Reports of Biochemistry & Molecular Biology
Vol.12, No.1, Apr 2023

Original article

www.RBMB.net

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

Hadeer Ahmed Mohamed Ibrahim[1], Abdelaziz Mohamed Hussein*[2], Mahmoud Gabr[1], Rasha Aly El-Saeed[3], Omar Abd-Alhakem Ammar[4], Ahmed Abdulatif Hassan Mosa[5], Abdel-Aziz Fatouh Abdel-Aziz[3]

Abstract

Background: The current work investigated the effect of melatonin on differentiation of adipose mesenchymal stem cells (AD-MSCs) into dopamine producing cells and its effect on autophagy process and alpha-Synuclein (α-Syn) secretion.

Methods: AD-MSCs were characterized by flow cytometry and divided into 4 groups; i) control group (AD-MSCs without any treatment), ii) M+MSCs group (MSCs treated with 1 µM 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 1µM 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.

Results: 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 another group ($P< 0.005$).

Conclusions: It is concluded that the melatonin promotes the differentiation of rat AD-MSCs into dopaminergic cells via induction of autophagy process and reduction of α-Syn secretion.

Keywords: Alpha synuclein, Autophagy, Dopaminergic cells, Melatonin, Mesenchymal stem cells.

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

1: Urology and Nephrology Center, Mansoura University, Mansoura, Egypt.
2: Department of Medical Physiology, Faculty of Medicine, Mansoura University, Mansoura, Egypt.
3: Division of Biochemistry, Chemistry Department, Faculty of Sciences, Mansoura University, Mansoura, Egypt.
4: Basic Science Department, Delta University for Sciences and Technology, Gamasa, Egypt.
5: Department of Neurology, Faculty of Medicine, Delta University for Sciences and Technology, Gamasa, Egypt.
***Corresponding author: Abdelaziz Mohamed Hussein; Tel: +20 1002421140; E-mail: menhag@mans.edu.eg.**
Received: 20 Sep, 2022; Accepted: 4 Feb, 2023

[DOI: 10.61186/rbmb.12.1.13]

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 (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).

Mesenchymal stem cells (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 specially 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 stem cells (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 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 (17). Also, it is suggested that melatonin may contribute significantly to regulation of osteogenic differentiation of MSCs. In addition, current evidence proved that melatonin preconditioning improves dramatically the proliferative, pro-survival, paracrine secretion of MSCs following transplantation (18). 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.

Materials and Methods

Chemicals and agents

Phosphate-buffered saline (PBS), DMEM-low glucose, fetal bovine serum (FBS), Penicillin-streptomycin antibiotic, neurobasal A medium and B27 supplement were purchased from Gibco, Grand Island, USA. Also, collagenase type I, trypsin/EDTA and melatonin were purchased from Sigma Aldrich, St. Louis, USA. FITC- or PE-conjugated antibodies against CD45, CD34, CD105, CD90 were purchased from BD Bioscience, San Jose, CA, USA. Rat Dopamine and α-syn. (7.5 ng/L-90 ng/L) ELISA kits were purchased from Biospes, Chongqing, China. RNeasy Mini Kit

[DOI: 10.61186/rbmb.12.1.13]

and RT^2 First Strand Kit were purchased from Qiagen, Germany.

Rat adipose-derived mesenchymal stem cells; isolation and expansion

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 six Sprague Dawley rats weighing 80-100 g. 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*., (19) 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 ºC. After that, the homogenized sample was centrifuged at 505 *g* at room temperature for 5 minutes. Finally, the supernatant was removed, and the cells pellet was suspended in 10 ml expansion media (DMEM-low glucose supplemented with 10% FBS and 1% Penicillin-Streptomycin antibiotic and cultured in 25 cm^2 tissue culture flask. The cells were cultured at density of $1x10^5/cm^2$ in 5% CO_2 incubator (HERAcell150i, Germany) at 37 ºC. The medium was changed every three 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 1×10^6 cells were stained with FITC- or PE-conjugated antibodies against CD45, CD34, CD105, CD90. Argon ion laser was used to analyze the labeled cells at a wavelength of 488 nm (FACS Calibur, 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 10×10^6 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 1 µM melatonin for 12 days. All experimental protocols were approved by the ethical committee of faculty of science, Mansoura University, code # PhD.19/10/2019.

Differentiation of AD-MSCs into dopamine producing cells

After passage 3, the cells were trypsinized with 0.25% trypsin/ EDTA and seeded in 75 cm^2 tissue culture flask at density of 2×10^6 cells. Twenty-four hours later, the culture medium was replaced with expansion media supplemented with 1 µM 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 1 µM 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% CO_2 incubator at 37 ºC (20).

