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Original Research

Evidence supporting neuroprotective effect of adipose derived stem cells on PC12 cells against oxidative stress induced by H_2O_2

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Abstract: Adipose-derived stem cells (ADSCs) are a population of cells derived from adipose tissue. ADSCs exhibit multilineage development potential and are able to secrete various factors, which influence adjacent cells. The present study examined the protective effect of ADSC's conditioned media (ADSC-CM) on PC12 cells exposed to H_2O_2, an oxidative injury model. After isolation, ADSCs were cultured and their osteogenic and adipogenic differentiation confirmed. Then, PC12 cells were co-treated with ADSC-CM and H_2O_2. Next, the effects of ADSC-CM on neurite outgrowth and cell differentiation in the presence of H_2O_2 were determined. Moreover, cell viability and apoptotic cell death percentage were evaluated using MTT assay, Hoechst staining and flow cytometry. Our results indicated the neuroprotective effects of ADSC-CM on morphological and morphometrical properties of neuron-like PC12 cells. Additionally, the profound decrease in percentage of apoptotic cells confirmed the protective effects of conditioned media from ADSCs that may be related to the release of trophic factors.

Key words: Adipose derived stem cells; Neuroprotective; PC12 cells; Oxidative stress.

Introduction

Adipose-derived stem cells (ADSCs) are a population of pluripotent mesenchymal stem cells that were isolated from subcutaneous adipose tissue and had the potential to differentiate into variety of cell lineages. Moreover, it has been reported that ADSCs possess the ability of production and secretion of trophic factors leading to repair and replacement of damaged neighboring cells (1, 2). Accordingly, therapeutic properties of ADSCs are considered to be related to their paracrine functions (3). Kim has reported that conditioned media from ADSCs (ADSC-CM) which contains various types of growth factors could be utilized for wound repair, replacement and regeneration (4). Also, the effect of ADSC-CM on reducing the proliferation and migration of B16 melanoma cells has been reported in culture and also in a mouse xenograft model (5). There are a few reports about the protective effect of mesenchymal stem cells on neural cells during oxidative injury (6).

Oxidative stress is known as a condition that in response to various stimuli, the body produces excessive amounts of free radicals including reactive oxygen species (ROS). Overproduction of ROS leads to DNA damage, lipid peroxidation and aberrant post-translational modification of proteins, which are involved in pathogenesis of neurodegenerative diseases (7, 8). One of the main consequences of oxidative stress is activation of intracellular molecules involved in programed cell death or apoptosis (9). Moreover, it has been shown that functions of numerous cytoplasmic proteins depend on ROS and oxidation state (10). In this regard, a substantial body of evidence has documented that cellular oxido-redox balance affects cytoskeletal proteins, which directly impacts cell morphology and morphometrics (11-13). As a matter of fact, neurons are extremely polarized cells possessing a cell body from which several dendrites emerge and an axon that makes functional communication networks with other neurons and glial cells (13). Therefore, regrowth and repair of neuronal neurites due to cytoskeletal rearrangement could be a criterion indicating development and polarization of these cells.

Accordingly, in the present study, we attempted to determine the protective effect of conditioned media obtained from ADSCs against injurious effects of oxidative stress induced by H_2O_2 in neuron-like PC12 cells. Differential morphologic criteria including neurite length and width, area of cell body and percentage of bipolar cells were assessed in CM-ADSC-treated H_2O_2 exposed PC12 cells. Furthermore, the percentage of apoptotic cells was quantified using flow cytometric methods.

Materials and Methods

Isolation and culture of ADSCs

Adult male rats were killed using CO_2. Pre-testes adipose tissue was removed and the obtained samples were digested with 0.1% collagenase type I (Sigma-Aldrich, St. Louis, MO) under gentle agitation for 30 min at 37 °C, and centrifuged at 1500 rpm for 5 min to obtain the stromal cell fraction. The pellet was filtered with 70 mm nylon mesh filter, and re-suspended in phosphate- buffered saline (PBS). The cell suspension was cultured at 37 °C in a control medium (Dulbecco's modified Eagle medium (DMEM), 10% fetal bovine serum (FBS), 100

units/ml of penicillin, 100 mg/ml of streptomycin). After 48 h, the culture media was changed in order to remove the debris and red blood cells. ADSCs were cultured and expanded in culture medium, and used for the experiments at passages 1-3. For collecting the AD-SC-CM for treatment, ADSCs were cultured in serum free medium. After 48 h, medium was collected, filtered and kept at -20 °C for future use.

