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# THE PRODUCTION OF NEURAL STEM CELLS AS A SUITABLE SOURCE FOR THE TREATMENT OF NEURODEGENERATIVE DISEASES

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**ABSTRACT**

Introduction: Spinal Cord Injury (SCI) is a disease that can disrupt many functions, including motor functions, today cell therapy is one of the treatments for these diseases. Adipose derived stem cells (ADSCs) from the mesenchymal stem cell group are easy and affordable access which can be differentiated into other cells. In this study, we differentiated the ADSCs by the neurospheres method into neural stem cells (NSCs).
Methods: In this study, adipose tissue was taken from rats and examined after digestion in the DMEM medium and surface markers of stem cells such as CD90, 29, and 45 were examined. ADSCs were distinguished into neurospheres in a neuron culture medium and then neurospheres were converted directly to NSCs. Nestin and NF-68 neuron markers were examined by immunocytochemistry.
Results: ADSCs expressed the mesenchymal stem cell markers and did not express the CD45 marker of the hematopoietic cells. Neurospheres produced from them also express the marker of neural stem cells such as nestin and NF-68 and the stem cells produced are also the same.
Conclusion: Therefore, ADSCs may be an ideal alternative easily accessible source of stem cells for the treatment of neurodegenerative diseases.



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## Introduction

Spinal Cord Injury (SCI) is damage to the spinal cord where the cord's three major functions, including movement, sensation and reflexes are partially or completely destroy (1). The incidence of this complication in the world is between 20 and 50 people per million population per year (2). But this figure in Iran is about 40 to 50 per million, and more than 3,000 people in the country every year suffer from spinal cord injuries(3), the incidence of which in men is more than that of women(4,5). Since the damage caused by these lesions is more likely to affect the younger population and causes a chronic and severe disability, the search for a way to treat this type of lesion is always considered (6).

The researchers have been interested to tissue and cell transplantation including embryonic spinal cord transplantation with or without growth factors (7), olfactory epithelial cell transplantation, embryonic stem cell transplantation, stem cell derived neuronal transplantation, and adult bone marrow stromal cells transplantation for therapeutic approaches(8, 9). Stem cells are cells that can retain the ability to divide for a very long time and, under favorable conditions and with suitable signals, can be distinguished from different types of cells of an organism. SCs have the ability to differentiate into adult cells with their specific shape and function, such as the cells of the heart, skin, nerve or bone (10).

The results of the other studies, along with the limitations of the use of embryonic stem cells and the limited resources of neural stem cells in therapeutic applications, have added to the importance of using mesenchymal stem cells (11).

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The important sources of mesenchymal cells are bone marrow and adipose tissue. Adipose derived stem cells (ADSCs) such as bone marrow stem cells have the potential to differentiate into ectodermic cells such as neural and glial cells, and therefore an appropriate source for treating neurodegenerative diseases (12).In this study, we used ADSCs to produce neurospheres and used the neurospheres to produce neuron-like cells for treatment of spinal cord injury.

## Materials and methods

### ADSCs Culture

Adipose tissue from inguinal region of Wistar rat (200–300 g) collected and washed extensively with phosphate buffer solution (PBS, pH 7.4) supplemented with 100 U/ml penicillin, and 100 mg/ml streptomycin. Washed tissue was minced and digested with 0.1% collagenase type I in Dulbecco's Modified Eagle Medium (DMEM) at 37°C for 30 min in shaking incubator, then to neutralize collagenase DMEM with 10% FBS added and centrifuged to separate mature adipocytes from a pellet consisting of a ADSCs. Pelleted cells were resuspended whit DMEM with 10% FBS and filtered through a 100 µm cell filter mesh (BD Biosciences) and were initially cultivated in DMEM, supplemented with 10 % fetal bovine serum (FBS, Gibco), 100 U/ml penicillin, and 100 mg/ml streptomycin. The cells were incubated at 37 °C and 5 % $CO_2$ in a 25-$cm^2$ plastic flask for 24h, and non-adherent cells were removed by replacement of the medium. The medium was changed every other day until cells became confluent.

### Neurospheres Formation and Expansion

Rat ADSCs in passage five were harvested with trypsin–EDTA and plated on a 25-$cm^2$ non-adherent plastic flask ($10^5$ cells/ml) in DMEM/F12 medium supplemented with 2 % B27 (Gibco), 20 ng/ml basic fibroblast growth factor (bFGF, Invitrogen), 20 ng/ml epidermal growth factor (EGF, Invitrogen), 100 U/ml penicillin, and 100 mg/ml streptomycin(13,14). The culture medium and growth factors changed every 48 hours. In this study, we did not use pipetting up and down and centrifugation of the neurospheres to produce single cells, but the neurospheres were cultured in the same medium supplemented with 5 % fetal bovine serum as the rounded form. Immunocytochemical evaluation was performed for nestin and NF-68 marker.

