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Differentiation of Adipose-Derived Mesenchymal Stem Cells into Neuron-Like Cells induced by using β-mercaptoethanol

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Abstract:

Background: Adipose derived-mesenchymal stem cells have been used as an alternative to bone marrow cells in this study. Objective: We investigated the in vitro isolation, identification, and differentiation of stem cells into neuron cells, in order to produce neuron cells via cell culture, which would be useful in nerve injury treatment. Method: Mouse adipose mesenchymal stem cells were dissected from the abdominal subcutaneous region. Neural differentiation was induced using β-mercaptoethanol. This study included two different neural stage markers, i.e. nestin and neurofilament light-chain, to detect immature and mature neurons, respectively. Results: The immunocytochemistry results showed that the use of β-mercaptoethanol resulted in the successful production of neuron cells. This was attributable to the increase and significant overexpression of the nestin protein during the early exposure period, which resulted in the expression of the highest levels of nestin. In comparison, the expression level of neurofilament light-chain protein also increased with time but less than nestin. Non-treated mesenchymal stem cells, considered as control showed very low expression for both markers. Conclusion: The results of this study indicate that adipose mesenchymal cells represent a good, easily obtainable source of bone marrow cells used to developing the differentiation process.

Key words: Adipose cells, Differentiation, Nestin, Neurofilament, Neurogenesis.

Introduction:

Mesenchymal stem cells (MSCs) exhibit an exceptional ability to differentiate into different cell types (1-3). Thus, the use of an approach involving MSCs is promising for the treatment of different neural diseases, because of their ability to produce neural progenitors (4). Neural progenitors and stem cells have been shown to differentiate into different neuronal phenotypes that can potentially be used to treat different human neural diseases (5). Bone marrow (BM) is the first source of choice for obtaining stem cells. However, as the associated technique requires us to extract stem cells from the bone marrow via aspiration (6), there is a need for the use of a less invasive method for stem cell extraction. Adipose tissues act as a source for the unlimited supply of mesenchymal stem cells (7). Adipose tissue is a loose connective tissue located in subcutaneous or visceral depots. It provides energy for metabolism and regulates thermogenesis, immune responses, and lactation (8).

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MSCs are one of the main components of adipose tissue, and have been isolated and detected in vitro using several methods, including those with low and high specificities (9). Adipose tissue mesenchymal stem cells (AMSCs) can adhere to aplastic culture flasks; thus, they can be grown in vitro as other types of MSCs that exhibit essential characteristics and the capacity to differentiate into multiple cell lineages, which renders them powerful and beneficial after their isolation (10). They have the phenotypic and genotypic characteristics of umbilical cord- and bone marrow-derived MSCs and they express mesenchymal cell markers CD105 and CD44 (11). Moreover, they have high proliferation rates and multiple differentiation potentials that enable them to exhibit the capabilities of umbilical cord- and bone marrow-derived MSCs (12). Adipose tissue-derived MSCs reportedly exhibit the potential to differentiate into multiple cell types, including neuron-like cells (13, 14). The differentiation properties of different kinds of MSCs are the same, even if they are obtained from different sources, such as from the umbilical cord, bone marrow, and adipose tissue; therefore,

the factors and differentiation media involved in the differentiation processes are the same (15). In a previous study conducted by our team, we used β-mercaptoethanol (BME) to induce neurogenesis in BM-MSCs, to produce neural progenitors, stem cells, and neurons (16). Moreover, nerve regeneration and repair are viable therapeutic approaches either by stem cells or by other approaches such as laser therapy (17). The aim of this research is the identification of a more accessible and alternative source of MSCs that would be highly promising, as it would allow neuronal cells to be produced and used for nerve injury treatment.