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 manufacture's instruction and the OD was measured using ELSA 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 manufacture's instruction. The concentration of

[DOI: 10.61186/rbmb.12.1.13]

total RNA produced was measured by Nanodrop 2000 spectrophotometer (Thermofisher, Massachusetts, USA). First stand cDNA was synthesized from 1 µg of total RNA by reverse transcription using RT^2 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-synclein (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 µL reaction volume that contains 12.5 µl 2X SYBR green master mixes (Qiagen, Germany), 1 µg 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 ºC for 15 min. followed by 40 cycles of denaturation at 94 ºC for 15 sec., annealing as in Table 1 for 1 min. and extension at 72 ºC for 1 min.

Table 1. List of primer sequence.

Primer		Sequence	Annealing Temperature (ºC)
MAP-2	**Forward**	5'-CAAACGTCATTACTTTACAACTTGA-3'	**56**
	Reverse	5'-CAGCTGCCTCTGTGAGTGAG-3'	
m-TOR	**Forward**	5'-GCAATGGGCACGAGTTTGTT-3'	**55**
	Reverse	5'-AGTGTGTTCACCAGGCCAAA-3'	
α-synclein	**Forward**	5'-TGCTGTGGATATTGTTGTGG-3'	**52**
	Reverse	5'-AGGTGCATAGTCTCATGCTC-3'	
GAPDH	**Forward**	5'-TATCGGACGCCTGGTTAC-3'	**55**
	Reverse	5'-CTGTGCCGTTGAACTTGC-3'	

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 ºC (21). Ultrathin sections (0.6-0.7 µm thick) were made by an ultramicrotome then, cut into 600-700 Aº 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 post-hoc test was used to find statistically significant different among the three studied groups. P value ≤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 5^{th} 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

[DOI: 10.61186/rbmb.12.1.13]

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).