Osteogenic differentiation

To induce osteogenic differentiation, 3×10^3 cells/cm² were plated in DMEM supplemented with 10% FBS, 10 mM β-glycerophosphate, 60 µM ascorbic acid, and 10^{-7} M dexamethasone (Sigma Aldrich, Belgium), and cultured for 3 to 4 weeks, replacing the medium every 3–4 days. To demonstrate osteogenic differentiation, the cultures were fixed and stained with Alizarin (Sigma-Aldrich, St. Louis, MO).

Adipogenic differentiation

To induce adipocyte differentiation, 3×10^3 cells/cm2 were cultured in DMEM supplemented with 10% FBS, 60 µM indomethacin, 10^{-6} M dexamethasone, 5 µg/ml insulin and 0.5 mM isobutylmethylxanthine (IBMX) (Sigma-Aldrich, St. Louis, MO). The cells were cultured, and the medium was replaced every 3-4 days. After 3 to 4 weeks of culture, cells, containing neutral lipids in fat vacuoles, were fixed in 10% formalin and stained with fresh oil red-O solution (Sigma-Aldrich, St. Louis, MO).

PC12 cell culture and treatment

PC12 cells were obtained from Institute of Pasture (Iran, Tehran). The cells were cultured in DMEM/F12 media supplemented with 10% fetal bovine serum (FBS) and penicillin and streptomycin (1%). Then, PC12 cells were treated with ADSC-CM (4:1 ratio of ADSC-CM to DMEM/F12 medium) and simultaneously exposed to H_2O_2 (150 µM) for 24 h (14).

Measurement of PC12 cell morphology

PC12 cells were seeded in 6-well plates. For morphological analysis, random images were acquired from each well, taking 20 images per well. A minimum of 50 cells per treatment were quantified. After co-administration of CPECs-CM and H_2O_2, 4 criteria were assessed: neurite length, cell body area, neurite width and percentage of bipolar neurons. Neurite length was defined as the sum of lengths of all primary branches and their associated twigs. The area of a cell body except its branches was defined as cell body area. To calculate neurite width, cell body area was divided by neurite length. Data analysis was done using the Cell^A software (14).

MTT assay

PC12 cells were cultured in 96-well plate. After treatment, 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well and incubated for 4 h. Then, supernatant was removed and dark blue crystal of formazan was dissolved in dimethyl sulfoxide. Absorption of the suspension was read at 590 nm, and cell viability reported as the percentage of control cells.

Hoechst staining

Nuclear staining was carried out by Hoechst 33258 staining. For this purpose, after treatments, cells were washed with PBS, stained with DNA dye Hoechst 33258 (5 µg/ml) for 10 min and rinsed with PBS. Fluorescence images were acquired using a fluorescent microscopy (Olympus, Japan). Apoptotic cells and cells with high membrane permeability displayed bright blue nuclei, while normal cells emitted only weak blue fluorescence.

Analysis of apoptosis rate by flow cytometry

For quantification of the rate of apoptosis, PC12 cells were harvested and washed twice with cold PBS and fixed with 70% ethanol. Then, cells were centrifuged at 200 g for 10 min and re-suspended in 100 ml PBS containing 200 mg/ml propidium iodide and 50 mg/ml RNase A at room temperature for 30 min. The fluorescence of cells was measured with a FACScan flow cytometer (Becton Dickinson, FACScan).

Cell viability assay

Live and dead cells were stained using the Eukolight™ Viability/Cytotoxicity assay (Molecular Probes). Culture medium was replaced with 2 mM calcein acetoxymethyl ester and 4 mM ethidium homodimer-1. Viable (green fluorescent by calcein) and non-viable (red fluorescent by ethidium) PC12 cells were captured in 10–20 random microscopic fields per condition per experiment.

Data analysis

All data are represented as the mean ± SEM. Comparison between groups was made by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test to analyze of the difference. Statistical significances were achieved when $P<0.05$ (n=3).

Results

Characterization of adipose-derived stem cells

In the primary culture, ADSCs isolated from rat pretestes adipose tissue showed a relatively homogenous population that exhibited a fibroblast-like morphology and expanded easily in vitro. During in vitro expansion with osteogenic medium, osteogenic differentiation of ADSCs and calcified extracellular matrix deposition was detected by Alizarin Red staining (Figure 1). Adipogenic differentiation was confirmed with an intracellular accumulation of lipid droplets by Oil Red O staining.