Materials and Methods:

Adipose Derived-Mesenchymal Stem Cell Isolation

The mice used in this study were provided by the Iraqi Center for Cancer and Medical Genetic Research (ICCMGR), at Mustansiriyah University (animal house unit). The ICCMGR scientific committee approved all the animal procedures. The adipose MSCs were prepared using the modified method described by Yamamoto and Sung (18, 19). Briefly, 6-9 weeks old donor male mice were killed by cervical dislocation. Their fur was washed with 70% alcohol, and the abdomen was exposed. The adipose tissue was separated from subcutaneous sites and cut into small fragments, and then transferred into a petri dish containing serum free minimum essential medium (MEM) (US biological, Massachusetts, USA), supplemented with 500 µg/ml streptomycin and ampicillin (Capricorn-Scientific, Germany). In the laboratory, under sterilizing conditions, the tissues were washed 3-4 times with phosphate-buffered saline (PBS) and suspended in PBS supplemented with 0.1% collagenase type I (US Biological, USA) that was pre-warmed at 37 ºC, then the tissue was incubated at 37 ºC for 45 minutes with shaking. Then, centrifugation was performed at 1500 rpm for 5 minutes. Then, pellets (containing the cell suspension) were cultured in a growth medium composed of MEM supplemented with 20% fetal bovine serum (FBS) (Cellgro, USA); it was also supplemented with 100 µg/ml each of penicillin and streptomycin. The cells were cultured in 25 cm^2 cell culture flasks and incubated with 5% CO_2 in 95% humidified air at 37 ºC.

Maintenance of Adipose MSCs

After 24 h, the cells were allowed to become attached to the culture flask surface overnight, and non-adherent cells were removed while changing the medium after 24 h, using growth media composed of 20% FBS MEM. The culture medium was changed every 3-5 days, until the cultured cells formed colonies of adherent cells, which took about a week to form a confluent monolayer of cells. Later, the monolayer of cells was passaged using 0.25% trypsin-EDTA (US biological, Massachusetts, USA) (20).

Immunophenotyping of AMSCs

The cells were seeded at a concentration of 3 $\times 10^5$ cells in an 8-chamber tissue culture slide (IWKA, Japan). After confluency was achieved, the cells were fixed for 10 minutes in 4% neutral buffered formalin.

The fixed slides were first blocked with 1% hydrogen peroxide (H_2O_2) for 10 minutes; then, they were incubated with 1.5% blocking serum for one hour (Santa Cruz Biotechnology, USA). Then, 1:100 diluted primary antibody (mAb anti-CD105 and mouse IgG mAb anti-CD44 (US biological, USA) was added and the solution was incubated overnight at 4 ºC in a humidified chamber. The anti-mouse IgG biotinylated secondary antibody was added after extensively washing cells with PBS. Then, the solution was incubated for one hour, and washed 2-3 times with PBS. Avidin conjugated to horseradish peroxidase (HRP) was added to each slide, which was then incubated in a humidified chamber for another 30 minutes and washed 2-3 times with PBS. The DAB chromogen solution mix was added to the slide for 20 minutes, and it was counterstained in hematoxylin stain for 2 minutes. The slides were covered and examined using a light microscope (Micros, Austria). The assay was repeated in triplicate.

Induction of neuron differentiation for adipose-MSCs

The adipose-MSCs obtained after the first passage step were induced to differentiate into neurons through exposure to 1 mM β-mercaptoethanol (BME) (Santa Cruz Biotechnology, USA) for 25, 27, 29, and 34 h; these exposure periods were classified as follows: in early differentiation media, cells were exposed to 20% FBS MEM media supplemented with 1 mM BME for 24 h. Then, they were subjected to a second round of exposure using late differentiation media, to induce neural differentiation, by exposing cells to serum-free MEM media supplemented with 5mM BME for different time periods (1, 3, 5, and 10 h) Lei et al., 2007 (21). The differentiated cells were fixed in order to perform the immunocytochemistry assay. Two different protein markers were detected during the differentiation process, using 1:50 diluted nestin (NES), (Santa Cruz Biotechnology,

USA) and neurofilament light-chain (NF-L) (diluted 1:100), (US biological, USA), and the stained cells were detected using a light microscope. The positive cells were counted, and the percentage was expressed as positive to non-positive cells for the specified marker.

Statistical Analysis

Statistical analysis of the immunocytochemistry assay data was performed using One Way ANOVA and the LSD test in IBM SPSS Statistics Software (version 20), for determining the average percentage mean values; the difference in the mean values was considered significant if $p<0.05$.