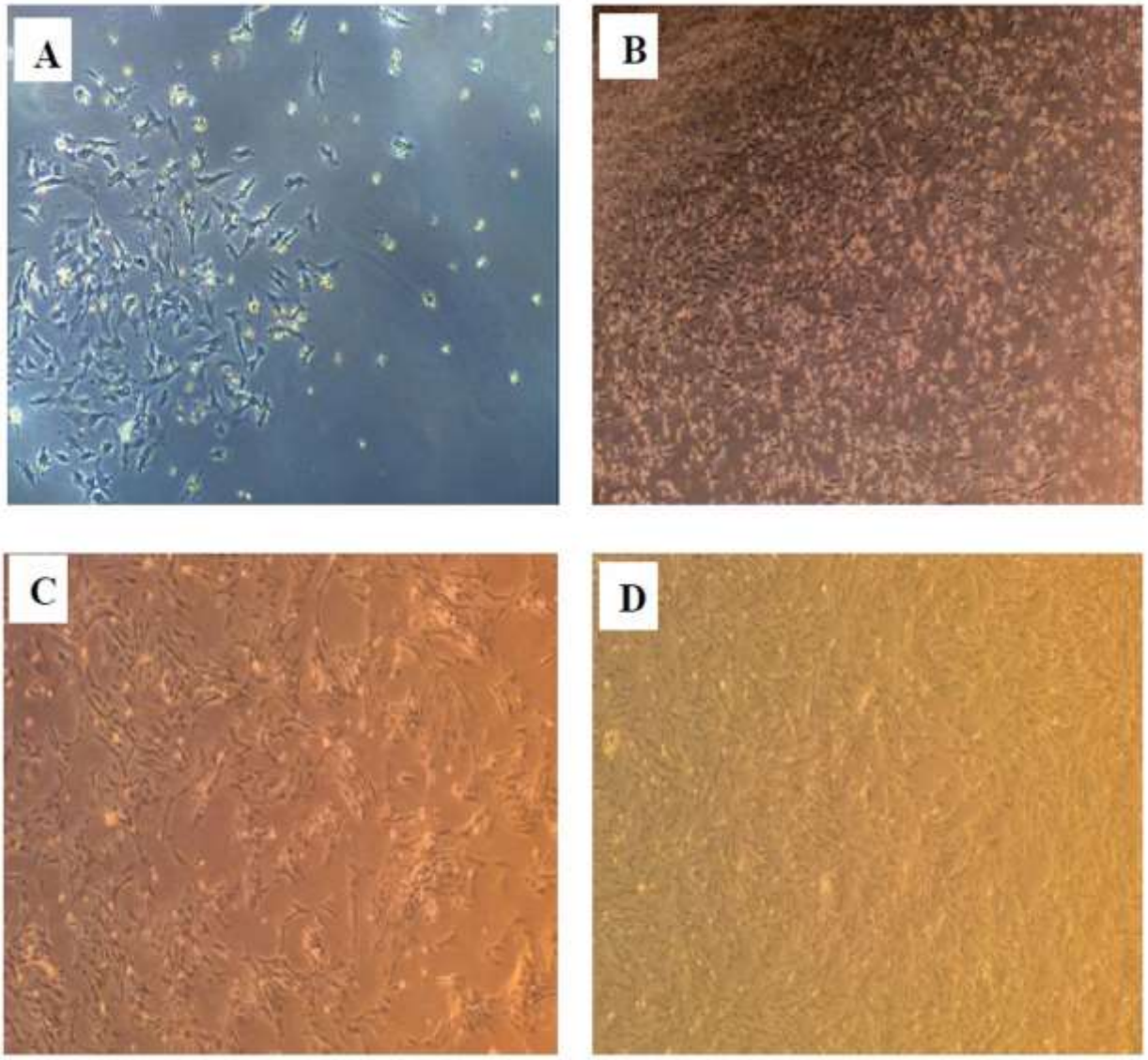

Fig. 1. Morphological changes in adipose derived-mesenchymal stem cells (AD-MSCs) during the culture. A: The shape of AD-MSCs after 5 days from isolation, B: Adherent AD-MSCs in the first passage. C: Adherent AD-MSCs in the second passage. D:

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).

[DOI: 10.61186/rbmb.12.1.13]

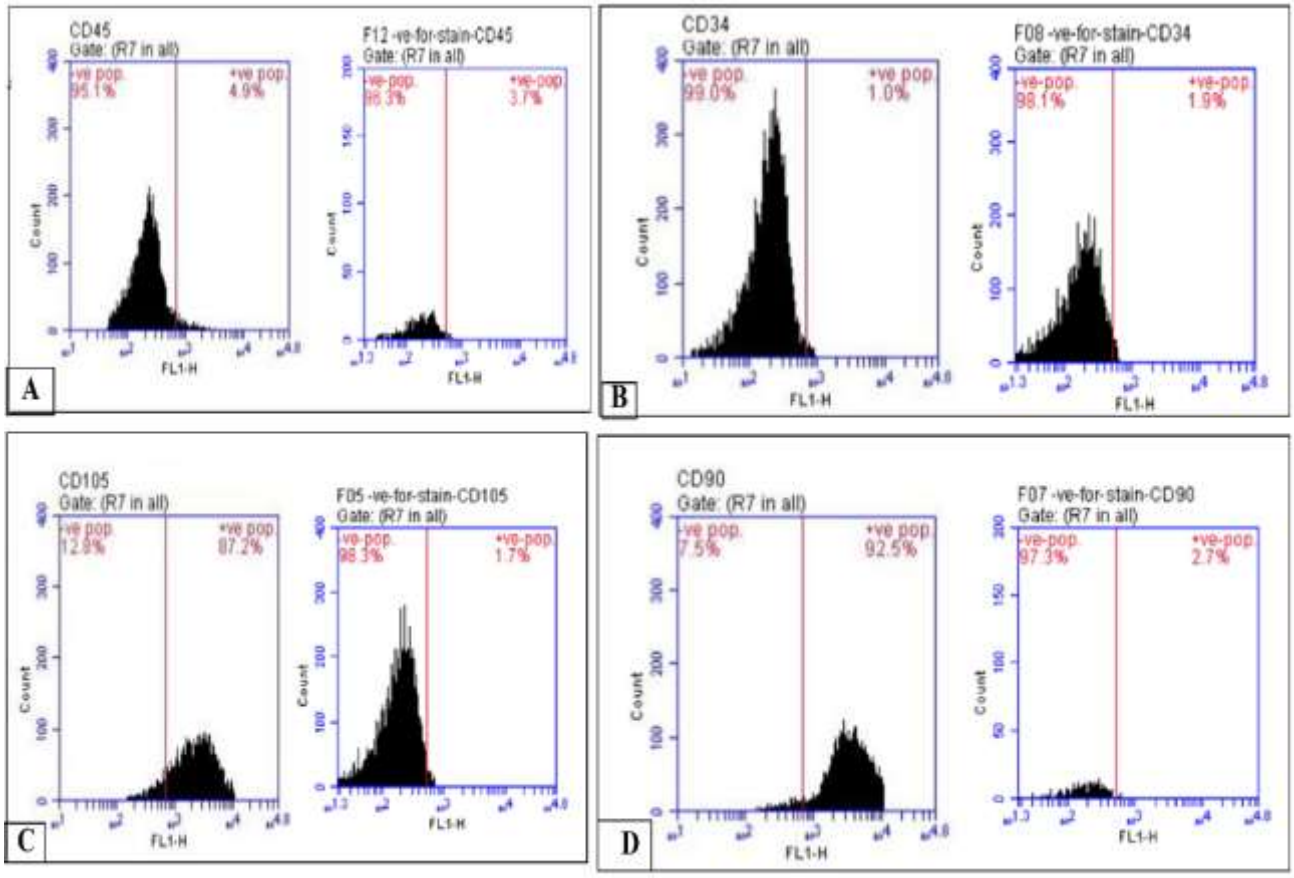

Fig. 2. Flow cytometry analysis for CD45, CD34, CD105 and CD90 surface markers with their negative control. Adipose derived-mesenchymal stem cells (AD-MSCs) were isolated from adipose tissue and analyzed after three cell passages. They are negative for hematopoietic markers CD45 and CD34 (A and B) and positive for the MSCs markers CD90 and CD105 (C and D). MSC, Mesenchymal stem cells; CD, cluster of differentiation.

