

# Differentiation of Human Umbilical Cord Derived Mesenchymal Stem Cells into Dopamine Producing Cells

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## Abstract

Human umbilical cord tissue namely Wharton's jelly is one of the rich sources of mesenchymal stem cells(MSCs). MSCs are pluripotent cells that can differentiate into different types of cells including neural and dopaminergic cells. Thus MSCs hold great promise for different neurodegenerative diseases such as Parkinson's disease which result from the impairment and failure in generation of dopamine neurons in the substantia nigra of the mesencephalon. This work was aimed to study the efficacy of WJ-MSCs to differentiate into dopaminergic (DAergic) neurons in presence of all trans retinoic acid (ATRA) by determination of dopamine level. Flow cytometry analysis showed that MSCs were positive for CD90 and CD73 and negative for CD45 and CD34. Immunocytochemistry staining showed that the cells treated with ATRA were strongly expressed for MAP-2 and showed less expression for cells treated with neurobasal media only. Results from ELISA indicated that dopamine levels were markedly increased in the ATRA-treated groups.

**Keywords:** Mesenchymal stem cells, Umbilical cord stem cells, Wharton's jelly, Dopaminergic neurons, All trans retinoic acid, Neurological diseases, Parkinson's disease.

## 1. Introduction:

Stem cells are unspecialized cells that are capable of self-renewal, potency and differentiation into different cells of the human body. According to their origin, they can be classified into embryonic stem cells (ESCs), adult stem cells (ASCs) and umbilical cord stem cells (UCSCs). [1]

Mesenchymal stem cells (MSCs) are a multipotent cell that are originated from the embryonic mesoderm during fetal development. It can be obtained from adult tissues such as bone marrow, adipose tissue and fetal/perinatal tissues such as placenta, umbilical cord, amniotic membrane. [2]

One of the most important sources for MSCs is human umbilical cord (HUC). Stem cells can be derived from various parts of the umbilical cord such as (i) inner amnion membrane (ii) outer amnion membrane (iii) intervacular compartments (iv) Wharton's jelly (WJ) and (v) perivascular region around the umbilical blood vessels. [3]

The richest region with MSCs in the umbilical cord is its tissue which called Wharton's jelly. WJ is the connective tissue surrounded the blood vessels of the cord and containing considerable amounts of hyaluronic acid and some sulfated glycosaminoglycans (GAGs) present in an insoluble collagen fibril. WJ is important in preventing compression, torsion and bending of the enclosed vessels, so it can supply the bidirectional blood flow between the fetal and maternal circulations. [4]

Wharton's jelly Mesenchymal Stem Cells (WJ-MSCs) can be considered as point to use un differentiated fetal cells that are multi-potential stem cells without any ethically problems. They are capable of plastic adherence, culturing with fibroblastic shape, cloning and expression of the cell surface markers of MSCs. [5]

WJ -MSCs have several favorite characteristics: (a) the obtaining procedure is noninvasive and painless with ethically noncontroversial source of MSCs, [6] (b) they have

the ability to proliferate and growth greater than adult MSCs, with high multipotency and without creation of teratomas, [7] (c) they are considered to be more innate than other different MSCs sources so they can be used in allogeneic and also in autologous transplantation due to their intermediate expression levels of major histocompatibility complex (MHC) Class I antigens and insignificant expression levels of MHC Class II antigens, which occur in the same manner with differentiated MSCs. This lead to prevent the complicated immune responses of allogeneic transplantation and so decreasing the need for strong immune suppression after treatment. [8]

WJ-MSCs can participate in solving many disorders such as neurological disorders, kidney injury, lung injury, orthopedic injury, liver injury, cancer therapy. [2] because they are able to differentiate into various lineages of three germ layer, including osteoblasts, chondrocytes, myocytes, adipocytes and dopaminergic (DAergic) neurons. [9]

Recently, using of WJ-MSCs in treatment of neural diseases provide a promising candidate in regenerative medicine due to: a) They have the ability to produce Proangiogenic and neuroprotective factors [10] b) after transplantation, they migrate into the damaged nervous tissues to enhance the recovery of this tissues and can be kept survival for long time [11] c) they regulate inflammatory response, decrease free radical production, reduce apoptosis, and promote endogenous neuronal growth, synaptic connections, and neural repair [12] , d) they can differentiate in vitro into neurons in presence of neural differentiation marker such as some chemokines, as basic fibroblast growth factor (bFGF) and epidermal growth factor(EGF) [13]. So this cells can be used in treatment of neurodegenerative diseases such as Parkinson's disease (PD).