Results:

Isolation and expansion of AMSCs in culture

The adipose derived MSCs were cultured successfully. After the primary culture process was performed for 24 h, a few cells were observed to adhere to the flask surface and form small colonies, usually after 48 h (Fig. 1- A and B). The morphology of the adherent cells was fibroblast-like, and they grew to form larger, separated colonies. Then, by the 5th day, the cells became more densely spread; the colonies expanded in size and achieved up to 80% confluency (Fig.1-C). At the first passage step, the AMSCs formed a homogeneous, confluent monolayer of fibroblastoid-like cells after 5-7 days (Fig.1- D).

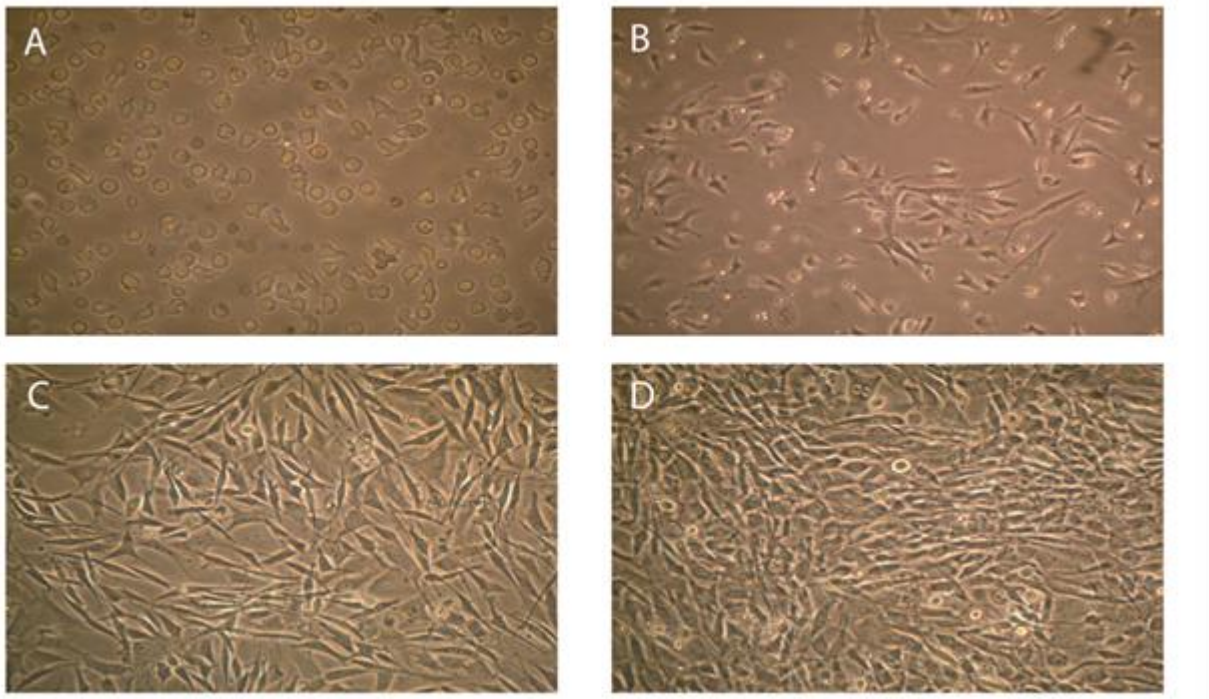

Figure 1. Morphological features of Adipose MSCs (A, after 24 h; B, after 48 h; C, after forming a monolayer; D, after forming a monolayer in passage 1). Phase contract pictures were obtained using an inverted microscope at a 10X magnification.

Immunophenotypic characterization of AMSCs

AMSCs were studied to determine their immunophenotype, via the immunocytochemistry analysis of the MSC markers CD105 and CD44. The AMSCs were stained in brown color, as they were positive for both CD44 and CD105 (Fig. 2A and B).