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 µm 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 µm 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).

[DOI: 10.61186/rbmb.12.1.13]

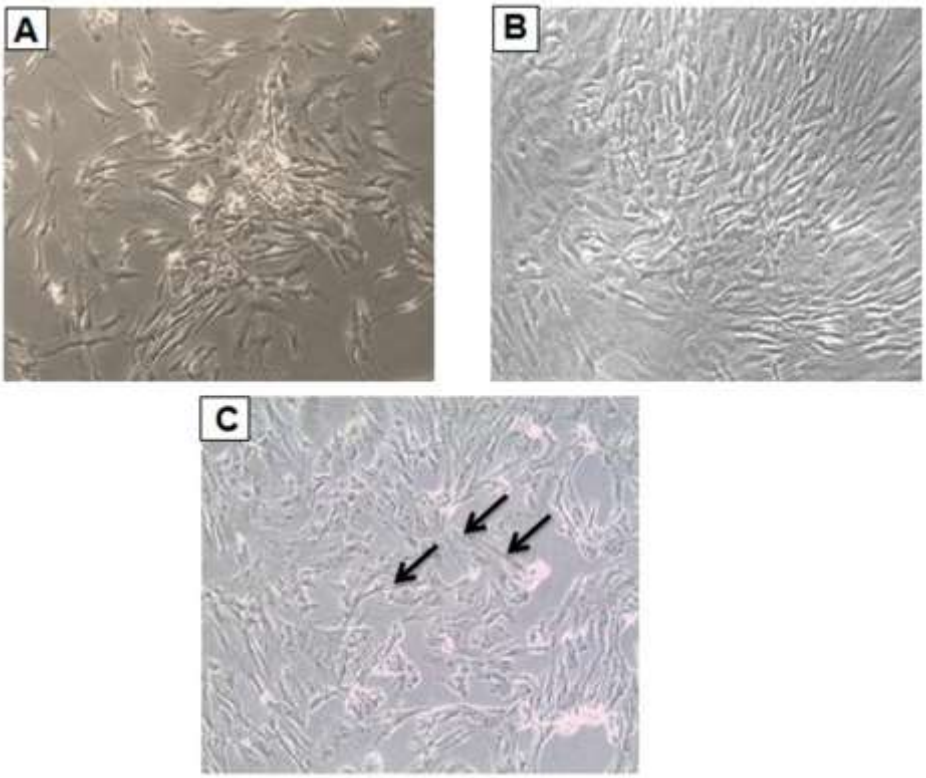

Fig. 3. Effect of melatonin on differentiation of adipose derived mesenchymal stem cells (AD-MSCs) into dopaminergic (DA)-ergic neurons. A: The cells overlapped and touching each other with neural-like processes after 12 days from induction with melatonin in M+MSCs group. B: The cells overlapped and 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. MSCs= mesenchymal stem cells, M= melatonin, DN= differentiated neurons.

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 another 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).

Table 2. Effect of melatonin on different genes expression.

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

Significant difference compared to corresponding [a]MSCs group, [b]MSCs+M group, [c] DN group and [d] DN+M group by one-way analysis of variance (ANOVA) followed by *post-hoc* multiple comparisons (LSD) at $P\leq 0.05$. MSCs= mesenchymal stem cells, M= melatonin, DN= differentiated neurons, MAP2 = Microtubule Associated Protein2; m-TOR, Mammalian target of rapamycin; α-Syn, alpha-synuclein.

[DOI: 10.61186/rbmb.12.1.13]

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. Regards to ELISA results, the DN+M group showed the highest concentration of dopamine when compared with another 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. The obtained results proved the effective role of melatonin to induce the neural differentiation and enhance the secretion of α-Syn.

Table 3. Effect of melatonin on Dopamine secretion.

Groups (mean ± SD)	***Dopamine Concentration*** (ng/L)
MSCs	1.67 ± 0.29
MSCs+M	11.22 ± 0.22[acd]
DN	14.30 ± 0.26 [abd]
DN+M	19.67 ± 0.38[abc]

Significant difference compared to corresponding [a]MSCs group, [b]MSCs+M group, [c] DN group and [d] DN+M group by one-way analysis of variance (ANOVA) followed by *post-hoc* multiple comparisons (LSD) at P≤ 0.05. MSCs= mesenchymal stem cells, M= melatonin, DN= differentiated neurons.