Parkinson disease (PD) is the most common movement disorder. It is characterized by hypokinesia, rigidity, resting

tremor and postural instability. It is caused by degeneration and loss of DAergic neurons and formation of intracellular Lewy bodies in the substantia nigra (SN) [11]. Current treatments for PD include levodopa, dopamine receptor agonists, enzyme inhibitors and deep brain stimulation (DBS) [14]. Therapies for PD have been mainly restricted on the relief of symptoms, and there is no effective and reliable model for studying the mechanism for this disease. Therefore, great efforts should be made to restore damaged DAergic systems using cell replacement therapy as an alternative approach. [15]

## 2. Material and Methods:

The study protocol was approved by the Ethics Committee of Mansoura University and all procedures were performed according to ethical committee approval.

### 2.1. Materials:

Phosphate buffer saline (PBS), Dulbecco's Modified Eagle Medium (DMEM), Pencillin-Streptomycin antibiotic, Fetal Bovine Serum (FBS), Neurobasal A- Medium(Gibco), Collagenase type I, Hyaluronidase, ATRA (Sigma), Anti-CD 90, Anti-CD 73, Anti-CD 34, Anti-CD 45 (BD Anti-Microtubule associated protein -2 (MAP-2), Human dopamine ELISA kit (Biospes)

### 2.2. Methods:

#### 2.2.1. Sampling procedure:

Umbilical cords were collected from Department of Obstetrics and gynecology, Mansoura University Hospital and transferred to Mansoura Research Center for Cord Stem Cells in faculty of medicine Mansoura University-Egypt, (MARC - CSC). Umbilical cords samples (No.=10) were collected from healthy infants born by cesarean section after informed consent obtained from mothers. The cords were transported into sterile container containing DMEM (low glucose), 100U/ml penicillin and 100mg/ml streptomycin antibiotics. The cords were stored at 4°C and processed within 2 hours after birth.

#### 2.2.2. Isolation of mesenchymal stem cells from Wharton's jelly:

The cord was cut into small pieces about 2-3 cm and immersed in phosphate buffer saline (PBS) several times to remove the blood as possible. Each piece was cut lengthwise and blood vessels were removed by using sterile scissors and forceps. The inner matrix containing Wharton's jelly was scratched by sterile slide or scalpel and cut into small pieces. The extracted WJ were then digested with enzymatic cocktail consisting of 4 mg/ml Collagenase Type I and 1 mg/ml Hyaluronidase solubilizing in DMEM (low glucose) medium and incubated for 1hr. in shaking water bath followed by centrifugation for 10 minutes at 2000 r.p.m. The pellet was incubated with 0.1% trypsin-EDTA for 30 minutes at 37 °C in shaking water bath followed by centrifugation for 10 min. at 2000 r.p.m. The cell pellet was re suspended in culture

medium containing DMEM (low glucose) supplemented with 20% FBS and 1% pencillin/ streptomycin antibiotic and transferred to 25cm<sup>2</sup> tissue culture flask. The samples were incubated in incubator at 37°C with 5%CO<sub>2</sub> level and examined every 3 days. [16]

#### 2.2.3. Culturing of WJ-MSCs:

After 3 days from culturing, the cells were examined and the media was changed. When the cultured cells reached 70% - 80% of confluence, the old culture medium was removed and the cells were washed with about 5 ml PBS. About 5 ml trypsin-EDTA was added to the flask and examined under microscope. When the adherent cells began to separate from each other, the trypsin EDTA was removed and the flask was incubated in incubator at 37°C for 3 minutes. About 10 ml of culture media was added to the flask and pipetted many times. Then they transferred to a new 75cm<sup>2</sup> tissue culture flask and completed to 25ml culture media. [16]

