been applied to manage the symptoms but there is no definitive treatment for neurodegenerative disorders(1). Due to the side effects of current therapeutic strategies, now-a-days stem cell therapy have been considered as a potential regenerative strategy for patients with neurologic disorders(2).

The stem cells technology has received considerable attention in recent years due to their potential for cellular and noncellular treatments(3). Stem cells have the ability to continuously renew themselves and can differentiate into many cell types(2). There are three types of stem cells that have therapeutic application: embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and adult stem cells (ASCs)(4). There are some limitations of the use of ESCs and iPSCs such as ethical complications, genetic instability and tumorigenicity(2). ASCs are found in many of the tissues and organs such as adipose tissue, bone marrow and amniotic fluid. However, ASCs have insufficient renewal and

differentiation properties compared to other stem cells(2) but these cells are immunocompatible and can produce large number of cells by in vitro cultures(4).

Recently among ASCs, mesenchymal stem cells (MSCs) are more considered due to easily accessible and great potential for regenerative medicine. MSCs are non-hematopoietic, fibroblast-like and plastic-adherent cells that form a heterogeneous population. This heterogeneity is determined with their differentiation capability, morphological differences and rate of proliferation(3). Human MSCs are identified through three criteria: 1) expression of CD73, CD90, and CD105, and the lack of expression of hematopoietic markers such as CD11b, CD34, CD45, CD79, CD19, and HLA-DR, 2) in vitro differentiation to osteoblasts, chondrocytes, and adipocytes and, 3) plastic adherence(3).

Adipose tissue is one of ideal sources for MSCs as large amount of tissue is easy to obtain with the least discomfort for patient and gives a high number of stem cells compared to bone marrow. Human adipose stem cells (hADSCs) have also nonmesodermal lineage differentiation potential such as neuronal lineage(4). Pervious data indicated that hADSCs were used in some degenerative diseases treatment such as osteogenesis imperfecta, dental structure degeneration, heart infarction and ischemic brain stroke(5).

Although, MSCs were widely applied for cell therapy, but there are some concerns about side effects of systemic application, such as favoring tumor growth and the possibility of MSCs differentiation into mesodermal lineages in inappropriate locations.

Based on literature, the therapeutic effects of MSCs work in two ways: the cell mediated effects (direct differentiation of MSCs) and paracrine mediated effects through soluble factors(6). On the other hand, recent experiments have indicated that the beneficial effects of MSCs are mediated mostly by paracrine secretion (MSCs secretome)(7, 8).

MSCs secretome includes miRNAs, growth factors and extracellular vesicles (EVs). Recently, the EVs were more attractive for non-cellular therapy. These EVs are categorized into microvesicles (100-1000 nm), Golgi vesicles or apoptotic bodies (50-5000 nm), and nanovesicles or exosomes (40-100 nm). This category is based on their size, morphology, origin, and mode of release(3, 7).

Stem cells, especially MSCs, use EVs to transfer growth, transcription, and anti-inflammatory factors to target cells. These factors have crucial rules for tissue regeneration and differentiation in local and distant tissues(3). The exosomes are currently being known as important mediators for cell-cell interaction and released by many types of cells such as T cells, B cells, neurons and stem cells(9) and exist in body fluids including urine, blood and cerebrospinal fluid(10).

Exosomes are surrounded by a phospholipid bilayer. The molecular signatures of exosomes are unique to each cell type and include proteins, lipids, coding and non-coding RNAs. RNA molecules within exosomes are the main messengers of the responses. For instance, coding mRNAs are functionally translated at recipient cells and microRNAs (miRNA) regulate gene expression following that mediate cell-cell

communication. Therefore, they can induce the phenotypic modifications and modulate the microenvironment(10, 11).

Exosomes are derived from lipid-raft microdomains. The formation of exosomes follows the endocytic-exocytic pathway, that contains early and late endosomes, lysosomes, and multivesicular bodies (MVBs). Exosomes originate from inward budding of late endosomes and subsequently form MVBs. Following the fusion of MVBs with the plasma membrane, exosomes are released outside the cell (3, 12).

MSC-exosomes are able to induce expression of cell cycle genes, growth factors like NGF(11), and neurotransmission, immune modulation, and differentiation(9). Furthermore, long half-life in circulation, low immunogenicity, and ability to cross the brain-blood barrier (BBB), make exosomes ideal tool for therapeutic applications(10).

