

Effect of Melatonin on Alpha Synuclein and Autophagy in Dopaminergic Neuronal Differentiation of Adipose Mesenchymal Stem Cells

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Abstract

The current work investigated the effect of melatonin on differentiation of adipose mesenchymal stem cells (AD-MSCs) into dopamine producing cells and also its effect on autophagy process and alpha-Synuclein (α -Syn) secretion. AD-MSCs were characterized by flow cytometry and divided into 4 groups (n = 6); i) control group (AD-MSCs without any treatment), ii) M + MSCs group (MSCs treated with 1 μ M melatonin for 12 days), iii) DN group (MSCs cultured in neurobasal A medium and essential neuronal growth factors for 12 days) and iv) DN + M group (MSCs cultured in neurobasal A medium and 1 μ M melatonin for 12 days). By the end of experiments, the dopamine and α -Syn levels using ELISA, the expression of MAP-2, m-TOR and α -Syn genes at the level of mRNA and detection of autophagosomes formation using transmission electron microscope were performed. We found that the isolated cells were MSCs due to their positivity expression for CD105 and CD90 and negativity expression for CD34 and CD45. The concentration of dopamine was significantly higher and α -Syn concentration was significantly lower in DN + M group when compared to other groups ($p < 0.005$). Also, this group showed the highly expression for MAP-2 gene and less expression for m-TOR and α -Syn genes ($p < 0.005$). Moreover, there was significantly increase in autophagosomes formation in this group than other group ($p < 0.005$). It is concluded that the melatonin promotes dopaminergic neural differentiation via induction of autophagy process and reduction of α -Syn secretion which may be become one of the possible ways to treat Parkinson's disease.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease which results from formation of intracellular Lewy bodies (LBs) (abnormal protein aggregates containing alpha-synuclein (α -Syn)) and degeneration of dopaminergic (DAergic) neurons in the substantia nigra (SN) (1). Dopamine is an essential neurotransmitter in the brain, and any dysfunction in its generation results in several psychiatric and neurological disorders (2–4). Alpha-Synuclein (α -Syn) is an amphipathic small protein that is mainly expressed in the presynaptic terminals of the central nervous system and regulates the release of neurotransmitters that plays a role in synaptic transmission (5). Aggregation of this protein is known to be strongly neurotoxic and lead to cell apoptosis especially for DAergic neurons (5).

Also, autophagy is a cellular mechanism that responsible for maintaining cell survival and homeostasis through digestion of aggregated proteins and damaged organelles by lysosomes. So, inducing the autophagy process can lead to remove and destruct the misfolded α -Syn aggregation which may be used as a potential and attractive therapeutic treatment for PD complications (6–7). Mammalian target of rapamycin (mTOR) signaling has indicated to be involved in autophagy and apoptosis process regulating besides its essential role in cell development and tissue repair apoptosis. Previous studies have revealed that mTOR inhibition through some medication, including Rapamycin could upregulate the autophagy process in neurons (8).

Moreover, although the currently available therapies can ameliorate the symptoms of this disease in its early stages, they are unable to prevent the degeneration of DAergic neurons. So, replacement of the damaged DAergic neurons with a fresh DAergic neurons derived from mesenchymal stem cells (MSCs) can provide an effective treatment for this disease because of their multilineage differentiation capability without ethical problems, easy acquisition, and autologous transplantation feasibility (3, 9).

MSCs can be obtained from different tissues as adipose tissue, skin, umbilical cord, and placenta. One of the most attractive and benefit sources is adipose due to the ease of obtaining samples during operative procedure as in liposuction, rapid growth, and longevity (10). Adipose derived MSCs (AD-MSCs) are multipotent stem cells that can be differentiated into adipocytes, osteoblasts, chondrocytes, myocytes and neuronal cells such as DAergic cells. Many studies have been conducted to characterize the differentiation potential of AD-MSCs into different cell types especially into neurons that can be used in the treatment of neurological disease (11–13). Moreover, Autophagy can work as a promoter or as a suppressor in the SCs differentiation that regulated by mTOR and the PI3K/AKT pathways. mTOR participates in protein synthesis while its down regulation results in an increase rate of autophagy in the cell. (14).

Melatonin (N-acetyl-5-methoxytryptamine) was first discovered in the 1950s as a neurohormone that secreted mainly from the pineal gland in the brain and also, from extrapineal tissues like gastrointestinal tract, oocytes, neurons, endothelial cells (15–16). It has several physiological functions as antioxidant, radical scavengers that mitigate the harmful effects of free radicals, anti-inflammatory and anti-apoptotic functions (17). It was documented that melatonin protects human AD-MSCs from oxidative stress and cell death. Different studies have shown that pretreatment with melatonin can enhance the homing of BMSCs after transplantation and improves therapeutic outcomes of BMSCs in the case of transplantation in liver fibrosis (18). Also, it is suggested that melatonin may contribute significantly in regulation of osteogenic differentiation of MSCs. In addition, current evidence proved that melatonin preconditioning improves dramatically the proliferative, prosurvival, paracrine secretion of MSCs following transplantation (19). So, in the current study, we aimed to evaluate the effect of melatonin on differentiation of AD-MSCs into DAergic neurons through activation of autophagy and reduction of α -Syn secretion which may be become one of the possible ways to treat Parkinson's disease.

Material And Methods

Chemicals and agents:

Phosphate-buffered saline (PBS), DMEM-low glucose, fetal bovine serum (FBS), Pencillin- streptomycin antibiotic, neurobasal A medium and B27 supplement were purchased from Gibco, USA. Also, collagenase type I, trypsin/EDTA and melatonin were purchased from Sigma Aldrich, USA. Anti- CD45, CD34, CD 105, CD 73 were purchased from BD Bioscience, USA. dopamine and α -syn. ELISA kits were purchased from Biospes, China. RNeasy Mini Kit and RT² First Strand Kit were purchased from Qiagen, Germany.