#### 2.2.4. Differentiation of WJ-MSCs into dopaminergic neurons:

After the third passage at approximately 60% confluence (2×10<sup>6</sup>/flask), the cells were divided into three groups. The first cells group were treated with differentiation medium consisting of neurobasal A medium, 10µl ATRA, 10% FBS, 1% pencillin/ streptomycin antibiotic and L- glutamine. The second cells group were treated with differentiation medium but without ATRA and served as a neuron control group. The third cells group were cultured in primary culture medium and served as a control group. The flasks were incubated in incubator at 37°C with 5% CO<sub>2</sub> and the media were changed every 3 days. After 12 days, the cells were used to determine specific markers for dopaminergic neurons. [17]

#### 2.2.5. Characterization of WJ-MSCs by flow cytometry:

After the third passage, about 5×10<sup>5</sup> were resuspended in 1 ml primary culture media. A 100µl cells were stained by a combination of antibodies: PE-conjugated CD73, CD90; FITC conjugated CD34, CD45 (BD Bioscience) and incubated in the dark for 30 min. at room temperature. A 2 ml stain buffer fetal bovine serum (BD Bioscience) was added to each tube and centrifuged at 2000 r.p.m for 10 minutes. The supernatant was discarded and 500µl stain buffer was added to each tube. The cell-surface antigens expression was assayed. [18]

#### 2.2.6. Immunocytochemistry staining for MAP-2:

After 12 days from the induction to the differentiation medium, the ATRA treated group and controls groups were fixed with 4% para formaldehyde for 15 min. at room temperature and washed three times with 1X PBS. Then they were fixed with methanol for 10 minutes and incubated with blocking solution to block endogenous peroxidase for 10 minutes. Fixed cells were incubated with mouse anti-MAP-2 for 30-60 min. at room temperature then washed three time with wash buffer. The slides were incubated with enzyme conjugate at room temperature followed by DAB (Power stain 1.0 poly HRP DAB kit for mouse and rabbit, Genemed)

according to the manufacturer's instructions. The slides were then counterstained with hematoxylin, dehydrated and mounted. Positive reaction appeared as brown cytoplasmic stain. [19]

### 2.2.7. Determination of dopamine level by dopamine Enzyme Linked Immunosorbent Assay (ELISA):

After 12 days from the induction to the differentiation media, the cells from treated group and different control groups were trypsinized and the cell suspension was diluted with PBS make the cell concentration reached 1 million / ml. The cells were damaged and intracellular components were released through repeated freeze-thaw cycles. Then they were centrifuged at 2000-3000 r.p.m. for 20 min and supernatant was collected for ELISA following the manufacturer's instructions (Human dopamine ELISA kit, Biospes). [17]

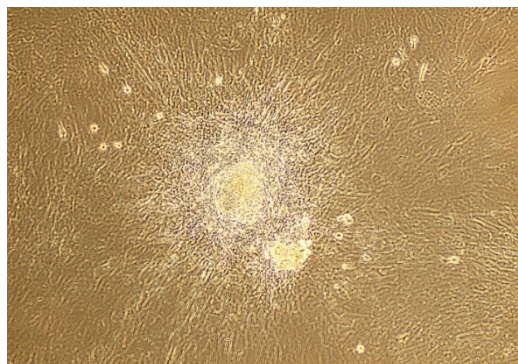
### 2.2.8. Statistical analysis:

The data of dopamine assay were given as mean  $\pm$  SD using a two-tailed Student's t-test. The statistical software SPSS16.0 was used. The difference between the mean was considered highly significant when  $P < 0.001$ .

