Isolation of Neural Stem Cells from Olfactory Mucosa and Olfactory Bulb of Adult Albino Rats

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Abstract: Background: Olfactory stem cells (OSCs) can be isolated from the olfactory mucosa and proliferate in culture media allowing studying their characters and differentiation into one or more specialized cells to be used in treatment of various diseases. **The aim** of this study is to isolate neural stem cells from the olfactory mucosa and olfactory bulb of the adult male albino rats and characterize the isolated cells regarding their morphology and expression of MSCs markers. **Materials and methods:** The OSCs have been isolated from the olfactory mucosa and olfactory bulb of the adult male albino rats, cultured and characterized morphologically and immunohistochemically. **Results:** OSCs were successfully isolated cultured in DMEM F-12 media supplemented with 10 % FBS and 1% penicillin/streptomycin. The cells were identified by their characteristic morphology having spindle shape cytoplasmic processes, their plastic. Adherence tendency and colony formation till the 3rd passage. Immunohistochemically they also characterized showing positive expression for CD44 and Nestin and negative expression for CD34. **Conclusion:** The results of this study explained that, the OSCs can be successfully isolated from the olfactory mucosa and olfactory bulb of the adult albino rats, cultured to proliferate in suitable medium and show the morphological and phenotypic characterization of mesenchymal stem cells.
[Osama M. A. M. Elzahaby, Rania A. Galhom, Wageh Mansour Abdel-Hay, Mamdouh Abdel-Halim Ghaly. **Isolation of Neural Stem Cells from Olfactory Mucosa and Olfactory Bulb of Adult Albino Rats.** *Nat Sci* 2018;16(9):37-45]. ISSN 1545-0740 (print); ISSN 2375-7167 (online). http://www.sciencepub.net/nature. 6. doi:10.7537/marsnsj160918.06.

Key words: Isolation, culture, characterization, morphology, phenotype, olfactory stem cells.

1. Introduction

The olfactory mucosa lines the postero superior aspect of the nasal septum and the undersurface of the cribriform plate. Its epithelium shows continuous renewal by neurogenesis to replace the damaged cells by the various types of infection and pollution that exposed all the time. **(1, 2)** For this purpose it exhibits a large population of stem cellsto cope with this function throughout life (**3, 4**). The OSCsare present in the basal layer of the olfactory epithelium, lamina propria and the olfactory bulb showing the characteristics of mesenchymal stem cells (MSCs) and neural stem cells as well, however the lamina propria stem cells showclose relation to the bone marrow mesenchymal stem cells (BM-MSCs), so they are also named olfactory ecto-mesenchymal stem cells (OE-MSCs) (**5, 6 & 7**).

Olfactory stem cells derived from the olfactory mucosa can be cultured on suitable culture to proliferate and generate neurospheres that are able to give rise to neurons and glial cells, so, they are useful for cell therapy of various neurological diseases, specially the easily accessible mucosa that can be easily biopsied from the nasal cavity **(8, 9)**.

Olfactorybulb derived stem cells provide an attractive tool for developing transplantation-based therapy of neurodegenerative diseasesdue to their ability to self-renew and their neuronal phenotype differentiation **(10, 11).** These cells are derived from the neural stem cells lying the lateral ventricular walls of the brain which differentiate continuously by neurogenesis after birth giving rise to neuroblasts and the newly formed cells find its way to the olfactory bulb throughout life (**12**) and develop synapses with the other neurons of the olfactory bulb to be included in the olfactory pathway (**13**). Also, the original cells of the olfactory bulb undergo neurogenesis that is well characterized in rodents and adult monkeys (**14**).

Olfactory stem cells derived from the olfactory mucosa and bulbs are fusiform, fibroblast-like cells. During their initial colony forming unit like fibroblasts (CFU-F), the mucosal stem cells are negative for hematopoietic surface markers; CD 34, CD 45 and CD14 but positive for a variety of markers including Stro-1, CD 44, CD 29, CD 105, CD 73, CD 166 and CD 90 **(15, 16)**, while the olfactory bulb stem cells express CD29, CD44, CD90, CD105 and CD166 but not CD34 and CD45, consistent with the characteristics of MSCs and are capable of differentiation into mesenchymal lineages such as osteocytes, chondrocytes and adipocytes (**17**). Identification and studying themesenchymal like characteristics of these cells is an important issue to be used in the future as a cyto therapy for neurodegenerative and neurodevelopmental diseases. So, the aim of our study was to isolate neural stem

cells from the olfactory mucosa and olfactory bulb of the adult male albino rats and characterize the isolated cells regarding their morphology and expression of MSCs markers.