Rat adipose-derived mesenchymal stem cells; isolation and expansion

In this study we isolated MSCs from rat adipose tissues. All animal studies were approved by the Ethical Committee of Mansoura University. Adipose tissue samples were collected from 6 Sprague Dawely rats weighing 80-100gm. All rats were euthanized by diethyl ether and the white adipose tissue of epididymis from each rat was removed under sterile conditions. The isolation procedures were done according to Hasani *et al.*, (20) as follow: The adipose tissue was washed two times with PBS. Then, the adipose tissue was digested with 0.075% collagenase type I and incubated for 45 minutes in shaking water bath at 37°C. After that, the homogenized sample was centrifuged at 1200 rpm at room temperature for 5 minutes. Finally, the supernatant was removed and the cells pellet was suspended in 10ml expansion media (DMEM-low glucose supplemented with 10% FBS and 1% Pencillin- streptomycin antibiotic and cultured in 25 cm² tissue culture flask. The cells were cultured at density of 1x10⁵/cm² in 5% CO₂ incubator (HERAcell150i, Germany) at 37°C. The medium was changed every 3 days and the cells were sub-cultivated using trypsin/EDTA at 90% confluency.

Characterization of AD-MSCs by flow cytometric analysis:

In order to evaluate the isolated cells was MSCs, the cultured cells were harvested after passage 3 and about 1×10⁶ cells was stained with FITC- or PE-conjugated antibodies against CD45, CD34, CD 105, CD 73 (BD Bioscience, USA). The cell analysis was performed using a FACS Calibur flow cytometer (Becton, Dickinson, United States) (14).

Study design:

After ensuring the isolated cells were MSCs, the cells were divided into 4 groups and each group contain 10×10⁶ cells as follow: a) control group: it was MSCs without any treatment, b) Melatonin and MSCs group (M + MSCs): it was MSCs treated with 1 μM melatonin for 12 days, c) Differentiated neurons group (DN): it was MSCs cultured in neurobasal A medium and essential neuronal growth factors for 12 days, d) Differentiated neurons and Melatonin group (DN + M): it was MSCs cultured in neurobasal A medium with essential neuronal growth factors and 1μM melatonin for 12 days.

Differentiation of AD-MSCs into dopamine producing cells:

After passage 3, the cells were trypsinized with 0.25% trypsin/ EDTA and seeded in 75 cm² tissue culture flask at density of 2 ×10⁶ cells. Twenty-four hours later, the culture medium was replaced with expansion media supplemented with 1μM melatonin for MSCs + M group. For DN group, the culture media was changed with neurobasal A medium /expansion medium (1:1) containing 2% B27 and in DN + M group, the cells were cultured in neurobasal A medium/expansion medium (1:1) containing 2% B27 combined with 1μM melatonin. The control group was cultured with expansion medium only. The media of each group was changed every 3 days and the cells were incubated for 12 days in 5% CO₂ incubator at 37°C (21).

Investigation of Dopamine and α -Synuclein concentration by Enzyme-Linked Immunosorbent Assay (ELISA):

To evaluate the effect of melatonin on differentiation of AD-MSCs into DAergic cells, the amount of dopamine and α -synuclein released in all groups were determined by using rat dopamine and α -synuclein ELISA kits. The assay was performed according to their manufacturer's instruction and the O.D was measured using ELISA reader (Tecan Infinite F50, Austria).

Gene expression by Quantitative Real time Polymerase Chain Reaction (RT-qPCR):

Total RNA was extracted from cells of all groups using RNeasy Mini Kit according to the manufacturer's instruction. The concentration of total RNA produced was measured by Nanodrop 2000 spectrophotometer (Thermofisher, Massachusetts, USA). First stand c.DNA was synthesized from 1 μ g of total RNA by reverse transcription using RT² First Strand Kit. The Quantitative Real Time PCR (RT-qPCR) analysis was performed on Step one plus real time PCR (Applied biosystem, USA) to compare gene expression for the following primers: DAergic neurons (Microtubule associated protein-2 (MAP-2)), autophagy (Mammalian Target of Rapamycin (m-TOR)) and α -synuclein (Table 1). The relative gene expression was determined using Glyceraldehyde-3-phosphate (GAPDH) as an internal control and normalized to that of the control AD-MSCs group.

In brief, amplifications were performed in a total of 25 μ L reaction volume that contains 12.5 μ L 2X SYBR green master mixes (Qiagen, Germany), 1 μ g of cDNA template, 10 pmol of each primer and to a final volume by nuclease-free water. The program was described as follow: initial denaturation at 95 $^{\circ}$ C for 15 min. followed by 40 cycles of denaturation at 94 $^{\circ}$ C for 15 sec., annealing as in Table 1 for 1 min. and extension at 72 $^{\circ}$ C for 1 min.

Transmission electron microscopy:

To study the effect of melatonin on autophagy by autophagosomes formation, the cells of all groups were harvested at the end of differentiation, fixed overnight with 3% glutaraldehyde at 4 $^{\circ}$ C and the technique was performed according to (22). Ultrathin sections (0.6–0.7 μ m thick) were made by an ultramicrotome then, cut into 600-700A $^{\circ}$ and stained with lead citrate and uranyl acetate. The autophagic structures in the cells were viewed using a transmission electron microscope (JEOL JEM-2100 at 160 KV, Electron Microscope Unit, Mansoura University, Egypt). The autophagic structures were examined in 200 cells for each group.

Statistical analysis

Statistical analysis was done using SPSS software (version 20). One-way analyses of variance (ANOVA) with Tukey posthoc test was used to find statistically significant different among the three studied groups. P value \leq 0.05 was considered statistically significant.