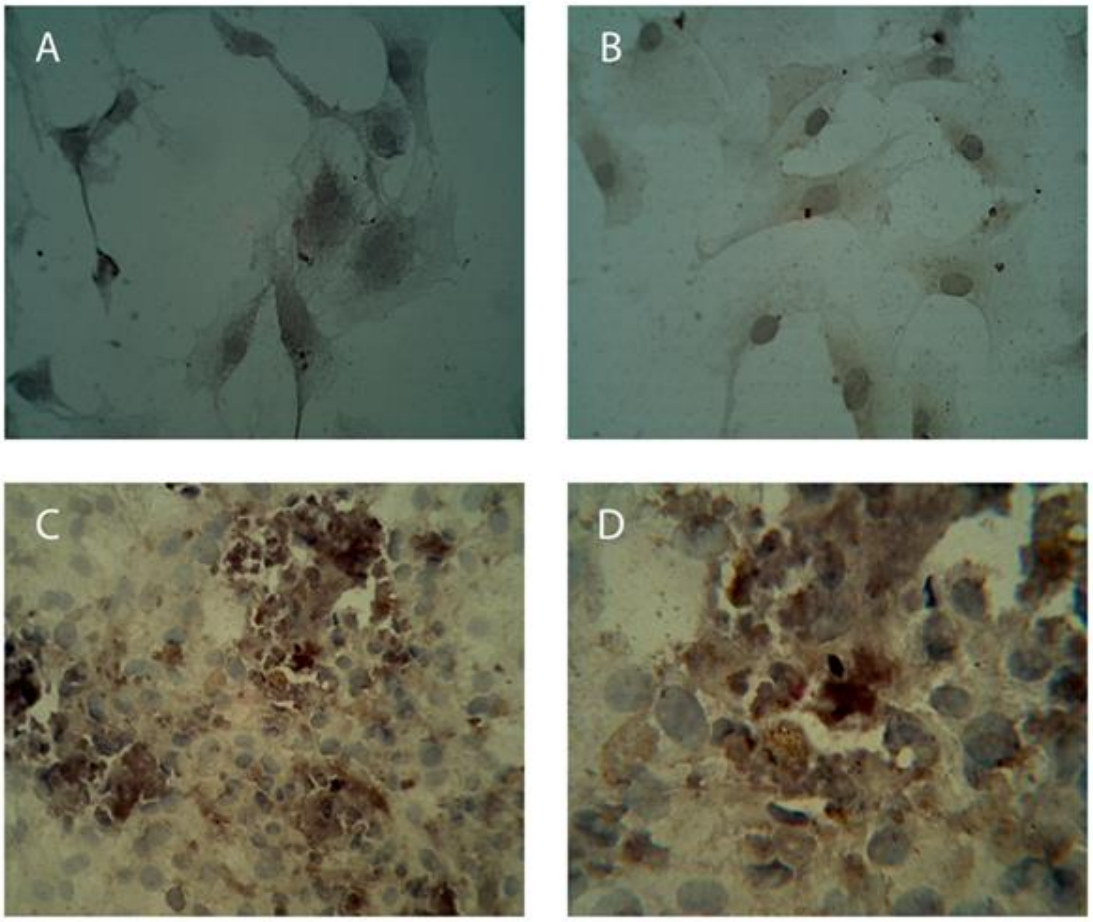

Figure 2. Immunophenotypic assay of AMSCs at the first passage step. (A, negative control at 40X; B, cells positive for CD105, 40X; C, cells positive for CD44 at 10X; D, cells positive for CD44 at 40X).

Neural induction from AMSCs

The first passage of AMSCs was carried out to achieve neural cell differentiation, using BME. The morphology of AMSCs after the differentiation process was more spherical than that observed before differentiation, during the first 24 h (early differentiation media) (Fig. 3A and B). Late differentiation media were used at 25 h, 27 h (Fig. 3C and D), 29 h (Fig. 3E and F), and 34 h, and cells became differentiated, larger in size, and finally formed branches of neuronal cells (Fig. 3 G and H). ICC assay analysis revealed that protein expression was upregulated, when the amounts of NES and NF-L expressed were 85% and 23%, after undergoing differentiation for 25 h, in comparison to the levels observed with non-differentiated cells (Fig. 4A and B). Furthermore, the protein expression levels of both markers were increased further at 27 h and 29 h (Fig. 4C and D, respectively), and the difference in levels was significant if $p < 0.05$. Higher protein expression levels were observed at 29 h (90% and 40.65% for NES and NF-L, respectively), compared with those observed with undifferentiated MSCs (as a negative control), as shown in Fig. 5.