2. Materials and Methods

Equipments and materials for tissue culture

Animals:

Ten adult male Sprague Dawley albino rats with an average weight 200 – 250 gm. were sacrificed for collection of olfactory mucosa and olfactory bulbs for stem cells culture. They were housed in separate cages in the animal house of Faculty of Medicine; Al Azhar University, according to the guidelines of care of laboratory animals. They were fed ad libitum and allowed free access to water.

Glass ware:

Bottles used for preparation and storage of medium and other solutions, pipettes graduated and Pasteur's, plugged and unplugged, treys, racks, surgical instruments used in dissection and dressings used in disinfecting the hood were sterilized by autoclaving at 160 °C for one hour (two cycles).

Equipments for cell culture:

Tissue culture dishes (35 mm diameter): for culturing and sub-culturing of the olfactory stem cells.

Easy flasks 25 cm2 and 75 cm2: for culturing and sub-culturing of the olfactory stem cells and growth medium storage. (Both Petri dishes and flasks were nontoxic, biologically inert, and optically transparent surface that allowed cells to attach and to grow).

Sterile tissue culture grade pipettes: 5ml, 10ml and 25ml: for preparing, adding and aspiration of media.

Nylon mesh filter: 100 um.

Laminar flow cabinet: (NUAIRE, Biological Safety Cabinets Class II Type, USA).

Standard air/ CO2 incubator: (NUAIRE CF Auto flow CO2 Water jacketed Incubator, USA).

Reagents for cells isolation, culturing and identification:

Dulbecco's modified Eagle's medium with Ham's F12 (DMEM-F12): (Dulbecco's Modified Eagle's Medium with 4.5g/L Glucose, with L Glutamine, Cat. N: BE 12-604 F, Lonza. B 4800 Verviers, Belginum) was in sterile 500 ml bottle, used as a culture medium to grow the olfactory stem cells extracted from the olfactory mucosa. It was stored at 4 °C.

Fetal bovine serum (FBS): was sterile filtered and cell culture tested. The solution was in sterile 100 ml bottle and was stored at –80 °C.

Penicillin-streptomycin solution contained Penicillin: (10,000 units/ml Penicillin G sodium) and streptomycin (10,000 µg/ml streptomycin in sulphate) it was sterile in 100 ml bottlesand was stored at –80°C.

Phosphate buffered saline (PBS): was sterile and filtered for cell culture in 500 ml bottles.It was stored at 4°C.

Trypsin/EDTA solution: (0.25% trypsin-0.05% ethylene diamine tetra-acetate (EDTA) suspension), 25300, Gibco. It was stored at – 80°C.

Collagenase enzyme solution type I: was purchased from Sigma-Aldrich Co.

Preparation of the working complete medium

The final composition of 100 ml of the complete medium was:

DMEM F12:89 ml.

FBS: 10 ml.

Penicillin G /streptomycin: 1ml.

Reagents for immunohistochemistry:

*Monoclonal antibodies for CD34, CD44, and nestin (Sigma) were used for characterization of the OSCs.

Microscopes:

Light microscope: (Leica with camera, Germany).

Inverted phase contrast microscope: (Olympus BX 50 with premiere 88-500 digital camera, Japan).

Isolation of stem cells from the olfactory mucosa and olfactory bulbs:

Ten adult male 6 months aged albino rats weighting about 200-250gm were anaesthetized by ether & sacrificed and the olfactory mucosa **(18)** and the olfactory bulbs **(19)** were collected under complete aseptic condition by skin dissection, skull decapitation and removal of the cribriform plate of ethmoid in the laminar flow cabinet. The collected tissues were cut into small pieces in a sterile petri-dish then incubated in falcon tubes containing 0.1 % collagenase type I (0.1g collagenase type I dissolved in 100 ml PBS) for 30 minutes at 37°C in the water bath**.** The cells washed twice more by re-suspending them in PBS, filtered by 100 um nylon mesh filter and centrifuged for 10 minutes at 1800 rpm.