Table 4. Effect of melatonin on α-synclein secretion.

Groups (mean ± SD)	***α-synuclein Concentration*** (pg/ml)
MSCs	0.08 ± 0.14
MSCs+M	3.13 ± 0.31[acd]
DN	5.56 ± 0.90 [abd]
DN+M	1.15 ± 0.07 [abc]

Significant difference compared to corresponding [a]MSCs group, [b]MSCs+M group, [c] DN group and [d] DN+M group by one-way analysis of variance (ANOVA) followed by *post-hoc* multiple comparisons (LSD) at P≤ 0.05. MSCs= mesenchymal stem cells, M= melatonin, DN= differentiated neurons.

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 increased 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.

[DOI: 10.61186/rbmb.12.1.13]

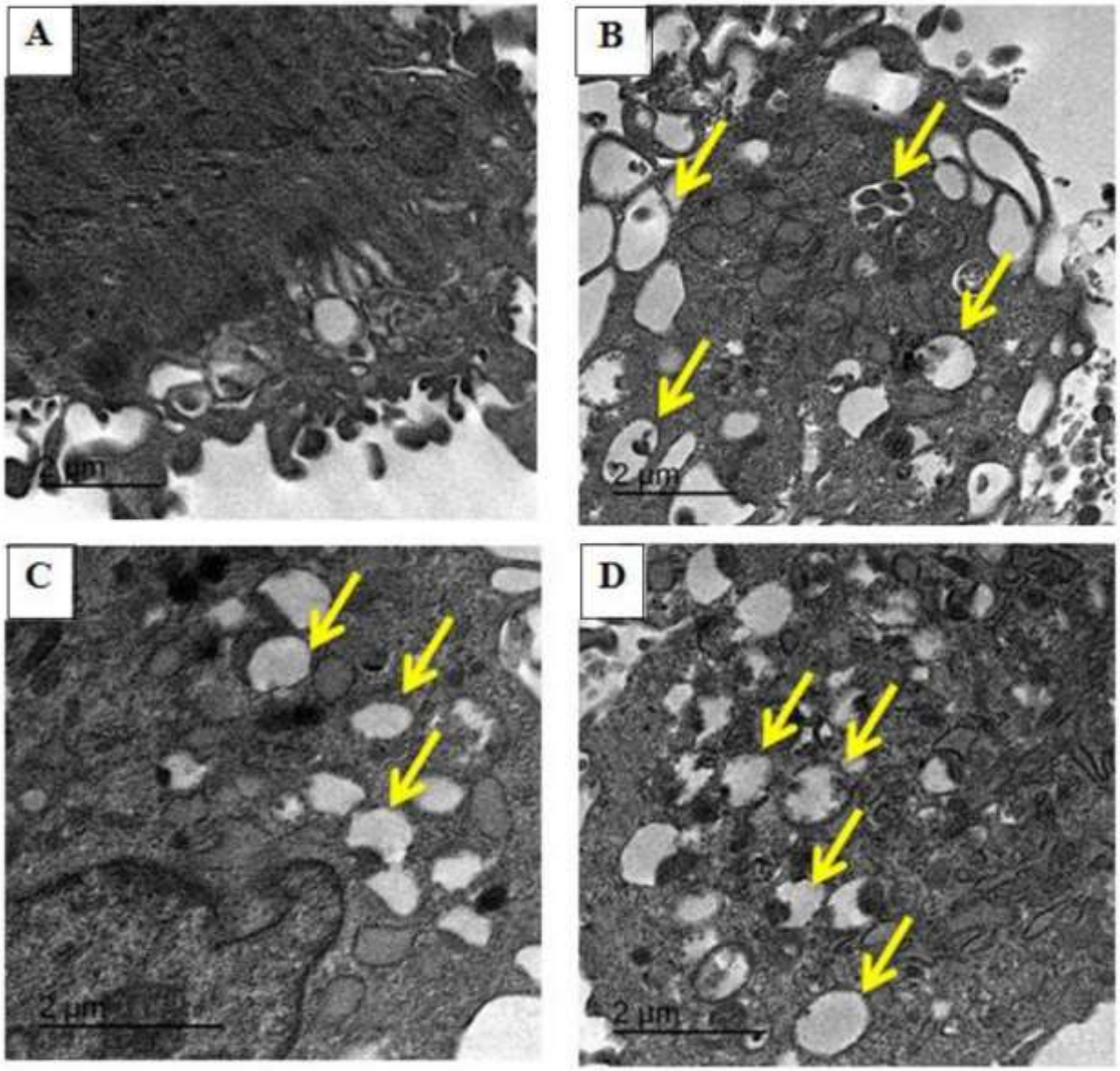

Fig. 4. Electron micrographs of the autophagic ultrastructures. A: The autophagic ultrastructures in MSCs group. B: The autophagic ultrastructures in MSCs+M group. C: The autophagic ultrastructures in DN group. D: The autophagic ultrastructures in DN+M group. MSCs= mesenchymal stem cells, M= melatonin, DN= differentiated neurons.