## 3. Results:

### 3.1. Morphological changes of cultured WJ-MSCs:

Mesenchymal stem cells were extracted from umbilical cord tissues by mixed enzymatic explant method within 2 hours of umbilical cord collection. During the first few days of culture, the primary isolated cells were appeared as clumps and colonies with a heterogeneous shape, round and flat cells and fibroblast like cells with short and long processes which continued to proliferate. Spindle shaped cells were observed on the 5<sup>th</sup> 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.1)

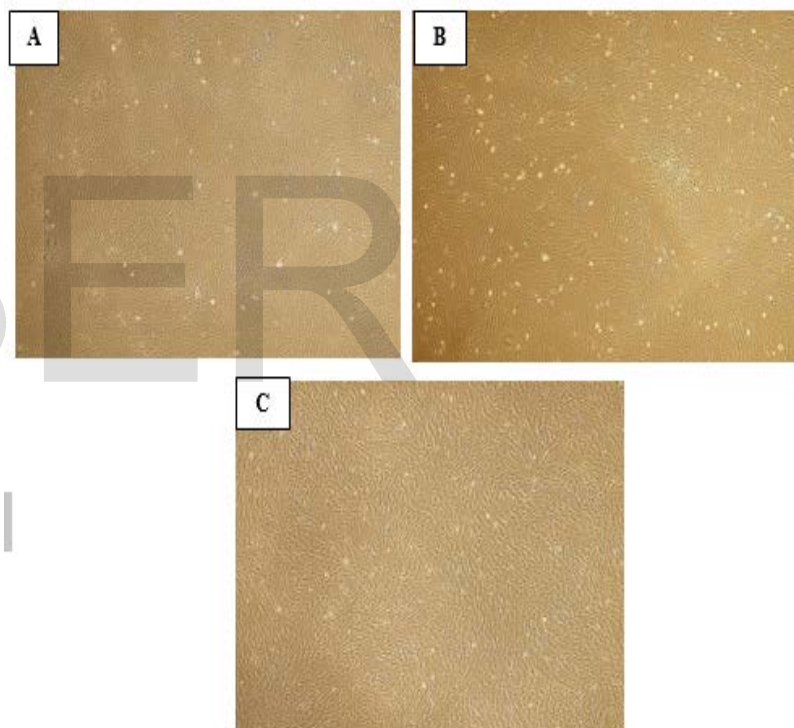


**Fig. (1):** Wharton's Jelly Mesenchymal Stem Cells after 5 days from isolation.

### 3.2. Culturing and Expansion of WJ-MSCs:

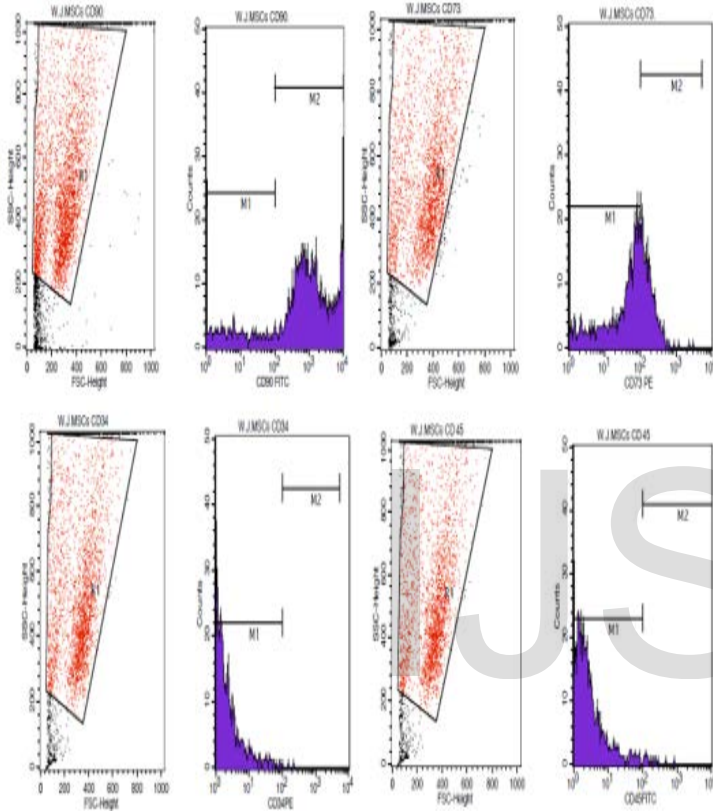
After 5 days of culture in the primary culture media, a few short fibroblasts like cells were adhered to the tissue culture flasks. When the cells were reached to 70-80% confluence after 10-12 days from primary culture, they were expanded, overlapped and sub cultured into several passages with long-spindle shaped fibroblast like cells. After the third passage, the cells were ready to be differentiated into neurons as shown in Fig. (2).