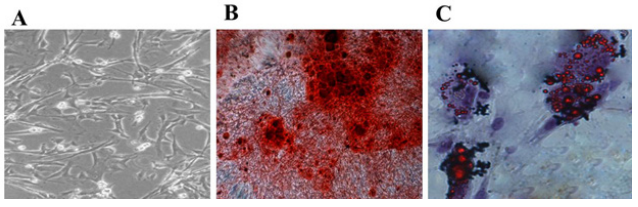

Figure 1. The morphology of ADSCs in culture (A) ADSCs exhibit properties of stem cells, in vitro multi-lineage differentiation potentials of ADSC were investigated. (B) Osteogenesis was demonstrated by Alizarin red S staining, (C) adipogenesis by Oil Red O staining.

ADSC-CM protected PC12 cells against H_2O_2 induced cell death

To assess the effect of ADSC-CM on PC12 cells, viability of cells treated with ADSC-CM and H_2O_2 was determined by the MTT assay. As shown in Figure 2., MTT assay revealed that cell viability of PC12 cells 24 h after co-treatment with ADSC-CM and H_2O_2 increased significantly compared to the cells incubated only with H_2O_2 (increased 1.59 fold compared to the H_2O_2 exposed cells). Based on our data, co-treatment of PC12 cells with ADSC-CM and H_2O_2 significantly decreased cell death. Also, the protective effect of ADSCM on H_2O_2-exposed cells was confirmed using LIVE/DEAD assay, as witnessed in Figure 3.

ADSC-CM increased PC12 cells neurite length after H_2O_2 -induced oxidative stress

As depicted in Figure 4, in cells co-exposed to ADSC-CM and H_2O_2, neurite length increased significantly compared to cells incubated only with H_2O_2. Neurite length in the group that received H_2O_2 and ADSC-CM at the same time after 24 h was 1.78 fold higher than H_2O_2 treated cells.

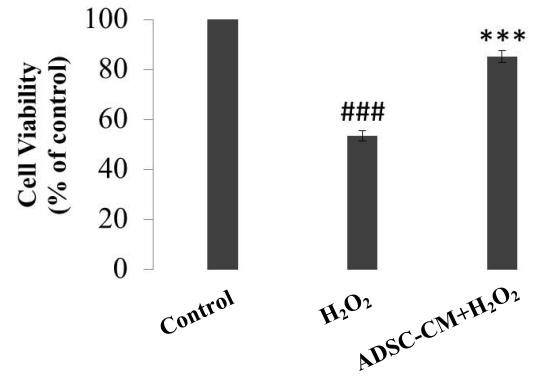

Figure 2. Effect of ADSC-CM on cell viability. ADSC-CM and H_2O_2 was added to PC12 cells at the same time. After 24 h, cell viability was determined by MTT assay. Viability was calculated as the percentage of living cells in treated cultures compared to control cells (n=3). ***P<0.001 significant different from H_2O_2 groups; ### P<0.001 significant different from control group.

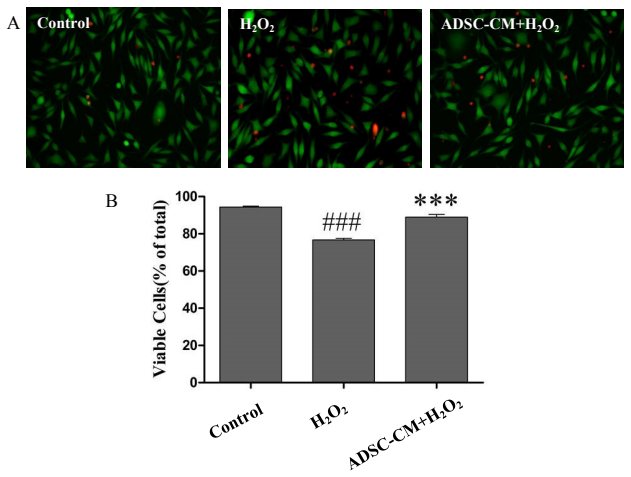

Figure 3. The fluorescence images for assessment of cytotoxicity using the LIVE/DEAD assay. Healthy cells show green fluorescence, whereas dying or dead cells with compromised membranes appear red. Percentage of viable cells of each field (10–20 random microscopic fields per condition per experiment). was calculated in treated cultures (H_2O_2 and/or ADSC-CM+H_2O_2 exposed cells) compared to control cultures. *** P<0.001 significant different from H_2O_2 groups; ###P<0.001 significant different from control group.