Results

Morphology and Proliferation of AD-MSCs:

Immediately after culturing, the cells were appeared as clumps and colonies with a heterogeneous shape. Spindle shaped cells were observed on the 5th day adherent to the tissue culture flasks. The round and flat cells were gradually decreased by changing the primary culture media every 3 days and fibroblast like cells were remained only in the tissue culture flasks (Fig. 1A). After 14 days from culturing, the proliferation rate of cells reached to 80–90% confluence and the culture consisted of a homogenous monolayer of fibroblast-like cells (Fig. 1B). Then, the cells were expanded, overlapped and sub-cultured into several passages with long- spindle shaped fibroblast like cells (Fig. 1C). After the third passage, the cells were ready to differentiate into neurons as shown in (Fig. 1D).

Confirmation of AD-MSCs by flow cytometry:

The flow cytometric examination indicated that isolated cells were strongly expressed the specific cell surface markers of MSCs such as CD90 (92.5%) and CD105 (87.2%) and negativity expressed the specific cell surface markers of hematopoietic stem cells such as CD45 (4.9%) and CD34 (1%). These results indicated that the isolated cells were MSCs and there was no contamination with cells from hematopoietic origin (Fig. 2).

Induction of differentiation towards dopaminergic neurons:

After passage 3, the effect of melatonin on dopaminergic neuron differentiation derived from AD-MSCs were examined and compared with negative control group and M + MSCs group. For M + MSCs group: within 8–10 days after treatment with 1 μ m melatonin, the morphology of AD-MSCs changed into spindle-shaped cells that appeared to be more elongated and with neural-like processes interacting with each other (Fig. 3A). For DN group: after 8–10 days from being exposed to induction medium, the cells became thinner and had shorter extension as shown in (Fig. 3B). For DN + M group: after 6 days from the induction with 1 μ m melatonin, the AD-MSCs showed a bipolar spindle shape. Their cytoplasm retracted to the nucleus and forming condensed cell bodies with cytoplasmic extensions. At the end of induction time, the cell bodies increasingly converted to spherical and refractile shape with elongated and branched cell process that showed a neuronal appearance (Fig. 3C).

Effect of melatonin on different genes expression:

Based on qPCR, the best expression level for MAP-2, m-TOR and α -Syn genes was showed in DN + M group which proved the ability of melatonin in presence of neurobasal A medium to differentiate AD-MSCs into DAergic neurons completely, increase the autophagy process and enhance the expression of α -Syn. In brief, the expression of MAP-2, the specific gene for neural differentiation, was significantly increased in DN + M group compared to other groups ($P < 0.0001$). In the other hand, the expression level of m-TOR, specific gene for increasing the autophagy process, was found to be markedly reduced in DN +

M group when compared with other group ($P < 0.0001$). Moreover, the DN + M group was recorded a highly significant decrease in α -syn expression level when compared with other groups ($P < 0.0001$). (Table 2)

Effect of melatonin on secretion of Dopamine and α -Synuclein level

The dopamine and α -Syn concentration was measured by ELISA technique to investigate the effect of melatonin on the chemical function of the DAergic neurons derived from AD-MSCs. Depending on ELISA results, the DN + M group showed the highest concentration of dopamine when compared with other group ($p = 0.00$) (Table 3). While, there was a marked reduction in α -Syn concentration in DN + M group than that in MSCs + M group and DN group ($p < 0.05$) but it was still higher than that in MSCs group (Table 4). The obtained results proved the effective role of melatonin to induce the neural differentiation and enhance the secretion of α -Syn.

Effect of Melatonin on Autophagy

According to electron microscope results, the MSCs group showed nearly no autophagosomes and served as a control group (Fig. 4A). The DN + M group exhibited the highest autophagic ultrastructures when compared with MSCs group, MSCs + M group and DN group (Fig. 4D). Moreover, there was a significant increase in autophagosomes in the MSCs + M group than that in MSCs group and DN group (Fig. 4B). Finally, the DN group exhibited increase number of autophagosomes when compared with MSCs group (Fig. 4C). So, it was proved that the melatonin was a good autophagy activator during differentiation of AD-MSCs into DAergic cells.

Discussion

As a result of lacking of effective treatment tools for PD, symptomatic relief and delay in the progression of the disease remain the choice for treatment by using pharmaceutical medications which become not effective in the advanced stages of the disease. So, the effective generation of DAergic neurons in vitro from MSCs will be one of the promising therapeutic approach in treatment of this disease by replacing the lost neurons with healthy and functional ones (3, 23).

In the present study, we isolated MSCs from adipose tissue due to their high proliferation rate that can be differentiated into different cell types such as osteoblasts, chondrocyte, adipocytes, myocytes and neuronal cells as DAergic cells. Also, they could be obtained using minimally invasive methods in comparison with other sources of MSCs, have less ethical issues, provide more homogenous stem cells with less variation in morphological features, and be ideal for the assessment of environmental changes (24). MSCs were enzymatically (collagenase) isolated and cultured from rat adipose tissues. After 5–6 days, the morphology of cells was changed from round into spindle shaped and MSCs with fibroblast shape were demonstrated from other cells by their ability to adhere to the polystyrene tissue culture flask. The cells were sub cultured into several passages at 70–80% confluence. This cell morphology has been

confirmed by many other studies which isolated MSCs from adipose tissue of different animal species as rat (20), rabbit (25), dog (26), horse (27–28), and human (29).

After that, AD-MSCs were confirmed by flow cytometry. The results showed that these cells were positive for MSCs surface markers as CD90 and CD105 with 92.5% and 87.2% respectively and negative for hematopoietic stem cells (HSCs) surface markers as CD 45 and CD34 with 4.9% and 1% respectively. This is in agreement with Hasani *et al.*, (20) who also isolated MSCs from rat adipose tissue. Several studies confirmed that the AD-MSCs showed high positivity for MSC markers as CD73, CD90, and CD105 and integrin markers as CD29 and CD44 but showed negativity for HSCs as CD45 or an endothelial cell marker as CD31 (30–31). There are some confirmed properties for MSCs that are suggested by the international society for cellular therapy including: plastic adherence, a specific set of cell surface markers (CD73, CD90, CD105) and absence of CD14, CD34, CD45, and human leucocyte antigen-DR (32). The isolated cells are agreed with these criteria so these cells are AD-MSCs.