Culture of the olfactory stem cells:

The cell pellet was resuspended in 10 ml complete medium that consists of 89% low-glucose Dulbecco's Modified Eagle's Medium with Ham's F12 (DMEM-F12) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were seeded in Petri-dishes at a density of 1×10^6 per dish in complete mediumandwere incubated in CO_2 incubator (humidified atmosphere containing 5% CO_2), at 37°C. After three days, washing was done and the non-adherent cells were removed and the medium was replaced with a fresh complete medium. The culture was monitored daily and the medium was changed every 3 days. After 10–14 days, when the colonies were evident, cells were passaged using

0.25% trypsin and 0.05 mmol/l EDTA. The OSCs Cultures were passaged twice a week and used at passages 1, 2, or 3 **(20)**.

Sub-culturing technique:

Each cultivation of cultured cells represents a passage. The media was removed and the cell sheet was washed by 10 ml PBS for 2-3 times. PBS was removed and 1ml of trypsin-EDTA was added to the Petri-dish to cover the cellsuniformly. To help cell separation gentle shaking of the dish was done then incubated at 37°C in the CO_2 incubator and dissociation follow up each 5-10 minutes using the inverted microscope. Once the cells started to detach from the substratum, dish shaking was done to accelerate dissociation. When complete detachment occurs; all the cells were rounded and floating, counteraction of the activity of trypsin-EDTA was done by adding 5ml of complete medium. Helping cell clusters breaking, the cell suspension was put in a falcon tube and gentle repeated pipetting was one. A sample of 0.5 ml aliquot was used for a cell count using the haemocytometer. The cells were subcultured in Petri-dishes at a density of 1×106 per dish in complete medium and used as a stoke for sub-culture (**21**).

Viability testing:

The trypan blue exclusion assay is done at the beginning and the end of culture to determine the survival of cells as following: The cells were trypsinized and the cell suspension was prepared in complete medium, (89 % DMEM-F12 + 10% FBS + 1% penicillin/streptomycin). A test tube containing a mixture of 100 µl of cell suspension and 100 µl of a solution of 0.4% trypan blue was prepared. This method is based onthe principle that viable cells do not take up certain dyes, whereas dead cells do as the trypan blue is a membrane lipid insoluble dye that is visible when it leaks into cells that have damaged cell membranes. After 2 minutes, a drop from the mixture was taken by a Pasteur pipette and added to a haemocytometer chamber. The viable cells appear transparent like bright circles while the dead cells appear blue in color, often with irregular edges (**22, 23**).

Characterization of the olfactory stem cells:

Characterization of the OSCs was done using immunocytochemicalmarkers specific for stem cells according to the criteria of the international society of cytotherapy **(24).** Including Nestin **(25)**, CD34 and CD44 markers **(26)**:

The medium of the third passaged cells was aspirated from the 35mm Petri dishes and the adherent OSCs were washed twice with PBS. The adherent cells were fixed by adding mixture of acetone/methanol (1:1) for 10 minutes. The acetone/methanol mixture was aspirated and the cells were washed with PBS. The dishes containing OSCs were treated with 0.3% hydrogen peroxide in methanol (30 min) to abolish endogenous peroxidase. The cells were incubated for 30 minutes at room temperature with CD34, CD44, and nestin monoclonal antibodies (1:200 dilutions in PBS) then washed twice by PBS. The cells were incubated with peroxidases conjugated rabbit anti-mouse IgG secondary antibody for 30 minutes at 37°C. Final wash three times with PBS was performed. A negative control was performed using only the secondary antibody to exclude any cross-reaction. The cells were examined with phase contrast microscope and CD34, CD 44, and nestin reaction was observed by the bright field. Positive reactions appeared as brownish discoloration in the cytoplasm.

Statistical analysis:

Parametric data including the number of viable cells and the number of positively stained ones were represented as mean ± standard error (SE). One-way analysis of variance (ANOVA) was used to compare the means of all the counted cells from all passages. The Tukey-Kramer post-hoc test was used to test the significance between them. The value of $P<0.05$ was statistically significant.