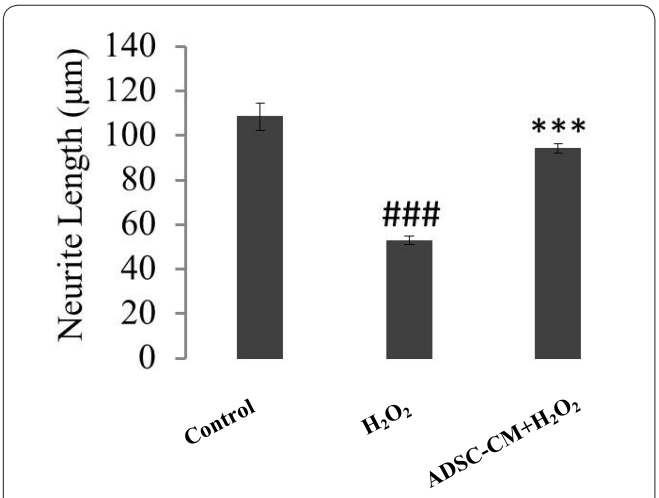

Figure 4. Effect of ADSC-CM on the PC12 cells morphology, Neurite length was defined as the sum of lengths of all primary branches and their associated twigs. *** P<0.001 significant different from H_2O_2 groups; ### P<0.001 significant different from control group.

ADSC-CM decreased PC12 cells cell body area after H_2O_2 induced oxidative stress

Our results showed that cell body area increased in the present of H_2O_2 (7.43 fold in cells exposed to H_2O_2 in comparison with control cells). On the other hand, in ADSC-CM treated cells exposed to H_2O_2 cell body area reduced significantly compared to the cells incubated only with H_2O_2 as shown in Figure 5.

ADSC-CM decreased PC12 cells neurite width after H_2O_2 induced oxidative stress

Calculation of neurite width showed significant decrease in the groups receiving ADSC-CM and H_2O_2 after 24 h relative to stress (H_2O_2 treated) group. This value for the group treated with ADSC-CM and H_2O_2 simultaneously was 21% compared to the stress group after 24 h (Figure 6).

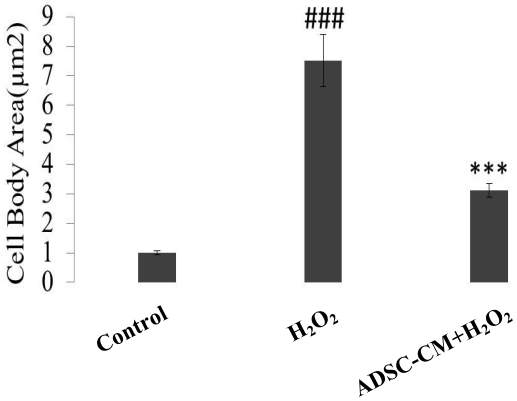

Figure 5. Effect of ADSC-CM on the morphology of PC12 cells exposed to H_2O_2. The area of a cell body except its branches was defined as cell body area. *** P<0.001 significant different from H_2O_2 groups; ### P<0.001 significant different from control group.

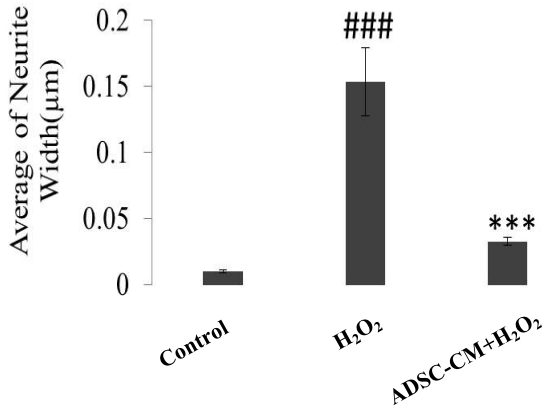

Figure 6. Effect of ADSC-CM on the PC12 cells morphology, neurite width was calculated by dividing cell body area to neurite length. *** P<0.001 significant different from H_2O_2 groups; ### P<0.001 significant different from control group.

ADSC-CM decreased percentage of bipolar neurons after H_2O_2 induced oxidative stress

As a marker of undifferentiation, the percentage of bipolar neurons also increased in H_2O_2 exposed cells (1.94 fold after 24 h exposure to H_2O_2 compared to the control cells). However, the percentage of bipolar neurons significantly decreased in the group treated with ADSC-CM along with H_2O_2 compared to H_2O_2 exposed cells (56% decrease 24 h after exposure to H_2O_2) (Figure 7). Furthermore, the number of multipolar cells increased in the presence of ADSC-CM as well (data not shown).