Different studies reported the ability of MSCs derived from different sources to differentiate into DAergic neurons. Khademizadeh *et al.*, (33) who proved that the human AD-MSCs were able to differentiate into DAergic neurons in presence of cocktail of differentiating factors (FGF-2, FGF-8, and Sonic HedgeHog (SHH)) in neurobasal medium and B27 supplement. Also, Urrutia and his colleagues who compared the MSCs capacity from different sources to the neural differentiation in the presence of differentiation factor and retinoic acid in alpha-MEM, they documented that the most applicable source of producing DAergic neurons was adipose tissue (34). Moreover, Thangnipon *et al.* (35) proved that amniotic fluid MSCs were differentiated into cholinergic neurons by using BMP-9 and N-benzylcinamide.

However, the potential role of melatonin in inducing neuronal differentiation in MSCs has not been reported. Several studies have shown that melatonin is a neuroprotective agent that induces neurogenesis in embryos. Melatonin combined with physical exercise potentiates adult rat hippocampus neurogenesis by enhancing cell survival (36), and administration of melatonin before and during sleep deprivation increases the number of neural precursor cells in adult rats (37). It has been reported that melatonin can induce neuronal differentiation in PC12 cells and induced pluripotent stem cells (38).

Our differentiation results showed that the ability of melatonin to differentiate the AD-MSCs into cells that appeared to be more elongated and with neural-like processes interacting with each other in MSCs + M group. Also, the DN + M group showed completely differentiated DAergic cells after 12 days from induction with melatonin and neurobasal media with essential growth factors. On the other hand, there was no completely differentiation in DN group, which proved that melatonin can induce AD-MSCs into DAergic neurons in presence of neurobasal media with essential growth factors.

These results are also proved by qPCR which indicated that in DN + M group, there was a marked expression of MAP-2 which is a specific marker for neurons and implicated in microtubule synthesis and stabilization by crosslinking with intermediate filaments which involved in stabilizing the dendritic shape during neuron development (39) while, in other treated groups, the expression level of this genes were reduced. Moreover, dopamine ELISA results were in the same line of these results where there was a

significant increase in dopamine concentration in DN + M group but it was declined in other groups. All above results are indicated that there was complete differentiated occurred in DN + M group in contrast to other treated groups. Our results are in agree with Phonchai *et al.*, (21) who documented that the melatonin has proliferative effects and differentiation abilities to induce the DAergic neuronal differentiation of human amniotic fluid MSCs.

Autophagy is shown to be crucial in many processes engaged in stem cells differentiation like digestion of redundant organelles and ubiquitinated proteins, protecting cells from DNA damages, preservation of ATP levels by recycling the metabolites of which their biosynthesis is highly energy consuming (40), surviving in nutritional stress conditions and providing nutrients required in activation of stem cells (41).

Different studies documented that the autophagy process is activated during differentiation of embryonic stem cells (ESCs), adult stem cells (ASCs), hematopoietic stem cells (HSCs), MSCs, and neuronal stem cells (NSCs). In 2018, Hasani, and his teamwork documented that there was downregulation for m-TOR gene, the main negatively regulator of autophagy process, during DAergic differentiation derived from human AD-MSCs. Also, other studies showed that there was a decline in mTOR during differentiation of MSCs using beta mercaptoethanol (42) and osteogenic differentiation (43). Currently, there was an evidence for the ability of melatonin to activate the autophagy during neural differentiation through reducing the expression of m-TOR level (44). These findings are consistent with our results which indicated that there was a significantly reduction in m-TOR gene expression level in DN + M group compared to other groups. This mean that the melatonin can activate the autophagy process during differentiation of AD-MSCs into DAergic cells.

Moreover, Autophagy is a highly conserved intracellular catabolic progress, which eliminates damaged organelles and long-lived proteins by isolating these cytoplasmic components in autophagosomes and delivering them to lysosomes for degradation. The results of transmission electron microscope which used to observe the autophagosome formation and analyze both the qualitative and quantitative change of autophagy, showed that there was a markedly increase in formation of autophagosome in DN + M group in contrast to other treated groups. These results are agreed with Liu *et al.*, (45).

The accumulation of misfolded α -Syn may be addressed as the most critical cause of cellular toxicity in PD pathology that finally led to the cell apoptosis (46). It has been recognized that malfunction of the natural destructive process, such as autophagy, may lead to the accumulation of toxic proteins (47). Hence, increasing autophagy may be pursued as a potential and attractive therapeutic approach to reduce PD complications by intracellular degradation of α -Syn (6). As a result of the ability of melatonin to increase the autophagy during neural differentiation (44), it also can reduce the secretion of α -Syn to remove its aggregation. based on gene expression and ELISA results in our study, there was a marked reduction in α -Syn level in DN + M group when compared with other groups.

In conclusion, our study showed that the effective role of melatonin on differentiation of MSCs derived from adipose tissue (AD-MSCs) into DAergic cells and also its effect on how it could modulate or enhance the autophagy process of the differentiated cells in parallel with decreasing the secretion of α -

Syn protein from the differentiated cells which may be an alternative way of cell replacement therapy to provide a perfect treatment for Parkinson's disease.