Transferring miRNAs by MSC-exosomes accelerates neural plasticity and functional recovery and also increases neuronal differentiation of neural progenitors. As well as MSC-exosomes have anti-apoptotic effects. Taken together, these data clarify that exosomes have potential to protect against neuronal diseases(12).

Also, exosome-based, cell-free therapies reduce the complications of cell-based therapies and improve patients' outcomes. The therapeutic advantages of exosomes can be attributed to promotion of endogenous repair signals in the injured tissue and immune regulation(12). Although the cell-free therapy by exosomes has a bright prospect, there are still some limitations and disadvantages to be investigated into. One of them is that they are static and more cannot be produced in vivo as may be possible when transplanting the cell itself. Further research is needed to explore the relationship between dosage, injection frequency, and the long-term therapeutic effect, and whether single or multiple administrations will have a negative effect, which is very important for the correct use of exosomes to treatment (13, 14).

PC12 cells are pheochromocytoma cell line of the rat adrenal medulla that are accepted as a classic in vitro model for neuronal differentiation, neurosecretion, and neuronal injury studies(15). After treatment with nerve growth factor (NGF), PC12 cells undergo neural-specific changes such as producing lengthy neurite extensions and increase of cholinergic receptors expression so differentiate into neurons. In results, these cells represent many neural markers(16).

In this study, we attempted to purify exosomes from supernatant of hADSCs and evaluate the effect of these exosomes on PC12 cells survival. Also, we determined effect of exosomes on neuronal differentiation of these cells by assessment of gene expression and representing neural markers.

Method And Material

1. Isolation and culture of hADSCs

Subcutaneous adipose tissue was obtained from three abdominal surgery candidates. The adipose tissue was legally achieved by collecting a written consent approved by Care Committee of Isfahan

University of Medical Sciences. After washing the tissue samples with phosphate buffered saline (PBS, Bioidea, 1038), the extracellular matrix was digested with 0.075% collagenase type I (Sigma, C9891), and the cell suspension was neutralized and centrifuged at 1600 rpm.

The pellet was resuspended in 75 cm² culture flask and was cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) with 1% penicillin/streptomycin (all from Bioidea, Iran), at 37°C and 5% CO₂. The culture medium was changed every 2 to 3 days. Once the cells were confluent (70-80%), they were trypsinized and sub-cultured at 1:2 dilutions. In this study, the cells from passage 3-5 were used.

2. Characterization of hADSCs

Surface markers of hADSCs were examined by flow cytometry to confirm stemness of the harvested cells. Cells were trypsinized, centrifuged and then resuspended at density of 1×10⁵ cells per 1ml. They were washed twice with 1% bovine serum albumin (BSA)/PBS, then incubated with antibodies against positive (CD44, CD90, from IQ product and DakoCytomation, respectively) and negative (CD14/ CD45, from IQ product) markers for 30 minutes. Primary antibodies were directly conjugated with fluorescein isothiocyanate (FITC) or R-phycoerythrin (R-PE). A FITC-conjugated mouse IgG isotype and a PE-conjugated mouse IgG isotype antibody were used as negative control. Flow cytometric measurements were performed on BD FACSAria flow cytometer (Becton–Dickinson, San Jose, CA).

3. Isolation of hADSC-exosomes

The cells were cultured to 70% confluency, and washed 3 times with PBS then, they were incubated with DMEM and 1% penicillin/streptomycin (in serum free media) for 24 hour and conditioned medium (CM) was collected. Exosomes isolation was carried out according to manufacturer's instructions of ExospinTM precipitation (Cell Guidance Systems). Briefly, to remove floating cells and debris, CM was centrifuged at 300g for 10 minutes followed by 16000 g for 30 minutes. The supernatant was collected and then incubated with precipitation buffer overnight at 4 C. The sample was centrifuged at 16000 g for 1 hour and the supernatant was discarded. The pellet was resuspended in 100 µL of PBS. Using the provided columns, the sample was purified and exosomes were eluted in 200 µL of PBS. Exosomes were used immediately or stored at -70°C for three months(7, 17).

4. Identification of hADSC-exosomes

4.1. Transmission electron microscope (TEM)

A transmission electron microscope was utilized to confirm the successful isolation of exosomes and examine the morphology of them. For this purpose, 20-µl of exosome dilution was loaded on a copper grid (staining for 1 minute). Then the sample was stained with 20-µl of 2% phosphotungstic acid for 1 minute and was dried for 10 minutes. Finally, the exosomes were observed with a transmission electron

microscopy (Zeiss, EM10C) and images were taken. The size of exosomes were measured by image J software (NIH, v1.52v [<http://rsb.info.nih.gov/ij/>])(18).