Discussion

As a result of lacking 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 approaches in treatment of this disease by replacing the lost neurons with healthy and functional ones (3, 22).

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

[DOI: 10.61186/rbmb.12.1.13]

assessment of environmental changes (23). The morphology of isolated cells appeared more spindle with fibroblast shaped and this is confirmed by many other studies which isolated MSCs from adipose tissue of different animal species as rat (19), rabbit (24), dog (25), horse (26, 27), and human (28).

In addition, flow cytometery confirmed that 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 agrees with Hasani *et al*., (19) 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 (29, 30). 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 (31,32). 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 not 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 was 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*., (20) who documented that the melatonin has proliferative effects and differentiation

[DOI: 10.61186/rbmb.12.1.13]

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 and osteogenic differentiation (42). Currently, there was evidence for the ability of melatonin to activate the autophagy during neural differentiation through reducing the expression of m-TOR level (43). 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 means 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*., (44).

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. It has been recognized that malfunction of the natural destructive process, such as autophagy, may lead to the accumulation of toxic proteins. 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 (43), 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.

Acknowledgements

The authors acknowledge Dr Basem Salah, director of Urology and Nephrology Center, for his support to do this work in the research facility at Urology and Nephrology center, Mansoura University.

Funding

The study is a self-funded and not funded from any funding agent elsewhere

Conflicts of Interest

The authors declare no conflict of interest.

[DOI: 10.61186/rbmb.12.1.13]

References

1. Askar MH, Hussein AM, Al-Basiony SF, Meseha RK, Metias EF, Salama MM, et al. Effects of exercise and ferulic acid on alpha synuclein and neuroprotective heat shock protein 70 in an experimental model of Parkinsonism disease. CNS & Neurol Disor-Drug Targ. 2019;18(2): 156-69.
2. Mehraein F, Sarbishegi M, Golipoor Z. Different effects of olive leaf extract on antioxidant enzyme activities in midbrain and dopaminergic neurons of Substantia Nigra in young and old rats. Histol Histopathol. 2016;31(4):425–31.
3. Alizadeh R, Bagher Z, Kamrava SK, Falah M, Hamidabadi HG, Boroujeni ME, et al. Differentiation of human mesenchymal stem cells (MSC) to dopaminergic neurons: A comparison between Wharton's Jelly and olfactory mucosa as sources of MSCs. J Chem Neuroanat. 2019;96:126-33.
4. Alizadeh R, Hassanzadeh G, Joghataei MT, Soleimani M, Moradi F, Mohammadpour S, et al. In vitro differentiation of neural stem cells derived from human olfactory bulb into dopaminergic-like neurons. Eur J Neurosci. 2017;45(6):773-84.
5. Hussein AM, Eldosoky M, El-Shafey M, El-Mesery M, Ali AN, Abbas KN, et al. Effects of Metformin on Apoptosis and Alpha Synuclein in Rat Model of Pentylenetetrazole-induced Epilepsy. Can J Physiol Pharmacol. 2019; 97(1):37-46.
6. Scrivo A, Bourdenx M, Pampliega O, Cuervo AM. Selective autophagy as a potential therapeutic target for neurodegenerative disorders. Lancet Neuro. 2018;17(9):802-815.
7. Chen HX, Liang F, Gu P, Xu PL, Xu HJ, Wang WT, et al. Exosomes derived from mesenchymal stem cells repair a Parkinson's disease model by inducing autophagy. Cell Death & Dis. 2020; 11(4):288.
8. Mostafavi H, Forouzandeh M, Bigdeli MR, Nadri S, Eskandari M. Neuroprotective Effects of Microfluidic Encapsulated Induced Conjunctival Mesenchymal Stem Cells Through Autophagy Modulation in a Parkinsonian Model Res Squ. 2021; 1-24.
9. Chung TH, Hsu SC, Wu SH, Hsiao JK, Lin CP, Yao M, et al. Dextran- coated iron oxide nanoparticle-improved therapeutic effects of human mesenchymal stem cells in a mouse model of Parkinson's disease. Nanosc. 2018;10(6): 2998-3007.
10. Alajez NM, Al-Ali D, Vishnubalaji R, Manikandan M, Alfayez M, Kassem M, et al. Which Stem Cells to Choose for Regenerative Medicine Application: Bone Marrow and Adipose Tissue Stromal Stem Cells – Similarities and Differences. J of Nat & Sci of Med. 2018;1(2):48-54
11. Moon MY, Kim HJ, Choi BY, Sohn M, Chung TN, Suh SW. Zinc Promotes Adipose-Derived Mesenchymal Stem Cell Proliferation and Differentiation towards a Neuronal Fate. Stem Cells Int. 2018; 2018:5736535
12. Chi K, Fu RH, Huang YC, Chen SY, Hsu CJ, Lin SZ, et al. Adipose-derived Stem Cells Stimulated with n-Butylidenephthalide Exhibit Therapeutic Effects in a Mouse Model of Parkinson's Disease. Cell Transplant. 2018;27(3): 456-470.
13. Muñoz MF, Argüelles S, Medina R, Cano M, Ayala A. Adipose-derived stem cells decreased microglia activation and protected dopaminergic loss in rat lipopolysaccharide model. J Cell Physiol. 2019;234(8):13762-13772.
14. Hasani S, Boroujeni ME, Aliaghaei A, Baghai K, Rostami A. Dopaminergic induction of human adipose-derived mesenchymal stem cells is accompanied by transcriptional activation of autophagy. Cell Biol Int. 2018;42(12):1688-1694.
15. Lerner AB, Case JD, Mori W, Wright MR. Melatonin in peripheral nerve. Nat. 1959; 183: 1821.
16. Govindasamy N, Chung Chok K, Ying Ng P, Yian Koh R, Moi Chye S. Melatonin Induced Schwann Cell Proliferation and Dedifferentiation Through NF-κB, FAK-Dependent but Src-Independent Pathways. Rep Biochem Mol Biol. 2022;11(1):63-73.
17. Mortezaee K, Khanlarkhani N, Sabbaghziarani F, Nekoonam S, Majidpoor J, Hosseini A, et al. Preconditioning with melatonin improves therapeutic outcomes of bone marrow-derived Mesenchymal stem cells in targeting liver fibrosis induced by CCl4. Cell Tissue Res. 2017;369(2): 303-312.