Declarations

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgments

Not applicable

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Tables

Table 1: List of primer sequence:

Primer	Sequence	Annealing Temp.
MAP-2 forward	5'-CAAACGTCATTACTTTACAACCTTGA-3'	56 °C
MAP-2 reverse	5'-CAGCTGCCTCTGTGAGTGAG-3'	
m-TOR forward	5'-GCAATGGGCACGAGTTTGTGTT-3'	55 °C
m-TOR reverse	5'-AGTGTGTTCCACCAGGCCAAA-3'	
α -synuclein forward	5'-TGCTGTGGATATTGTTGTGG-3'	52 °C
α -synuclein reverse	5'-AGGTGCATAGTCTCATGCTC-3'	
GAPDH forward	5'-TATCGGACGCCTGGTTAC-3'	55 °C
GAPDH reverse	5'-CTGTGCCGTTGAACTTGC-3'	

Table (2): Effect of melatonin on different genes expression

Genes	MSCs group	MSCs+M group	DN group	DN+M group
MAP2 (mean \pm SD)	1.0	2.40 \pm 0.49 ^{acd}	5.67 \pm 0.41 ^{abd}	12.91 \pm 0.15 ^{abc}
m-TOR (mean \pm SD)	1.0	0.55 \pm 0.07 ^{acd}	0.85 \pm 0.05 ^{abd}	0.21 \pm 0.03 ^{abc}
α -Syn (mean \pm SD)	1.0	1.13 \pm 0.02 ^{acd}	1.77 \pm 0.50 ^{abd}	0.44 \pm 0.08 ^{abc}

Significant difference compared to corresponding ^aMSCs group, ^bMSCs+M group, ^c DN group and ^d DN+M group by one-way analysis of variance (ANOVA) followed by *posthoc* multiple comparisons (LSD) at $p \leq 0.05$.

Table (3): Effect of melatonin on Dopamine secretion

Groups	Dopamine Conc. (ng/L)
MSCs group (mean \pm SD)	1.67 \pm 0.29
MSCs+M group (mean \pm SD)	11.22 \pm 0.22 ^{acd}
DN group (mean \pm SD)	14.30 \pm 0.26 ^{abd}
DN+M group (mean \pm SD)	19.67 \pm 0.38 ^{abc}

Significant difference compared to corresponding ^aMSCs group, ^bMSCs+M group, ^c DN group and ^d DN+M group by one-way analysis of variance (ANOVA) followed by *posthoc* multiple comparisons (LSD) at $p \leq 0.05$.

Table (4): Effect of melatonin on α -synuclein secretion

Groups	α-synuclein Conc. (pg/ml)
MSCs group (mean \pm SD)	0.08 \pm 0.14
MSCs+M group (mean \pm SD)	3.13 \pm 0.31 ^{acd}
DN group (mean \pm SD)	5.56 \pm 0.90 ^{abd}
DN+M group (mean \pm SD)	1.15 \pm 0.07 ^{abc}

Significant difference compared to corresponding ^aMSCs group, ^bMSCs+M group, ^c DN group and ^d DN+M group by one-way analysis of variance (ANOVA) followed by *posthoc* multiple comparisons (LSD) at $p \leq 0.05$.

Figures

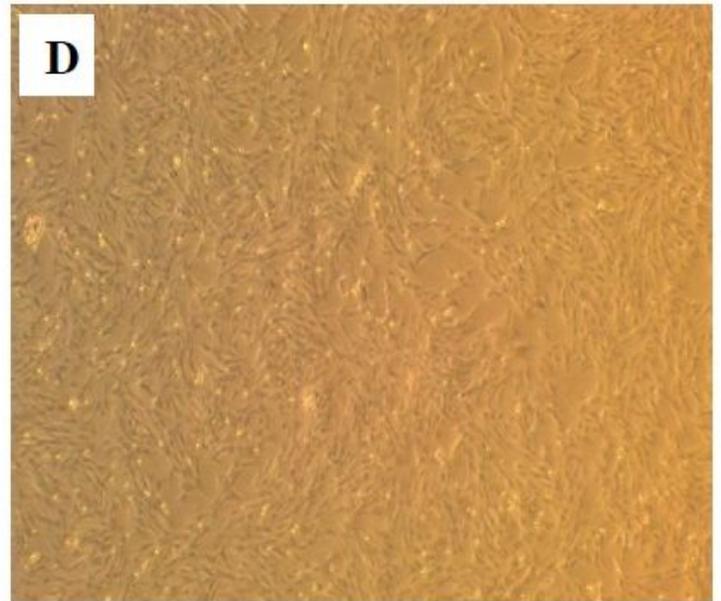
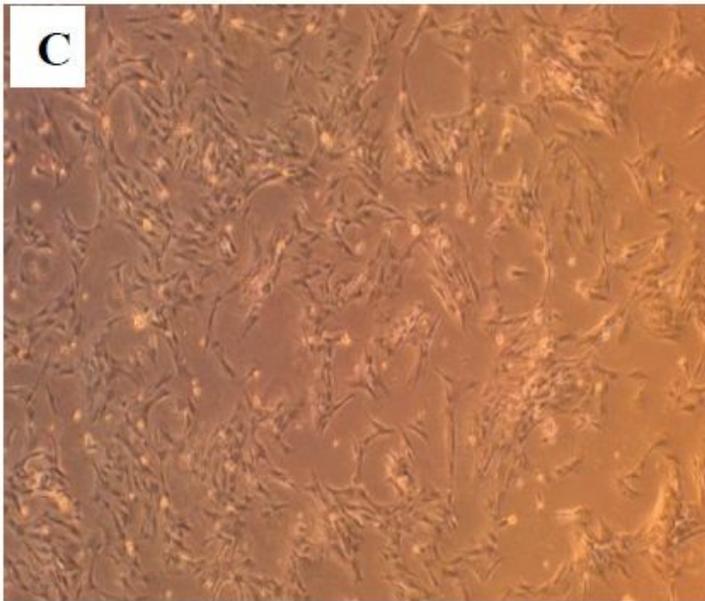
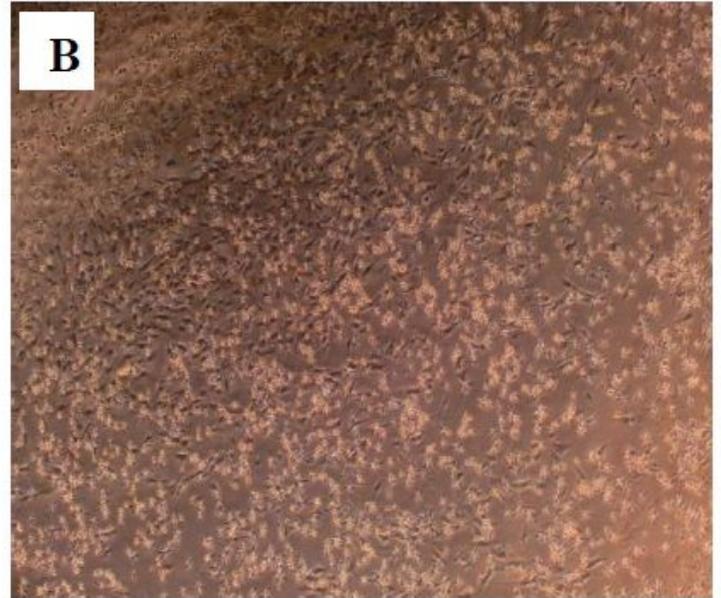
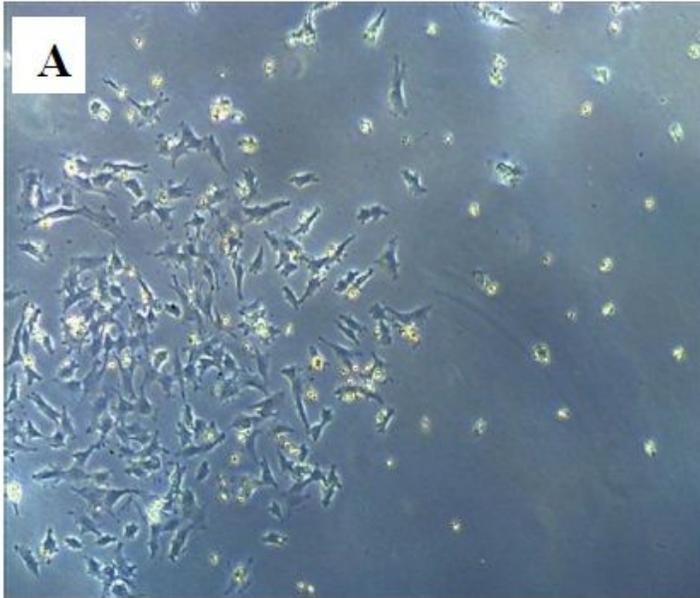