3. Results

This study was done in vitro by isolation of olfactory stem cells from the olfactory mucosa and olfactory bulb of adult male albino rats and culturing, proliferating them on suitable media. Ten adult male 6 months aged albino rats weighting about 200 and 250 gm. were used for cultivation of olfactory mucosa and olfactory bulbs. Culturing these stem cellswas done and the cells were expanded to form confluent cultures of adherent cells with a fibroblastic morphology.

Viability assay:

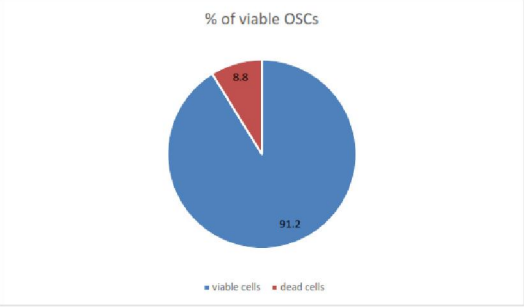

Chart 1: Percentage of viable OSCs from olfactory Mucosa and olfactory bulb of 10 male albino rats

Trypan blue is a vital stain used to selectively color dead cells blue. Due to selective permeability of the living cell membrane, trypan blue is not absorbed by living cells, but it can pass through the membrane

of dead cells. So that, the dead cells exhibit a distinctive blue color under a microscope. The viability of isolated OMSCs was 91.2 ± 3.4% (chart 1) through all passages without a significant difference ($p<0.5$).

Cultured olfactory stem cells:

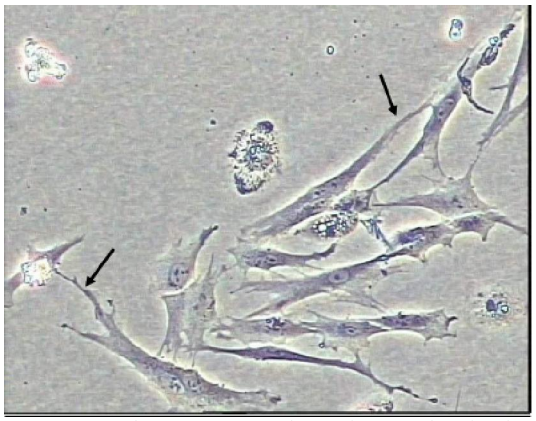

Figure 1: A phase contrast photomicrograph of primary cultured olfactory stem cells derived from rat olfactory mucosa and olfactory bulb 3 days after culture few population of cells begins to attach to the substratum and to gain processes (↑). Scale bar 100 µm. X100.

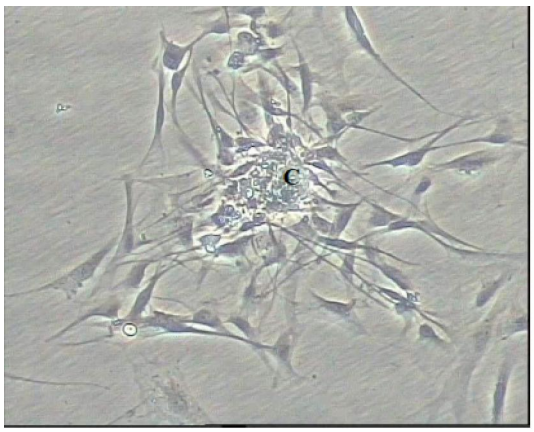

Figure 2: A phase contrast photomicrograph of primary cultured olfactory stem cells derived from rat olfactory mucosa and olfactory bulb 5days after culture showing the presence of a cell colony (C) and more than 30% confluency. Scale bar 100 µm. X100.

The OSCs adhere to the substratum after 3 days of cultivation showing a small population of single cells. The cells gained small processes and became spindle shaped, with single nucleus **(Fig. 1).** The non-adherent cells were removed with the 1st wash.