**Fig. (2):** (A) Adherent WJ-MSCs in the first passage. (B) Adherent WJ-MSCs in the second passage. (C) Adherent WJ-MSCs in the third passage.



### 3.3. Characterization of WJ-MSCs by flow cytometry analysis:

After passage 3, the cells were counted and  $50 \times 10^4$  cells/ml was taken to characterize and establish the purity of WJ-MSCs by flow cytometry. The obtained data indicated that these cells were strongly expressed the specific cell surface markers of MSCs such as CD90 (90%) and CD73 (64%) and weakly expressed the surface markers such as CD45 (0.65%) and CD34 (0.07%). These results indicated that the isolated cells were MSCs and there was no contamination with cells from hematopoietic or endothelial origin.



**Fig.3:** Flow cytometry analysis of WJ-MSCs surface markers expression.

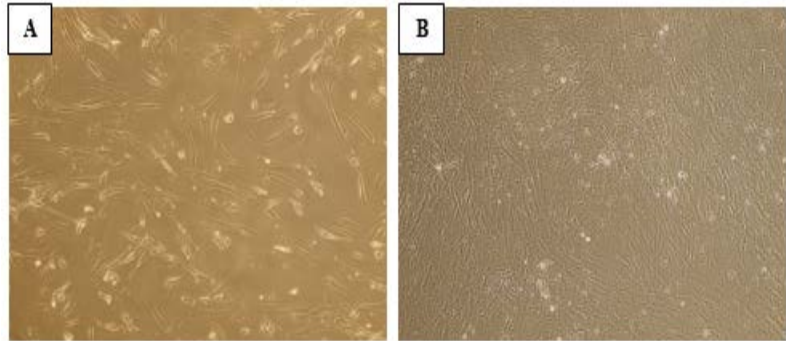
### 3.4. Differentiation of WJ-MSCs into dopamine producing cells:

When the cells were reached 55-60% confluence after passage 3, a group of cells were treated with neurobasal media only and other group was treated with neurobasal media in the presence of  $10 \mu\text{M}$  ATRA in order to determine the potential of WJ-MSCs to differentiate into neurons like cells.

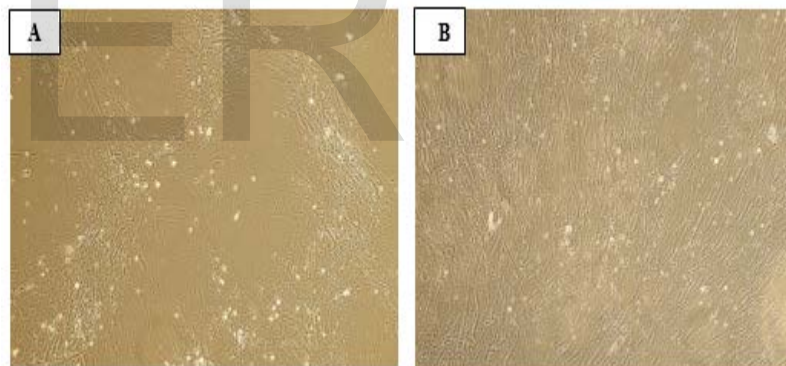
For ATRA treated group: In the beginning of induction, the WJ-MSCs showed a bipolar spindle shape. Within 5-6 days from the induction, the cells became thinner and had longer extensions followed by the formation of elongated and branched cell process with neurosphere like morphological change after 12 days as shown in **Figure (4)**.

For neuron control group: The cells became thinner and had shorter extension after 8-10 days as shown in **Figure (5)**. They did not completely differentiate into dopamine producing cells after 12 days.

For negative control group: The cells cultured in primary culture media shown fibroblast spindle shape like cells.



**Fig. (4):**  
(A): the cells became thinner with longer extension after 6 days from culturing with neurobasal media and ATRA  
(B): the cells became more elongated with branched cell processes after 12 days from culturing with neurobasal media and ATRA.