Figure 1

Morphological changes in AD-MSCs during the culture. **A:** The shape of AD-MSCs after 5 days from isolation, **B:** Adherent AD-MSCs in the first passage. **C:** Adherent AD-MSCs in the second passage. **D:** Adherent AD-MSCs in the third passage.

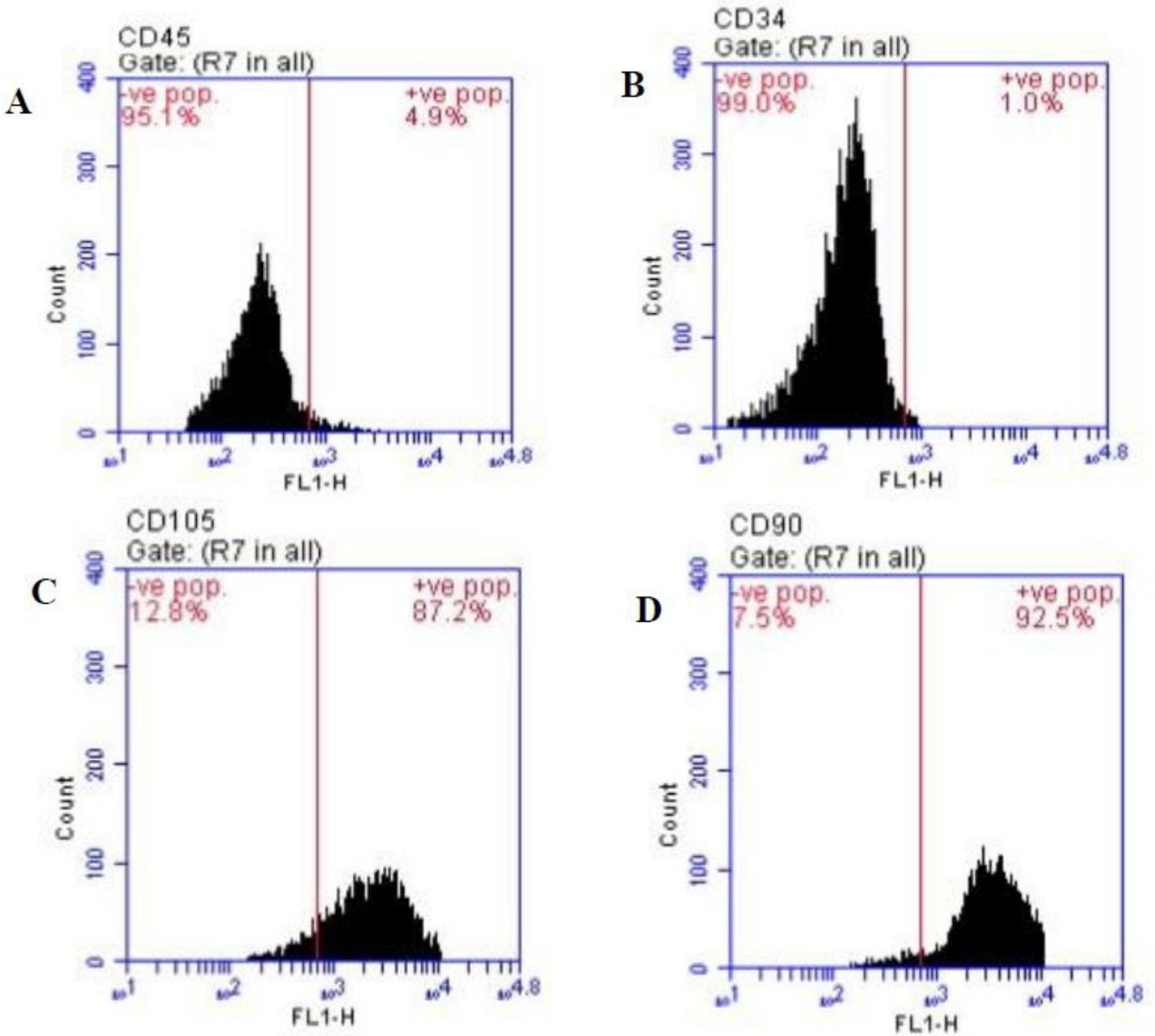


Figure 2

Flow cytometry analysis. AD-MSCs were isolated from adipose tissue and analyzed after three cell passages. (A and B) negative for hematopoietic markers CD45 and CD34, (C and D) Isolated MSCs were positive for the MSCs markers CD90 and CD105. MSC, Mesenchymal stem cells; CD, cluster of differentiation.