[DOI: 10.61186/rbmb.12.1.13]

18. Zhao L, Hu C, Zhang P, Jiang H, Chen J. Melatonin preconditioning is an effective strategy for mesenchymal stem cell-based therapy for kidney disease. J Cell Mol Med. 2020;24(1):25-33.
19. Mohammadi-Mahdiabadi-Hasani MH, Nabiuni M, Parivar K, Yari S, Sahebi AR, Miyan J. The Effects of Embryonic Cerebrospinal Fluid on The Viability and Neuronal Differentiation of Adipose Tissue-Derived Stem Cells in Wistar Rats. Cell J. 2020;22(2):245-252.
20. Phonchai R, Phermthai T, Kitiyanant N, Suwanjang W, Kotchabhakdi N, Chetsawang B. Potential effects and molecular mechanisms of melatonin on the dopaminergic neuronal differentiation of human amniotic fluid mesenchymal stem cells. Neurochem Int. 2019;124:82-93.
21. Morris JK. A formaldehyde glutaraldehyde fixative of high osmolality for use in electron microscopy. J of Cell Biol. 1965; 27(2):1A-149A.
22. Chia SJ, Tan EK, Chao YX. Historical Perspective: Models of Parkinson's Disease. Int J Mol Sci. 2020; 21(7):2464
23. Tirpáková M, Vašíček J, Svoradová A, Baláži A, Tomka M, Bauer M, et al. Phenotypical Characterization and Neurogenic Differentiation of Rabbit Adipose Tissue-Derived Mesenchymal Stem Cells. Gen. 2021;12(3): 431.
24. Zomer HD, Roballo KC, Lessa TB, Bressan FF, Gonçalves NN, Meirelles FV, et al. Distinct features of rabbit and human adipose-derived mesenchymal stem cells: Implications for biotechnology and translational research. Stem Cells Cloning Adv. Appl. 2018;11:43-54.
25. Zhan XS, El-Ashram S, Luo DZ, Luo HN, Wang BY, Chen SF, et al. A Comparative Study of Biological Characteristics and Transcriptome Profiles of Mesenchymal Stem Cells from Different Canine Tissues. Int J Mol Sci. 2019;20(6):1485.
26. Bourebaba L, Michalak I, Baouche M, Kucharczyk K, Marycz K. Cladophora glomerata methanolic extract promotes chondrogenic gene expression and cartilage phenotype differentiation in equine adipose-derived mesenchymal stromal stem cells affected by metabolic syndrome. Stem Cell Res Ther. 2019;10(1): 392.
27. Elashry MI, Gegnaw ST, Klymiuk MC, Wenisch S, Arnhold S. Influence of mechanical fluid shear stress on the osteogenic differentiation protocols for Equine adipose tissue-derived mesenchymal stem cells. Acta Histochem. 2019;121(3):344-353.
28. Machado AK, Homrich SG, Rodrigues CCR, Azzolin VF, Duarte MMMF, Pillar DM, et al. Human adipose-derived stem cells obtained from lipoaspirates are highly susceptible to hydrogen peroxide mediated cytogenotoxicity. Arch Biosci Health. 2019;1(1):11-28.
29. Bajek A, Gurtowska N, Olkowska J, Kazmierski L, Maj M, Drewa T. Adipose-Derived Stem Cells as a Tool in Cell-Based Therapies. Arch Immunol Ther Exp (Warsz). 2016;64(6):443-454.
30. Mo M, Wang S, Zhou Y, Li H, Wu Y. Mesenchymal stem cell subpopulations: phenotype, property and therapeutic potential. Cell Mol Life Sci. 2016;73(17):3311-21.
31. Hernández R, Jiménez-Luna C, Perales-Adán J, Perazzoli G, Melguizo C, Prados J. Differentiation of Human Mesenchymal Stem Cells towards Neuronal Lineage: Clinical Trials in Nervous System Disorders. Biomol Ther (Seoul). 2020;28(1):34-44.
32. Moazen B, Zarrinhaghighi A, Nejatollahi F. Selection and Evaluation of Specific Single Chain Antibodies against CD90, a Marker for Mesenchymal and Cancer Stem Cells. Rep Biochem Mol Biol. 2018;7(1):45-51.
33. Khademizadeh M, Messripour M, Ghasemi N, Momen Beik F, Movahedian Attar A. Differentiation of adult human mesenchymal stem cells into dopaminergic neurons. Res Pharm Sci. 2019;14(3):209-215.
34. Urrutia DN, Caviedes P, Mardones R, Minguell JJ, Vega-Letter AM, Jofre CM. Comparative study of the neural differentiation capacity of mesenchymal stromal cells from different tissue sources: An approach for their use in neural regeneration therapies. PLoS One. 2019;14(3):e0213032.
35. Thangnipon W, Puangmalai N, Suwanna N, Soi-Ampornkul R, Phonchai R, Kotchabhakdi N, et al. Potential role of N-benzylcinnamide in inducing neuronal differentiation from human