ADSC-CM suppressed H2O2-induced apoptosis in PC12 cells

The apoptosis rate was defined as the percentage of cells with sub-diploid DNA content (DNA fragmentation) determined by flow cytometry method (15). A significant increase in the apoptosis rate was found when cells were treated with 150μM concentration of H_2O_2 for 24 h (83.8%). However, when cells were treated with ADSC-CM for 24 h, the percentage of apoptosis notably decreased (29%) as illustrated in Figure 8.

On the other hand, apoptotic cells undergoing chromatin condensation visualized using the DNA-binding fluorescent dye Hoechst 33342. Nuclei of control cells appeared round to oval, with a separate pattern of blue fluorescence. After exposure to H_2O_2 for 24 h, cell nuclei became increasingly bright. Finally, cell size decreased and they fragmented into apoptotic bodies. In contrast, co-incubation with ADSC-CM appears remarkably preserved and attenuated apoptotic features, as shown in Figure 9.

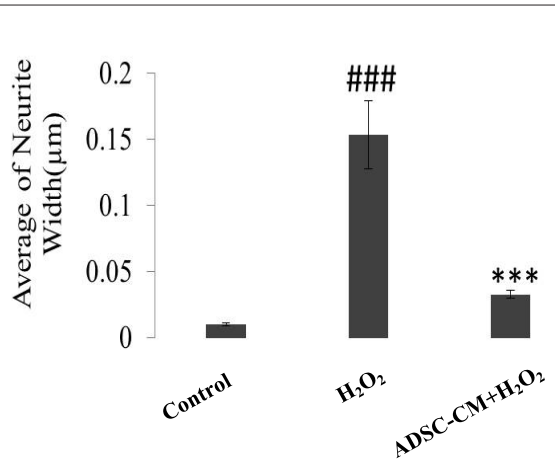

Figure 6. Effect of ADSC-CM on the PC12 cells morphology, neurite width was calculated by dividing cell body area to neurite length. *** P<0.001 significant different from H_2O_2 groups; ### P<0.001 significant different from control group.

Discussion

As one of the major causes of neuronal cell death, oxidative stress is predominantly involved in the pathophysiology of CNS-related disorders such as neurodegenerative diseases (16). For that reason, in the present study, H_2O_2 exposure was used to induce oxidative stress in neuron-like PC12 cells as a model of neurodegeneration. Our data clearly indicated that treatment of PC12 cells with ADSC-CM significantly decreased percentage of apoptosis induced by H_2O_2. Furthermore, the obtained data indicated that conditioned media collected from ADSCs improved morphology and morphometric characteristics of PC12 cells that were damaged by H_2O_2 exposure.

Nowadays, stem cell-based therapeutic methods have been known as a promising treating strategy for several types of human life-threatening conditions including liver diseases, cancer and central nervous system (CNS) disorders (17-19). Within the context of the nervous system, due to the limited capacity of the CNS to self-repair, CNS related diseases particularly neurodegenerative disorders have become one of the possible target applications for stem cell-based therapies (20).

A plethora of studies have indicated that oxidative stress induced by H_2O_2 elicits apoptotic death in PC12 cells via production of a large amount of ROS and disruption of the antioxidant defense system (21-23). In line with those previous observations, our results from the current study clearly indicate that exposure to

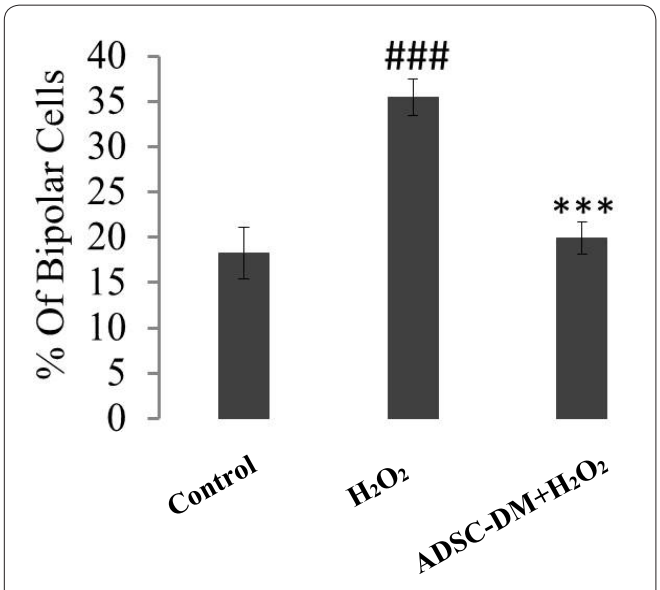

Figure 7. Effect of ADSC-CM on percentage of bipolar cells in the presence of H_2O_2 which is calculated by counting of bipolar cells in each field. *** P<0.001 significant different from H_2O_2 groups; ### P<0.001 significant different from control group.