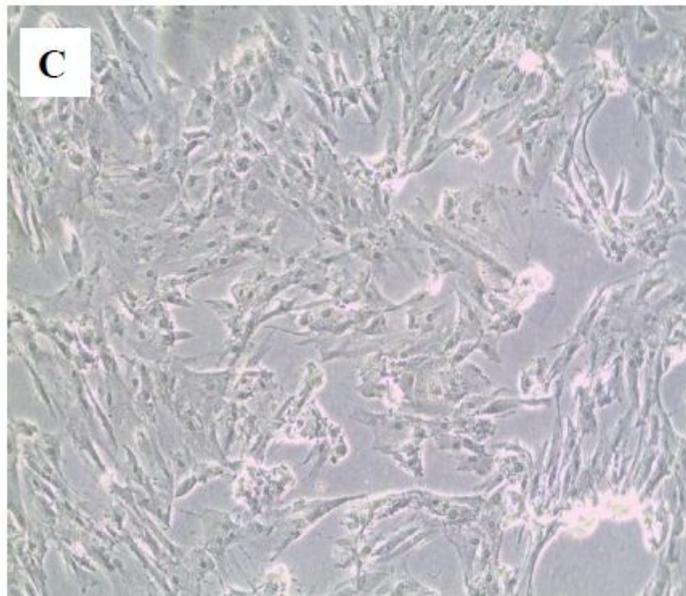
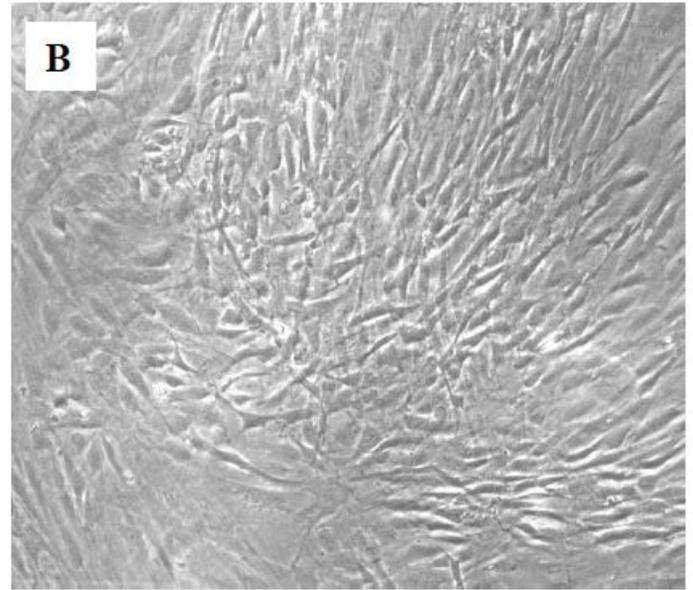
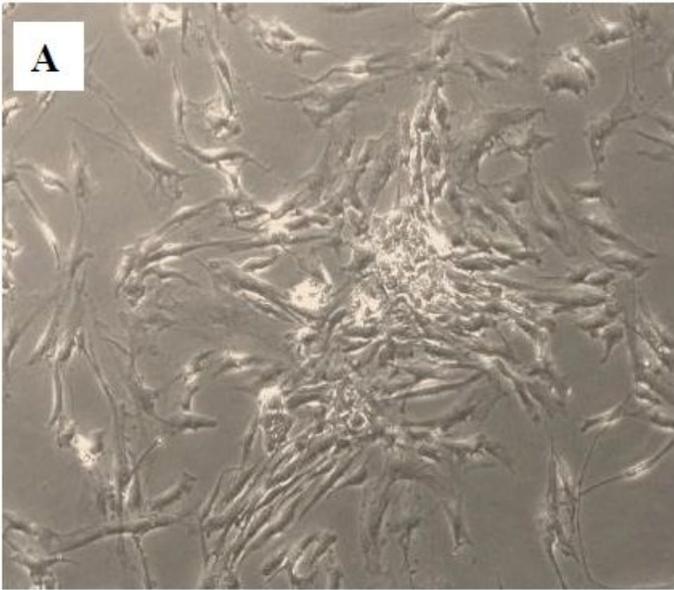


Figure 3

Effect of melatonin on differentiation of AD-MSCs into DAergic neurons. **A:** The cells overlapped and touching each other with neural-like processes after 12 days from induction with melatonin in M+MSCs group. **B:** The cells overlapped and touching each other with shorter extension after 12 days from differentiation in DN group. **C:** The cells became more elongated with branched cell processes after 12 days from differentiation in DN+M group.