4.2. Dynamic light scattering (DLS)

The presence of exosomes in CM was confirmed by means of DLS (Malvern Zen 3600 Instruments, UK). Size distribution of exosomes was measured according to the manufacturer's instructions (The sample was diluted 1:1000 in PBS)(19).

4.3. Bradford protein assay

The number of exosomes was evaluated by measuring the total exosome-associated proteins using the Bradford protein assay (Bio-Rad, Hercules, CA, USA) with comparison against a BSA. The dose of exosome was adjusted to 40 µg protein/suspended in 0.2 ml PBS (20).

5. Culture and differentiation of PC12 cells

PC12 cells (3000/cm²) were cultured in RPMI-1640 containing 10% horse serum, 5% FBS and 1% penicillin/streptomycin (16) as a culture medium. These cells were differentiated on tissue-culture dishes which were coated by Laminin/PDL(6) and assessed in four groups:

1. PC12 cells in culture medium as a control group (CON group)
2. PC12 cells in culture medium with NGF (100 ng/mL)(16) (NGF group)
3. PC12 cells in culture medium with exosomes (40 µg/mL)(21) (EXO group)
4. PC12 cells in culture medium with exosomes (40 µg/mL) and NGF (100 ng/ml) (NGF/EXO group)

The medium was changed every two days. Morphology of PC12 cells was observed by an invert microscopy (Nikon, PS-100).

6. Exosome tracking

To confirm transfer of isolated exosomes into PC12 cells, exosomes were purified using Exo-spin™ kit. Then exosomes were labeled with 20 µmol/L Acridine Orange (AO) for 90 minutes in dark place at room temperature (21°C). For removing free AO, the exosomes-AO were purified again. A dilution of exosomes with DMEM was prepared and added to PC12 cells. PC12 cells were incubated for 3 hours with exosome-AO. Subsequently, the cells were stained with DAPI to visualize cell nucleus. Cells were observed by a fluorescence microscope (Olympus, BX51, Japan)(22).

7. Cell viability assay

To determine cell viability, MTT assay was performed. Briefly, cells were differentiated in 24-well plates for 7 days. Then they were incubated with DMEM and 0.5mg/mL of MTT for 4 hours. After removing the

medium with MTT, 200 μ L DMSO was added. The optical density was measured at 540 nm by a microplate reader (Hyperion MPR 4+, Germany). MTT assay was performed at days 1, 3 and 7(16).

8. Immunocytochemistry

The differentiated cells were fixed using 4% paraformaldehyde for 30 minutes and permeabilized with 0.1% Triton X-100 for 1 hour. Then primary antibody for Map2 (Sigma-Aldrich) was added. The excess antibody was removed by washing with PBS and samples were incubated with secondary antibody conjugated with fluorescein isothiocyanate (FITC) (Sigma-Aldrich). Nuclei were stained by DAPI (Sigma-Aldrich) and samples were evaluated with fluorescent microscope (Olympus, BX51, Japan. Images were processed by image J software (NIH, MD, USA)(23).

9. RT-PCR and real-time PCR for gene expression analysis

After 7 days of differentiation, total RNA was isolated from PC12 cells by High Pure RNA Isolation Kit (Roche) according to the manufacturer's protocol. cDNA was synthesized from the RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) with oligo dT primers. RT-PCR was performed by 2 μ g total RNA. Primer sequences are shown in Table 1. Real-time polymerase chain reaction was performed by Maxima SYBR Green Rox qPCR master mix kit (Thermo Scientific) and the StepOne Plus™ quantitative Real time PCR detection System (Applied Biosystems). PCR reactions were performed in a total volume of 20 μ L. The PCR amplification conditions consisted of 10 minutes at 95°C followed by 40 cycles of denaturation step at 95°C for 15 seconds and annealing and extension for 1 minute at 60 °C. The mRNA expression levels of Map2, Gfap and β -Tub III were normalized with Gapdh (reference gene). The expression level of each target gene was calculated by $2^{-\Delta\Delta CT}$ (24).

Table.1: The sequences of primers used in the current study.