[DOI: 10.61186/rbmb.12.1.13]

amniotic fluid mesenchymal stem cells. Neurosci Lett. 2016;610:6-12.
36. Liu J, Zhou H, Fan W, Dong W, Fu S, He H, Huang F. Melatonin influences proliferation and differentiation of rat dental papilla cells in vitro and dentine formation in vivo by altering mitochondrial activity. J Pineal Res. 2013 Mar;54(2):170-8.
37. López-Armas G, Flores-Soto ME, Chaparro-Huerta V, Jave-Suarez LF, Soto-Rodríguez S, Rusanova I, et al. Prophylactic Role of Oral Melatonin Administration on Neurogenesis in Adult Balb/C Mice during REM Sleep Deprivation. Oxid Med Cell Longev. 2016;2016:2136902.
38. Shu T, Wu T, Pang M, Liu C, Wang X, Wang J, et al. Effects and mechanisms of melatonin on neural differentiation of induced pluripotent stem cells. Biochem Biophys Res Commun. 2016;474(3):566-571.
39. Yousefi B, Sanooghi D, Faghihi F, Joghataei MT, Latifi N. Evaluation of motor neuron differentiation potential of human umbilical cord blood- derived mesenchymal stem cells, in vitro. J Chem Neuroanat. 2017;81:18-26.
40. Riffelmacher T, Simon AK. Mechanistic roles of autophagy in hematopoietic differentiation. FEBS J. 2017;284(7):1008-1020.
41. Tang AH, Rando TA. Induction of autophagy supports the bioenergetic demands of quiescent muscle stem cell activation. EMBO J. 2014;33(23):2782-97.
42. Wan Y, Zhuo N, Li Y, Zhao W, Jiang D. Autophagy promotes osteogenic differentiation of human bone marrow mesenchymal stem cell derived from osteoporotic vertebrae. Biochem Biophys Res Commun. 2017;488(1):46-52.
43. Sotthibundhu A, Nopparat C, Natphopsuk S, Phuthong S, Noisa P, Govitrapong P. Combination of Melatonin and Small Molecules Improved Reprogramming Neural Cell Fates via Autophagy Activation. Neurochem Res. 2022;47(9):2580-2590.
44. Liu C, Zhou W, Li Z, Ren J, Li X, Li S, et al. Melatonin Protects Neural Stem Cells Against Tri-Ortho-Cresyl Phosphate-Induced Autophagy. Front Mol Neurosci. 2020; 13:25.

[DOI: 10.61186/rbmb.12.1.13]