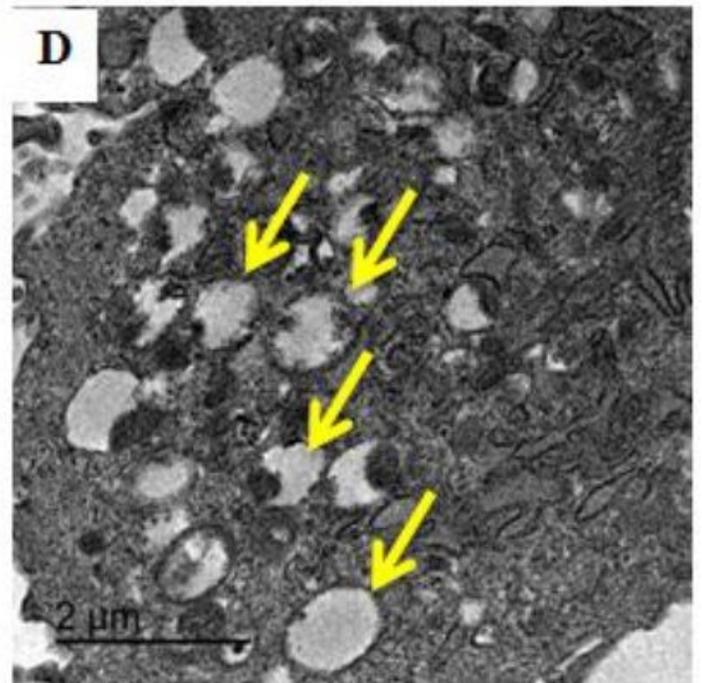
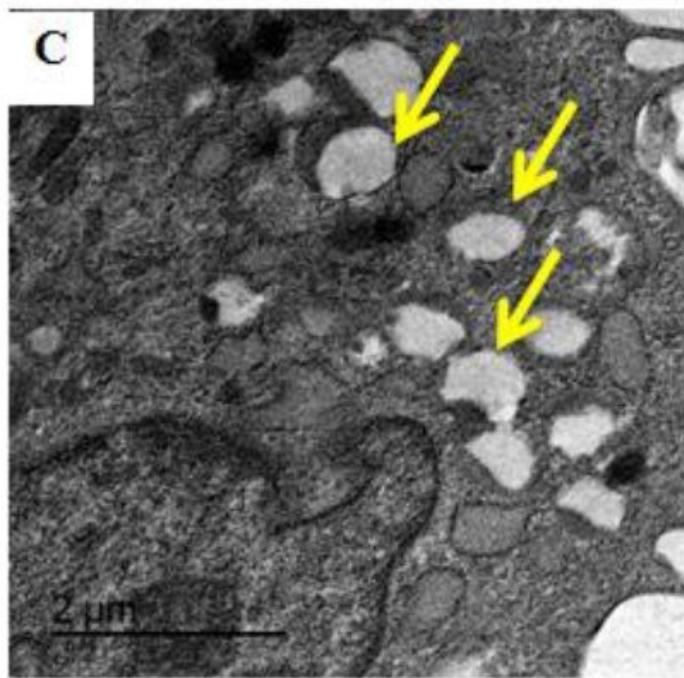
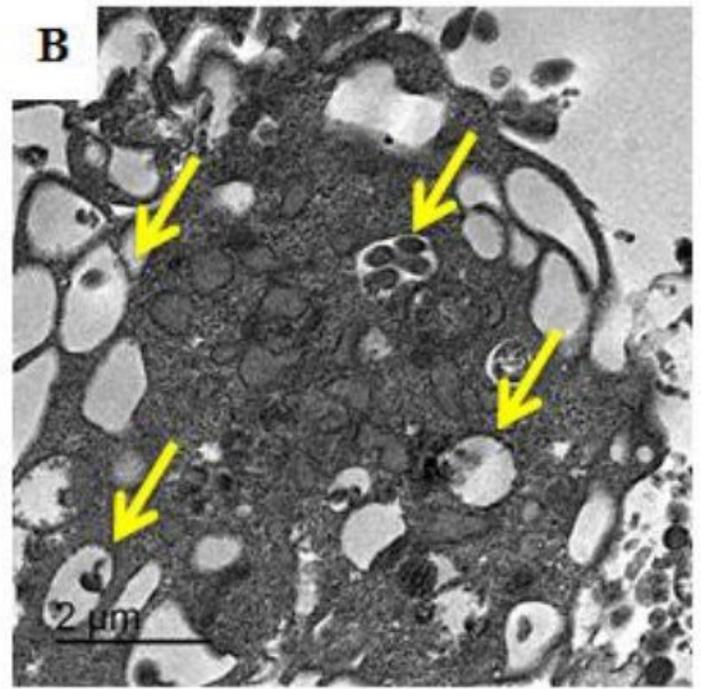
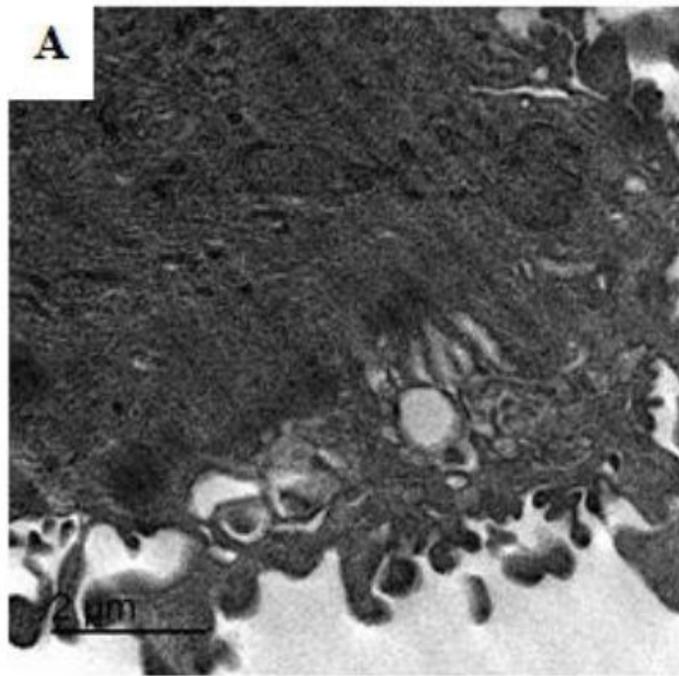


Figure 4

Electron micrographs of the autophagic ultrastructures. A: The autophagic ultrastructures in MSCs group. B: The autophagic ultrastructures in MSCs+M group. C: The autophagic ultrastructures in DN group. D: The autophagic ultrastructures in DN+M group.