Gene	Primer sequences	Size	Seq. ID
Map2-F	GACAGAGAAACAGCAGAGGAAGTG	188	NM_013066
Map2-R	TGTTCTGATGCTGGCGATGGT		
β -Tub III-F	ATAGACCCCAGCGGCAACTATGTG	174	NM_139254.2
β -Tub III-R	AGGCCTGAATAGGTGTCCAAAGGC		
Gfap-F	GATGGCGAGGTCATTAAG	249	NM_017009.2
Gfap-R	GGTGAGTATCCTGTTAGTTG		
Gapdh-F	CAAGTTCAACGGCACAGTCAAG	123	NM_017008.4
Gapdh-R	ACATACTCAGCACCAGCATCAC		

10. Statistical analysis

All data were analyzed by one-way ANOVA followed by Tukey's test with a significance threshold of $P < 0.05$. Data were expressed as mean \pm SEM (Standard Error of Mean). The statistical analyses were performed using SPSS (version 22.0; SPSS, Chicago, IL). Each experiment was repeated three times.

Result

Characterization of hADSCs

At present study, hADSCs were used at 3-5 passages. These cells showed a fibroblast-like morphology (Fig. 1A). To assess the stemness characteristics of hADSCs, the cells were incubated with FITC-conjugated primary antibodies for 45 minutes at 4 °C and were fixed by paraformaldehyde 2%. Finally, surface markers were analyzed by flow cytometry. Flow cytometry assessment determined that the cells were positive for the mesenchymal stem cell markers CD44 (99.98±1.01%), CD90 (99.98±0.56%) and were negative for the hematopoietic stem cell markers CD14/45 (2.56±0.74%), (Fig. 1B-C).

Characterization of hADSC-exosomes

The presence of exosome in cells culture supernatants was assessed by transmission electron microscope (TEM) and Dynamic light scattering technique (DLS). Obtained images from TEM determined that purified vesicles were almost round-shaped membrane structures with 30-100 nm in diameter (Fig. 2A-B). Also, DLS measurements showed that the size distribution of these vesicles, almost ranged from 30 to 100 nm (Fig. 2C) and confirmed TEM results.

Internalization of exosomes by PC12 cells

To investigate whether hADSC-exosomes can enter into PC12 cells, exosomes were stained with AO and then incubated with PC12 cells. The cellular uptake of exosomes was examined by fluorescence microscopy (Fig. 2D). This assessment revealed the spots in cytoplasm of PC12 cells were AO-stained exosomes that localized at the cell cytoplasm.

hADSC-exosomes enhance cell viability and proliferation

To determine whether hADSC-exosomes can enhance cell viability and proliferation of PC12 cells, these cells were incubated with exosomes for 7 days and then MTT assay was performed at days 1, 3 and 7. In this study, the viability and proliferation of PC12 cells significantly increased in the EXO groups as compared to the NGF (P<0.01) and CON (P<0.001) groups (Fig. 3A).

At day 7, there was a significant decreasing in cell viability of the EXO and NGF/EXO groups compared to day 3 when their proliferation rate was compared and normalized to their CON groups. But this decrement was significantly superior in comparison to the CON and NGF groups (P<0.001) (Fig. 3B).

hADSC-exosomes induce neuronal differentiation

To evaluate the effect of hADSC-exosomes on neuronal differentiation, PC12 cells were treated with the exosomes for 7 days. the neurites outgrowth could be seen obviously in differentiated cells by invert microscope (Fig. 4). By using ICC and qRT-PCR, the neural differentiation of PC12 cell was confirmed for expression of Map2 (Fig. 5).

As shown in (Fig. 4A-B), PC12 cells treated with the EXO and NGF showed neuronal morphology with neurite-like extensions (Fig. 4C-D). Immunostaining showed the cells were treated with the EXO and NGF were positive for MAP2 unlike the CON and NGF/EXO groups (Fig. 4C). The results obtained from qRT-PCR also confirmed immunostaining results and the expression of MAP2 indicated a significant increment in the EXO and NGF groups compared to other groups (Fig. 4,5).

In addition, the expression of *Map2*, *β-Tub III* and *Gfap* genes in differentiated cells in different groups were evaluated by qRT-PCR (Fig. 5).

The results of qRT-PCR showed a significant increase ($P < 0.001$) in *β-Tub III* gene expression in the treated groups compared to the CON group. Expression of this gene was significantly increased in the NGF and EXO groups compared with the NGF/EXO group ($P < 0.001$). Meanwhile, the most expression of *β-Tub III* was observed in the NGF group.