### NSCs Formation

After neurosphere culture for a week, neurospheres were cultured in DMEM/F12 supplemented with 2 % B27 (Gibco), 20 ng/ml bFGF, 20 ng/ml EGF, and 5 % FBS in six-well adherent plates (Sigma-Aldrich) and passaged up to three times. Immunocytochemical evaluation was performed for nestin and NF-68 and Oct4 marker.

### Immunostaining

Cells were cultured on cover slides and fixed in 4 % paraformaldehyde in PBS for 20 min at room temperature, For immunofluorescence, cells were blocked with 3% bovine serum albumin in PBS for 30 minutes and for nuclear markers cells were permeabilized with 0.4 % Triton X-100 and washed twice in PBS then incubated with primary anti-CD90 and anti-CD29 and anti-CD45 (for ADSCs) and anti-nestin, anti-NF-68 and anti-Oct4 (for ADSCs-derived NSCs and neurospheres) monoclonal antibodies (all from Abcam) overnight. Primary antibody washed three times with PBS and followed by incubation with FITC-conjugated rabbit anti-mouse antibody (secondary 1:1,000, Millipore) and Nuclei were counterstained with Propidium iodide (PI).

## Result

### ADSCs Culture

The results of ADSCs culture showed that, at earlier hours, cells floated and small droplets of fat were observed in the medium and at 24 h began to attach to the dish surface. After 24 hours, fibroblastic cells were observed and after each wash, the amount of fat granules was lower and more cellular colonies were observed. (Fig. 1A). At 7–8 days, the adherent cells became confluent and could be passaged once every 5 days. ADSCs were found to express CD90 and CD29 (specific mesenchymal cells marker) (98.7 % ± 3.2) (Fig. 1D,E) and were negative for immunofluorescence staining with CD45 (specific hematopoietic cells marker) (Fig. 1F).

### Formation and evaluation of neurospheres

The fatty cells that were trapped in the third passage were cultured in non-stick flasks in the neurospheres culture media mentioned above. The cells formed a small float colony after 6h (Fig.2A). On the third day they were observed Small spheroids (Fig.2B) On the fifth and seventh days, the diameter of the neurospheres increased and the tendency to connect to each other to form larger neurospheres was observed(Fig.2C,D).Neurospheres immunofluorescence staining result was positive for nestin , NF-68 and Oct4(Fig.E,F,G).

### Formation and evaluation of NSCs

We transferred the neurospheres to the six-well adherent plates without transforming them into single cells. In this context, (Fig. 3A) the neurospheres had been found to have been bound to the floor of the plate after 24 hours, and after 48 hours, the NSCs migrated from the neurospheres to the surface of the plate(Fig. 3B,C). The ADSCs-derived NSCs from spheroids strongly were positive for nestin and NF-68 immunostaining (99.23 % ± 0.17) (Fig. 3D,F).

**Figure 1.** represents the morphology and characterization of the adipose-derived stem cells (ADSCs) using phase-contrast images and immunocytochemistry method. A shows the primary culture of ADSCs, while B and C show the first and third passages, respectively (scale bar = 500 µm). D shows the immunostaining of ADSCs with anti-CD29 primary antibody (mesenchymal stem cells marker), E shows the immunostaining for CD90 (mesenchymal stem cells marker), and F CD45 (haematopoietic cells marker). G, H and I represent the phase contrast images of D, E and F, respectively, (scale bar = 200 µm).