After 5 days of seeding, the cells proliferate showing increased number with relatively elongated processes. Cell colonies were evident **(Fig. 2).**

One week after plating, the population of cells became long, showing spindle shape, with a fibroblast-like appearance, with the appearance of large colonies **(Fig. 3).**

Two weeks after plating, the cultured cells reached about 80 -100 % confluency, showing different morphological characters being: spindle, star shaped, polygonal or fibroblast-like **(Fig. 4).**

The OSCs were polygonal or spindle shaped, with long processes after re-plating during the first **(Fig. 5),** second **(Fig. 6)**. And third passages **(Fig. 7)** and showed a single phenotypic population at these passages. All the samples were similar regarding the colony formation and cellular morphology.

Olfactory stem cells stained with Giemsa showed well-defined boundaries with central rounded nuclei and granular cytoplasm **(Fig. 8)**.

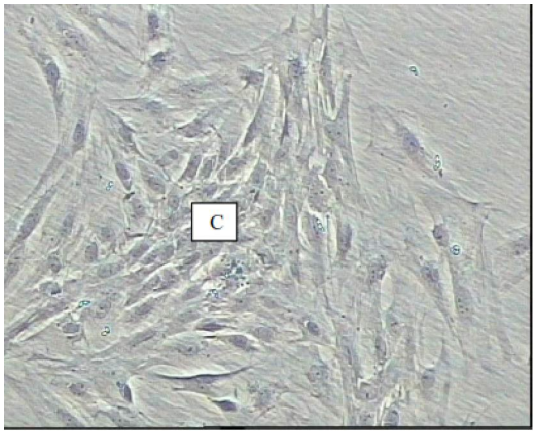

Figure 3: A phase contrast photomicrograph of primary cultured olfactory stem cells derived from rat olfactory mucosa and olfactory bulb one week after culture. The cells show large colony (C). Scale bar 100 µm. X100.

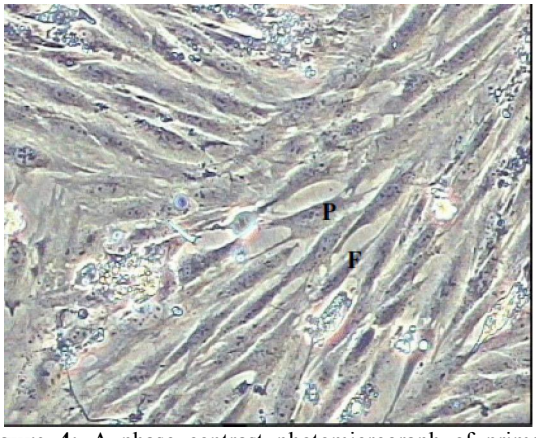

Figure 4: A phase contrast photomicrograph of primary cultured olfactory stem cells derived from rat olfactory mucosa and olfactory bulb 2 week after culture. The cells are confluent. Most of The cells are polygonal (P) and some are fibroblast-like (F). Scale bar 100 µm. X100.

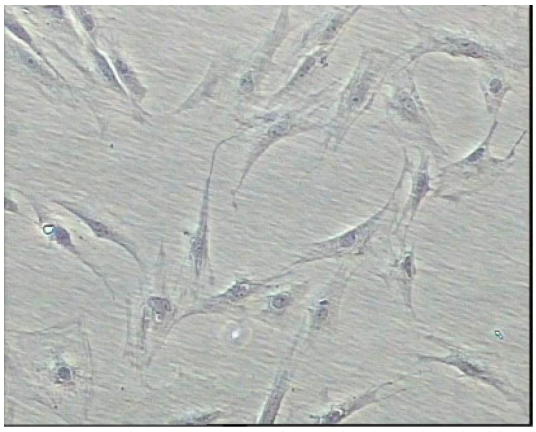

Figure 5: A phase contrast photomicrograph of olfactory stem cells derived from rat olfactory mucosa and olfactory bulb during the first passage showing numerous cells with granular cytoplasm with homogenous morphology of the cells. Scale bar 100 µm. X100.