On the other hand, the highest expression of *Map2* was detected in the EXO group that was significant ($P < 0.001$) compared to other groups. Moreover, the expression of *Map2* in the NGF group indicated a significant increase compared with the CON ($P < 0.05$) and NGF/EXO groups ($P < 0.01$).

The expression of *Gfap* was significantly superior in the NGF group compared to other groups ($P < 0.001$). While there was a significant increase ($P < 0.001$) in expression of this marker, in the NGF/EXO group in comparison with the EXO and CON groups.

Discussion

The nervous system has limited regeneration capacity. Therefore, treatment of neurological diseases is associated with difficulties. There are some evidences showing that stem cell therapy has been more considered as one of the therapeutic strategies for the neurological diseases(1, 2). hADSCs can be differentiated into multiple cell lines(25) and used to treat neurological degenerative disorders. Recent studies have shown that the stem cells secreted paracrine factors have therapeutic effects. These paracrine factors include cell-derived vesicles (nanovesicles and microvesicles) and soluble factors. Exosomes are nanovesicles that transfer proteins and gene products such as miRNA and mRNA to target cells so that influence their behavior(6, 7). These vesicles are secreted and taken up by most cell types. Exosomes have received increasing attention as paracrine mediators of stem cells in several disease models such as neurodegenerative diseases (11). As well as exosomes promote functional recovery and neurite outgrowth in neurons and astrocytes and also enhance neurogenesis and neurite remodeling(10). In addition, exosomes have the potential to promote neuroprotection, neuronal differentiation and neuroregeneration (19). In the present study, hADSC- exosomes were isolated and identified, and then we investigated the effects of hADSC-exosomes on cell viability and proliferation of PC12 cells. We also examined the role of exosomes on neural differentiation by expression of *Map2*, *β-Tub III* and *Gfap*. The results demonstrated that hADSC-exosomes enhanced the cell survival and proliferation in PC12 cells. As well as our findings indicated that exosomes could promote neuronal differentiation of these cells.

Exosomes are nanovesicles released by fusion of the multivesicular body with plasma membrane (11, 26). During exosome biogenesis, via endocytosis process, a part of the cytoplasmic membrane is folded inwards and the early endosome is formed. The late endosome is formed by invagination of the membrane of the early endosome. These late endosomes, which include intraluminal vesicles (ILV), are known as multivesicular bodies (MVBs). MVBs attach to the plasma membrane and, through exocytosis, the exosomes are secreted into the extracellular environment. In the direct pathway, exosomes are formed directly from the cell membrane (3, 12, 27). To uptake exosomes by the target cell, the exosomes enter the cell through the process of endocytosis or by binding to receptors (28, 29) (Fig. 6).

Previous studies have shown that these vesicles are lipid bilayer structures with spherical shape and size of 30-100 nm(11, 25). In the present study, exosomes were successfully isolated from hADSCs using a commercially exosome purification kit and were identified by DLS and TEM. The particle sizes were in the range of 30-100 nm with round morphology.

Exosomes are uptaken by other cells so they can affect the target cells(11). Reports suggest that following endocytosis, exosomes transfer genetic information (DNA and RNA) and protein to target cells and mediate signal transduction in these cells(22, 26). Hu et al. confirmed the physical entry of labeled exosomes into the cell cytoplasm of different cell lines(26). Our experiments found that AO labeled hADSC-exosomes can be internalized by PC12 cells and their genetic material was detected within cytoplasm of the cells, similar with previous observations (20).

MSCs-exosomes can transport potential regulatory components including proteins, mRNAs, and miRNAs into target cells thus enhance cell growth(11, 25, 30, 31). In this regard, Shabbir et al co-cultured labeled exosomes of MSCs with normal and diabetic chronic wound fibroblasts and confirmed that MSC derived-exosomes promote the cell growth in a dose-dependent manner. They found that cellular proliferation impaired significantly, when they used exosome-depleted medium. Due to their lipid bilayer shell, exosomes can effectively transfer signals and their contents into target cells. Exosomes can activate signaling cascades in target cells, including ERK, AKT, and STAT3, which increase expression of some growth factors, like HGF, IGF1 and NGF(11). Interestingly, Choi et al. found that ASC-exosomes increased the fibroblast population in S-phase. This study demonstrated the ASC-exosomes that stimulated cell-cycle, have regenerative potentials and play a pro-survival role (30). Furthermore, the studies on nervous tissue demonstrated that MSC-exosomes treatment increases the number of newly formed neuroblasts and endothelial cells in traumatic brain injury model(31), promotes neuroprotection and inhibits the apoptotic cascade in familial amyotrophic lateral sclerosis(7). We examined the survival effect of hADSC-exosomes on PC12 cells by MTT assay. In accordance with previous studies, there were a significant increased proliferation and cell viability in group treated with exosomes.