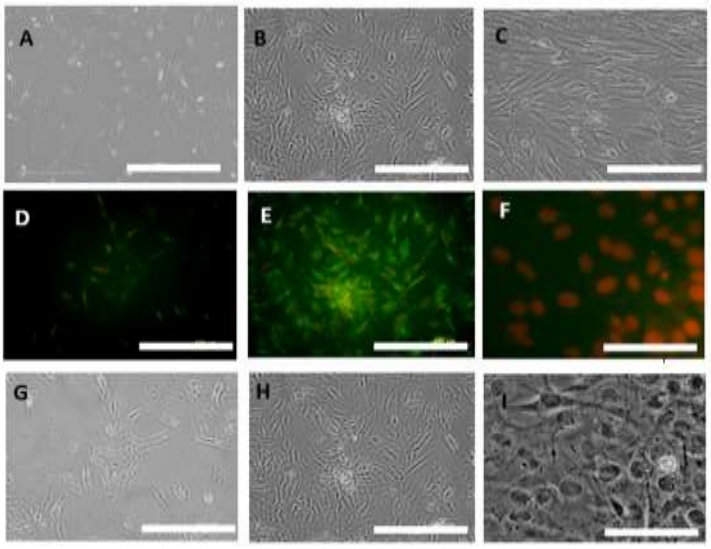
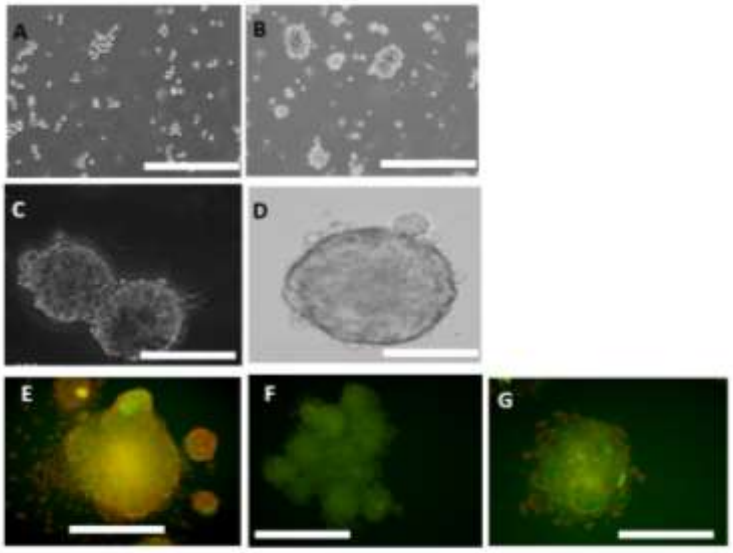
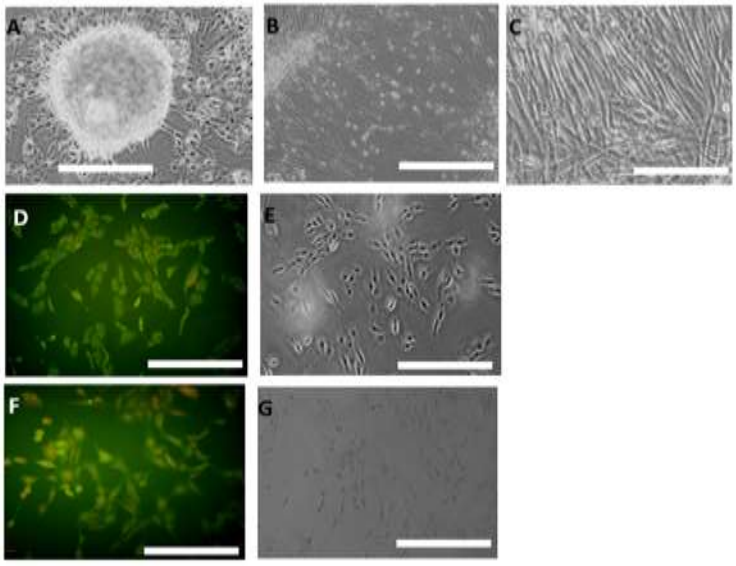



**Figure 2.** represents the morphology and characterization of the neurospheres using phase contrast images and immunocytochemistry method. A represents the phase contrast images of the neurospheres after 2 h, while, B, C and D represent them after 1, 3 and 5 day(s), respectively (scale bar = 500 µm). E shows the immunostaining of the neurospheres with anti-nestin primary antibody, whereas F and G show immunostaining for anti-neurofilament 68 and Oct4 primary antibodies (scale bar = 200 µm).

**Figure 3.** represents the morphology and characterization of the neural stem cells (NSCs). A demonstrates the cultured neurospheres in an adherent flask at 24 h, while B and C show the morphology of the NSCs after 3 and 7 days, respectively (scale bar = 500 µm). D and F represent the immunostaining of the NSCs with primary antibodies against nestin and NF-68 respectively. E and G represent the phase contrast images of D and F respectively (scale bar = 200 µm).






**Discussion**

In this study, we produced neural stem cells expressing neural stem cell markers like Nestin and NF-68 and Oct4, which were derived from neurospheres derived from adipose derived stem cells.

ADSCs are appropriate and accessible source for cell therapy that are available to a large extent (15).In our study, ADSCs were grown in culture and passaged easily.Characteristics of ADSCs the morphology, fibroblastic adaptation after three days with the findings of other researchers was consistent (16).we showed that ADSCs expressed CD90 and CD29 mesenchymal stem cells markers (17), and but not to CD45, hematopoietic cell marker (17); that are consistent with the findings of other researchers. In many studies using ADSCs as a source of cells for the treatment of degenerative disease of the nervous system and cell therapy have mentioned (18, 19 ,20).In our investigation, we differentiated the ADSCs into neuronal neurospheres in an induction culture medium, and in an immunocytochemistry study, markers of nerve stem cells such as NF-68, Oct4 and nestin these findings are in accordance with other investigations (21,22). In the following, neurospheres-forming cells were cultured in nerve culture medium in a different way without centrifuging and transforming them into single cells and producing neural stem cells from them. NSCs were released from the environment of the neurospheres around the flask and looks spindle and fibroblasts like cells as reported by the others(20).On the other hand, NSCs which is consistent with the findings of the others, immunostained for nestin and NF-68(23,21).The cells produced by this method may have less damage than the centrifuge and pipetting method and can be distinguished for the production of specific classes of neuronal cells, such as motor neuron, Schwann cells, and other types of neuronal cells. Of course, this requires extensive research into the production of NSCs.

**Conclusion**

In this study, adipose derived stem cells were differentiated into neurospheres, and neurospheres were converted directly to the NSCs directly without manipulation. These cells produced with the lowest cost could be a suitable source for the treatment of neurodegenerative diseases.

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