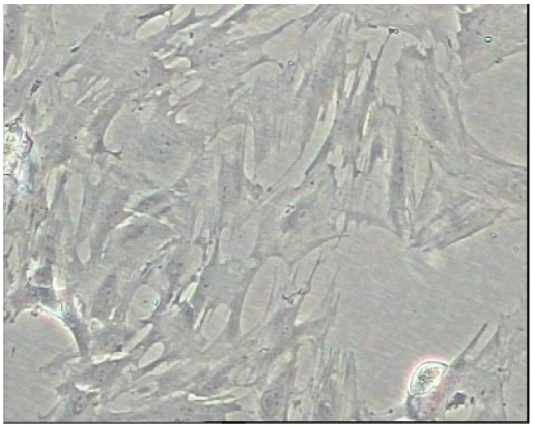

Figure 6: A phase contrast photomicrograph of olfactory stem cells derived from rat olfactory mucosa and olfactory bulb 2 days after the second passage showing numerous cells with granular cytoplasm with homogenous morphology of the cells and about 80 % confluency. Scale bar 100 µm. X100.

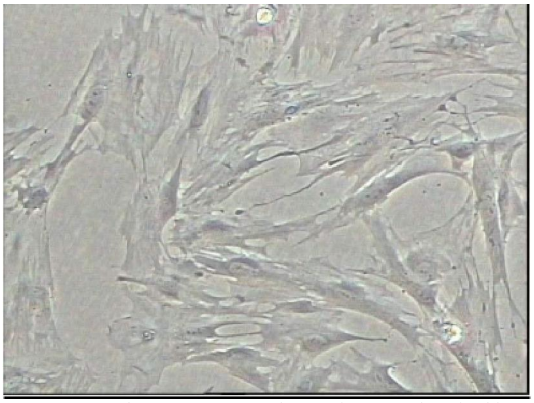

Figure 7: A phase contrast photomicrograph of olfactory stem cells derived from rat olfactory mucosa and olfactory bulb 2 days after the third passage showing homogenous morphology of the cells with about 90 % confluency. Scale bar 100 µm. X100.

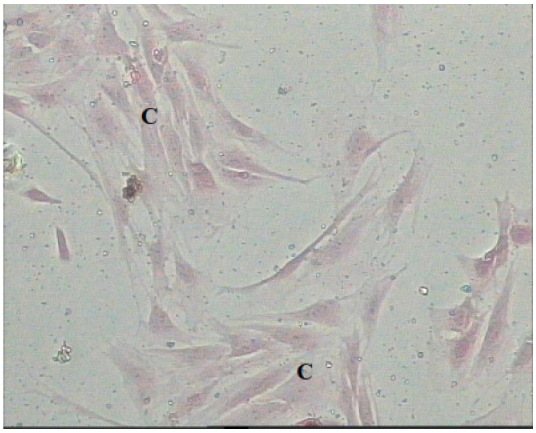

Figure 8: A photomicrograph of olfactory stem cells derived from rat olfactory mucosa and olfactory bulb. stained with Giemsa during the second passage. The cells are well defined with single nucleus and granular cytoplasm with the presence of a cell colony (C). Scale bar 100 µm. X 100.

Characterization of the olfactory stem cells

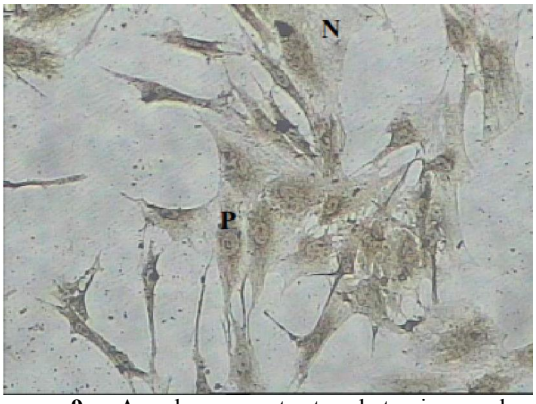

Figure 9: A phase contrast photomicrograph of Immunostaining for surface antigen CD44 in a monolayer of rat olfactory stem cells during the second passage. Most of the cells are positively stained (P) with few cells are negatively stained (N). Scale bar 100 µm. X100.

Identification and characterization of the stem cells derived from olfactory mucosa and bulbs was proven immunohistochemically using monoclonal antibodies against CD34, CD44, and Nestin with the cells of the third passage. The cells were positive for CD44 **(Fig. 9)** and Nestin **(Fig. 10)** in the form of brown cytoplasmic staining. Conversely, they were negative for CD34 **(Fig. 11).** Ten stained fields for each stain were examined and the number of positivecells showed a non-significant difference between them ($p<0.1$). The isolated stem cells were not contaminated with other cell lineages, more than 95±3% readily differentiated into mesenchymal

lineages **(Table 1)** and most of the cells had colony-forming capacity, which was maintained up to the 3rd passage.