Exosomes contain different miRNAs, which can change gene expression. Thereby, they enhance neural differentiation by transferring a variety of proteins and growth factors. In this regard, Xin et al confirmed that content of MSC-derived exosome can modify the characteristics of recipient cells by regulating their gene expression. They revealed that exosome treatment after stroke, can increase angiogenesis,

neurogenesis, axonal density and neurite remodeling(32). In another study, newly generated neurons, oligodendrocyte progenitor cells and mature oligodendrocytes were significantly increased after exosome treatment(33). So results with the past data showed that MSC-exosomes have a pivotal effect on the stroke recovery (32, 33).

Zhang et al. assessed the effect of MSC-exosomes on Traumatic Brain Injury (TBI), and reported that exosome therapy significantly increased the number of newly formed immature and mature neurons, and increased angiogenesis. Recently, to examine the effect of MSC-exosomes on neurite outgrowth, rat primary DRG neurons were treated with exosomes and results showed that neurite outgrowth of the DRG neurons increased after co-culturing with exosome(34). The other study demonstrated that MSC-exosomes can activate STAT3, ERK and AKT signaling pathway in target cells which cause increase expression of a number of growth factors including NGF, IL-6, and SDF1(11).

In this context, Xin et al reported that MSCs communicate with neurons and astrocytes via secretion of exosomes that contain miRNAs like miR-133b, which enhances neurite outgrowth in neurons. Exosomes transfer miR-133b from MSCs to neurons and astrocytes, and lead to downregulate CTGF (Connective Tissue Growth Factor, a major inhibitor of axonal growth at injury sites in the CNS) expression, and promote neurite outgrowth(35). Our findings showed that exosomes accelerated neural differentiation in PC12 cells through upregulation of Map2 and β -Tub III expression that represent neural maturation of these cells. In addition, morphology of cells changed from fibroblastic-like cells to nerve cells and neurite outgrowth was observed in exosome-treated cells.

β -Tub III as an early neuron marker, is often used to verify the neuronal differentiation of neural stem cells in *in vitro* studies(36). Meanwhile, Garcia et al indicated that Gfap-expressing cells are mainly neuroblasts and neural progenitors that are responsible for neurogenesis, and while the most of mature neurons are not Gfap-expressing cells(37). But Map2 is expressed mainly in mature neurons and involved in nerve cell structure and regulate transport of organelles. In our study, higher expression of Map2 and lower expression of β -TubIII and Gfap in exosome group imply that exosome contents accelerated the differentiation of PC12 cells into mature neurons compared with NGF group. Although both NGF and exosome caused neural differentiation individually, but they could not synergically upregulate neural markers expression, which need more studies to elucidate. Our explanation for this finding might be related to the ubiquitinated proteins. It has been demonstrated that the ubiquitinated proteins (Cbl and Cblb) can be released into the extracellular space. These proteins have been suggested to have a negative effect on NGF/TrkA pathway and facilitate TrkA ubiquitination, internalization, and turnover(38, 39). Therefore, they can suppress the NGF effect.

Conclusion

According to limited regeneration capacity of nervous tissue after injury and various diseases, and the lack of proper treatments, the use of stem cell derived exosomes could be a proper solution for treatment of neurological disorders without side effects of cell therapy. Results of current study indicated that

hADSC-exosomes could enter into the target cells, increase cell growth and viability, and induce neural differentiation of PC12 cells. These effects are probably due to existence of growth factors, proteins and miRNAs that promote neural differentiation, the cell cycle and inhibit apoptosis.

Declarations

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Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics approval and consent to participate

The adipose tissue samples were legally achieved by collecting a written consent approved by Care Committee of Isfahan University of Medical Sciences (IR.MUI.REC.1396.3.571).

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Samira Shariati Najafabadi, Noushin Amirpour and Hossein Salehi. The first draft of the manuscript was written by Samira Shariati Najafabadi and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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