**Fig. (5):**  
(A): the cells became thinner with shorter extension after 8 days from culturing with neurobasal media.  
(B): the cells overlapped and touching each other with shorter extension after 12 days from culturing with neurobasal media.

### 3.5. Characterization of dopaminergic neurons derived from WJ-MSCs:

Immunocytochemistry staining showed the cells were expressed one of the neurons specific markers monoclonal antibody protein-2 (MAP-2). The cytoplasm of cells treated with ATRA were strongly expressed for MAP-2 and few cells treated with neurobasal A media only were showed less expression (Fig. 6A, 6B). WJ-MSCs served as a control were stain negative for MAP-2 (Fig. 6C). These data indicate that WJ-MSCs could differentiated into neurons like cells.

To determine whether WJ-MSCs can be able to produce dopamine after 12 days from induction to neural differential conditions, the dopamine levels were measured in each group of cells by ELISA. Results indicated cells treated with ATRA showed a highly significant increase ( $P < 0.0001$ ) in dopamine concentration as compared with negative control group and group of cells induced to neurobasal A medium only These data indicate that WJ-MSCs treated with ATRA in presence of neurobasal A medium can produce dopamine in high concentration. (Table 1, Fig.7).

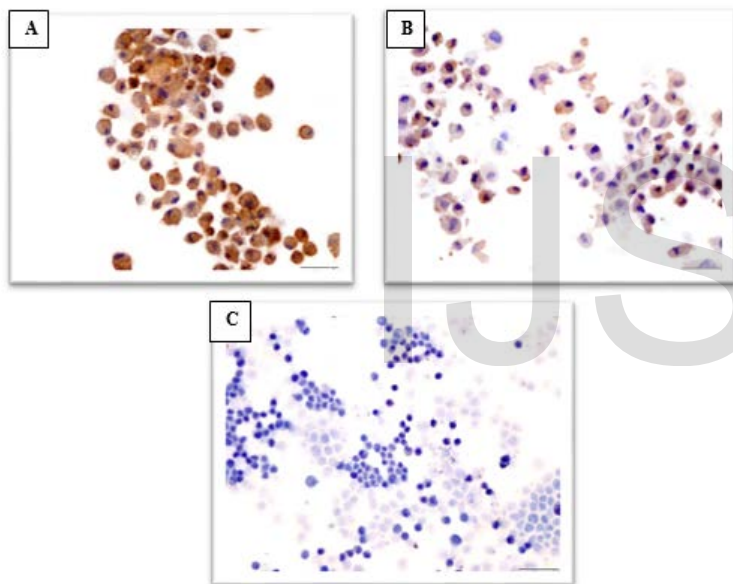
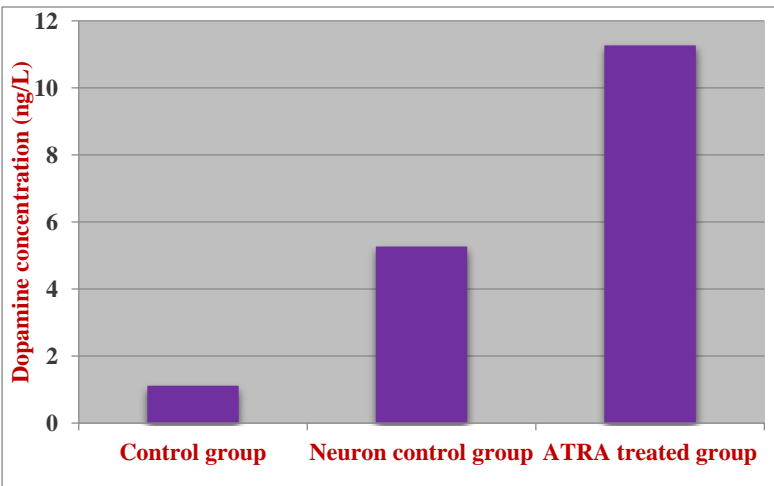


Fig. (6): After 12 days of culturing WJ-MSCs under neural differentiation.

A: cells treated with ATRA were strongly expressed MAP-2.