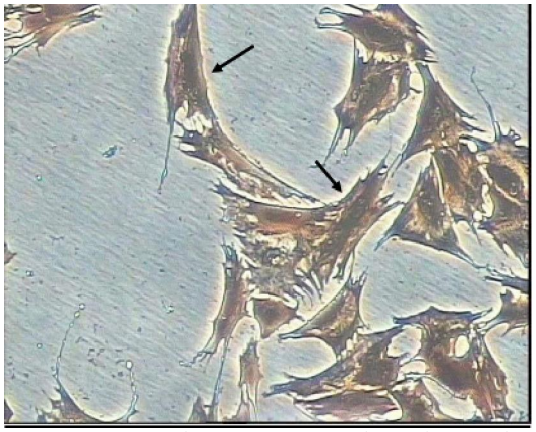

Figure 10: A phase contrast photomicrograph of Immunostaining for surface antigen nestin in a monolayer of rat olfactory stem cells during the second passage. The cells (↑) are positively stained. Scale bar 100 µm. X 100.

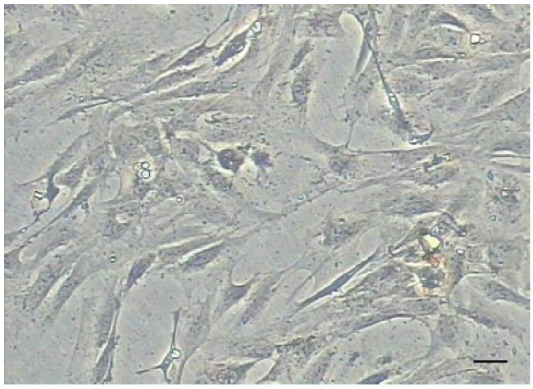

Figure 11: A phase contrast photomicrograph of Immunostaining for surface antigen CD34 in a monolayer of rat olfactory stem cells during the second passage. The cells are negative for CD34. Scale bar 100 µm. X100.

Table 1: Percentage of positively stained olfactory stem cells separated from olfactory mucosa and bulb of adult male albino rats

Stain	Percentage ± standard deviation
CD44	96±3%
Nestin	95±2%
CD34	5±2%

4. Discussion

Stem cells field of research has emerged rapidly having great interest among clinicians and scientists with the name of "regenerative medicine **(27),** as stem cells can be isolated from many tissues either from human or animals producing curative treatment by replacing the damaged cells **(28)**.

Our study presents a detailed protocol for isolation, culture and characterization of OMSCs from the olfactory mucosa and olfactory bulb of the adult male albino rats and this was in correlation with the results of the studies done by **Kuijtenet al., 2014** who detected the olfactory mucosa derived mesenchymal stem cells (OM-MSCs) in the olfactory mucosa of rats **(29), Grittiet al., 2002** who isolated the OSCs from the olfactory bulbs of rodents **(30),** and **Curtiset al., 2007** who detected that, the human neuroblasts migrate from the wall of lateral ventricles to the olfactory bulb **(31).**

Delorme et al., 2010 described the OSCs as a highly proliferative cell type and this was confirmed in our study during culture, although we harvested a very small tissue pieces, several millions of cells were derived in less than one month duration and we approve that rat OSCs can be successfully amplified by successive cell passages **(7).**

The OSCs were characterized immuno-histochemically in our study by being positive for Nestin and CD44 and this was correlated with **Sayuriet al., 2010** and **Jean-Claudeet al., 2014** results who demonstrated that the OSCs are positive for Nestin **(25, 20).** Also, the sphere formation and immuno-positivity for Nestin are two characteristics of the OSCs recognized by **Delorme et al., 2010 (7).** Moreover, the OSCs were proved to be positive for CD44 by **Hosseiniet al., 2015(26)**.