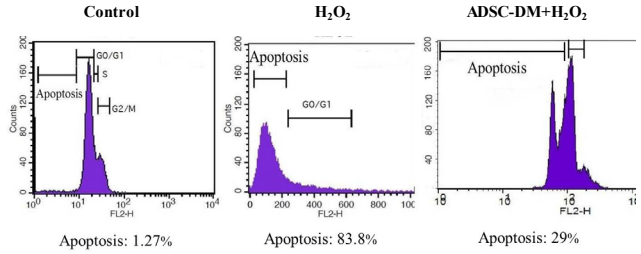

Figure 8. The level of apoptosis was determined by flow cytometric analysis of DNA content.

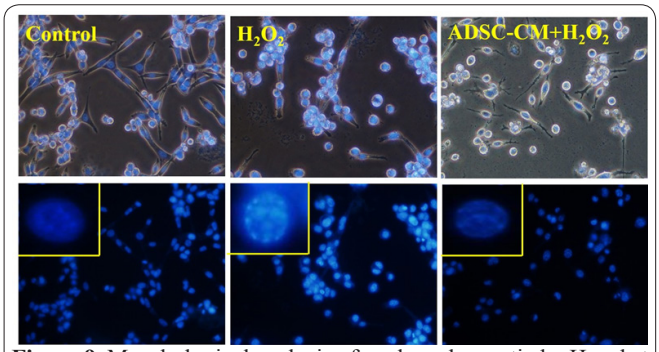

Figure 9. Morphological analysis of nuclear chromatin by Hoechst 33342. PC12 cells were treated with H_2O_2 in the absence or presence of ADSC-CM.

H_2O_2 significantly increased the number of dead cells in PC12 cells, whilst treatment of these cells with ADSC-CM significantly decreased the percentage of dead cells. Furthermore, our data revealed that morphometric properties of stressed cells improved after treatment with conditioned media obtained from ADSCs. Increase in neurite length and decrease in cell body area was vividly demonstrated in the presence of ADSC-CM. In line with our study, it has been reported by Hao and colleagues that treatment with ADSC-CM following glutamate excitotoxicity protects cortical neurons by suppression of neuronal cell apoptosis as well as by promotion of nerve regeneration and repair. They also indicated that the number of growth associated protein-43 positive neurites significantly increased in ADSC-CM treated cells (24). Similarly, morphometric analysis in our study confirmed the growth and differentiation of neurites in PC12 cells. In a literature review by Prockop, it has been shown that the healing effects of mesenchymal stem cells is not only due to their intrinsic 'stemness' but is also because of their ability to produce and secrete a vast profile of trophic agents including neuro-regulatory peptides, cytokines, neurotrophin factors and chemokines which have neuroprotective effects (25). Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), basic fibroblast growth factor (bFGF), vascular necrosis factor-α (TNF-α) and interleukins (ILs) 6, 7, 8 and 11 are amongst growth factors and interlukins secreted by mesenchymal stem cells including ADSCs (19, 26, 27). At least, 7% of molecules secreted by ADSCs have been reported to be associated with cytoskeletal structures explaining the effect of ADSC-CM on improvement of neurite outgrowth in PC12 cells exposed to H_2O_2 (19).

Additionally, it has been reported that ADSCs are able to produce and secrete vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF) and transforming growth factor-beta 1 (TGF-β1) which promote regeneration against injury in neighboring cells (28). It has been revealed that growth factors including NGF and bFGF by manipulation of intracellular signaling pathways presumable associate with the differentiation and regeneration of neural cells (29).

In conclusion, our data in line with previous studies demonstrated the striking neuroprotective effects of ADSC-CM on morphological and morphometrical properties of neuron-like PC12 cells. Additionally, the marked decrease in percentage of apoptotic cells confirmed the protective effects of conditioned media from ADSCs against oxidative stress induced by H_2O_2 that may be related to the release of trophic factors. However, the exact molecular mechanism of ADSC-CM protection against oxidative stress need to be elucidated.

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