B: cells treated with neurobasal media only were less

Groups	control group	Neuron Control group	ATRA group
Dopamine (ng/L)	1.12 ± 0.31	5.26 ± 0.24	11.26 ± 0.38
Mean ± SD (n=10)			

expressed MAP-2.

C: Cells of control group showed unspecialized cells stained negative for MAP-2

Table 1: The differences in dopamine concentration in different groups of cells:

Fig. 7: Dopamine concentration in different groups of cells

#### 4. Discussion:

In the present study we investigate the efficacy of mesenchymal stem cells isolated from human umbilical cord Wharton's jelly to differentiate into dopaminergic neurons in the existence of all trans retinoic acid which may provide a promising candidate for treatment of Parkinson's disease.

The most common important cause for Parkinson's disease is the continuous degradation and death of mesencephalic nigral DA. The pharmacological and surgical treatment of this disease such as substitution of L-dihydroxyphenylalanine (L-DOPA or levodopa) are associated only with improving the symptoms of early stage and delay the symptoms of advanced stages without stopping the continuous degradation of dopaminergic neurons. So the treatment of this disease is provided with stem cells transplantation that can differentiate into DAergic neurons and give the required dopamine level. [19]

Stem cells are responsible for adult cells regeneration throughout life due to its self-renewal property. MSCs are found in adult tissues such as bone marrow, adipose tissues, dental pulp and in neonatal tissues such as amnion, placenta, umbilical cord [20]. Umbilical cord Wharton's jelly was recently considered as an alternative source of MSCs. WJ-MSCs have the ability to specialize into ectoderm (neural cells), mesoderm (adipocyte, osteocyte, chondrocyte, smooth muscles etc.) and endoderm (Pancreatic progenitor) [2]

According to the previous studies which indicated that umbilical cord Wharton's jelly are an ideal source for MSCs due to noninvasive collection procedure, high proliferation and differentiation ability, identical expression of surface cell markers, immunomodulatory, non-tumorigenic and known to secrete proangiogenic as well as neuroprotective factors [21], [22], [23], [24], [25]. MSCs transplantation provide an important effect in treatment of PD because they can differentiate into DAergic cells which can replace the damaged DAergic neurons and they have a trophic effect that is intermediated by different trophic factors. [26]

In the current study MSCs were isolated from 10 samples within 2 hours after collection of umbilical cord. Wharton's jelly was digested by using enzyme cocktail containing of collagenase type I and Hyaluronidase for 1 hr. followed by trypsin for 30 min. to isolate MSCs. Then these cells were cultured with DMEM complete media in humidified incubator at 37 °C in 5% CO<sub>2</sub>. The cells were examined every 3 days and the media was changed with a new complete media. During the first few days from culturing, the isolated MSCs were heterogeneous. After 5-6 days, 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. [16]

After passage three, the cultured cells were analyzed by flow cytometry which proved that this cells were MSCs without any contamination from hematopoietic or endothelial cells. [18]

At 50-60% confluence with density of 2×10<sup>6</sup> MSCs, the cultured cells were divided into three groups and induced to differentiation media. After 12 days from the induction to the differentiation media, the ATRA treated group were completely differentiated into dopaminergic neurons, markedly expressed MAP-2 which is a specific marker for neurons and dopamine were secreted with high concentration. But the neuron control group which induced only to neurobasal media showed small percentage of dopaminergic neurons with not completely differentiated and less expression for MAP-2 and dopamine level. The negative control did not differentiate.

The results of our study proved that the mesenchymal stem cells extracted from Wharton's jelly are an excellent source of stem cells for differentiation into neurons with their typical structure and function in presence of ATRA.

#### 5. Conclusion:

Wharton's jelly mesenchymal stem cells provide an alternative source for treatment of Parkinson's disease due to its ability to differentiate into dopaminergic neurons in presence of ATRA and neurobasal medium with marked dopamine level.

#### Conflict of interest statement:

This study was funded by the science and Technology Development fund "STDF"

#### Acknowledgments:

The authors are grateful to all patients for participating in our study. Thanks to all team workers at MARC-CSC in faculty of medicine Mansoura University-Egypt.

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