In our study, the OSCs harvested on complete media composed of (89% DMEM-F12 supplemented with 10% FBS and 1% Penicillin-Streptomycin). we use DMEM-F12 medium because it is stable, improves cell viability and growth, potentially increases the proliferation of stem cells and minimize toxic ammonia build-up simulating **Pagano et al., 2000** and **Ercolin et al., 2016** who used DMEM-F12 to culture OSCs derived from olfactory mucosa and olfactory bulb **(32, 33 & 34)** and other types of stem cells such as human embryonic stem cells **(35),** human-induced pluripotent stem cells **(36),** neural stem cells **(37)** andhair-follicle bulge stem cells **(38).**

We added FBS to the culture media as it is the most widely used serum-supplement for *in vitro* cell culture of eukaryotic cells**(Van der Valk et al., 2010) and (Jochems et al., 2002**) because of its very low antibodies level, its high growth factors that allows proliferation in various different cell culture applications and its richness of variety of proteins that allow cultured cells maintenance in a medium where they can survive, grow and divide **(39, 40).**

To prevent bacterial growth we added 1% Penicillin-Streptomycin to the culture medium **(41).** In addition, DMEM F12 supplemented with 15% bovine fetal serum and 1% Penicillin-Streptomycin was used

by **Ercolin et al., 2016** to culture the OSCs obtained from the rabbit olfactory mucosa **(42).**

In the present study, most of the OSCs shows the characters of mesenchymal stem cells as they were attached to the substratum of the plastic Petri dishes with polygonal with fibroblast-like morphology possessing small body and few long cytoplasmic processes and formed cell colonies in the first culture and these characters were in correlation with the International Society for Cellular Therapy information's that has proposed that, the different types of mesenchymal stem cells including the OSCs show plastic adherent properties under normal culture conditions and has a fibroblast-like morphology **(43).** The results of **(Girard et al., 2011)** study confirmed the polygonal and fibroblast-like morphology of the cells with few long processes and small cell body during the culture of the isolated OSCs from the olfactory mucosa of rats and human **(44).** Also, the results of **(Ercolin et al., 2016)** study detected that the OSCs were able to adhere to the culture plastic dishes showing fibroblastic morphology with colonies formation, so it can be classified as MSCs **(42).**

The proliferation and clonogenic capacity of stem cells is detected by formation of colonies. As regard the OSCs colonies that formed in our study, it were characterized by the fibroblast like features of the cells with long processes and this was in correlation with the results found by **(Mumm et al., 1996)** who detected that, the stem cells culture derived from the mice olfactory epithelium formed colonies of fibroblast-like cells with small cell bodies and long processes **(45),** and **(Soleimani et al., 2004)** who detected that, the OSCs culture derived from the rats olfactory bulb presented colonies of spindle shaped cells in the primary culture **(46).**

The formed OSCs colonies in our study looks like the colonies formed by MSCs derived from the human placenta **(47)**, human umbilical cord MSCs **(48)**, BM-MSCs **(49)** and the culture of the multipotent stem cells derived from the mucosa of human fallopian tubes **(50),** as all showed fibroblast like features and the latter cells developed similar colonies of fibroblast-like cells with few long processes.

The human embryonic stem cells when cultured give haematopoietic colony forming cells that develop sphere colonies of small rounded cell bodies with no or short cell processes **(51),** while the muscle stem cells that are derived from the skeletal muscles when cultured show colonies of satellite cells with small bodies and numerous processes **(52).**

In the current study, the number of viable cells was determined by the trypan blue assay according to **(Avelar-Freitas et al., 2014) (53),** the viability of the OSCs was more than 94.2 ± 3.4% through the different passages and this was in correlation with consistent with the results of **(Diaz-Solano et al., 2012)** study who used the human olfactory mucosa derived mesenchymal stem cells to enhance the survival, proliferation and differentiation of human hematopoietic stem cells **(54).**

Finally, according to our study results, the stem cells of the olfactory mucosa and olfactory bulb of the adult albino rats can be successfully isolated and can efficiently proliferate when cultured on suitable media to produce large number of olfactory stem cells.

Conclusion

The olfactory mucosa and olfactory bulbs of the adult male albino rats contain olfactory stem cells that can be isolated and cultured on DMEM-F12 medium to proliferate and expand in culture to give large number of neural stem cells with the hope of the possibility to use these cells in management of neuro degenerative diseases as they share the morphological and phenotypic characteristics of mesenchymal stem cells.

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