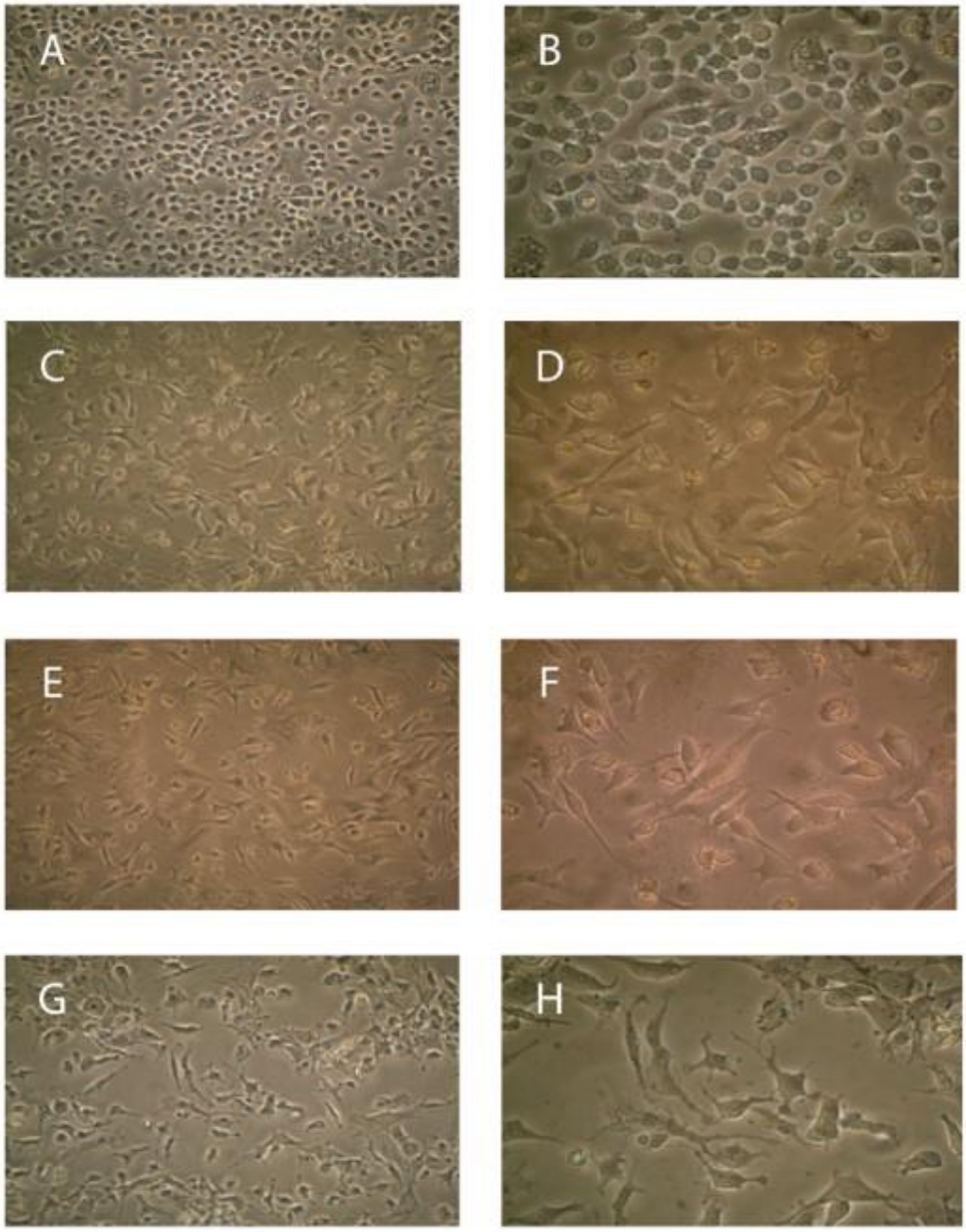

Figure 3. Adipose MSCs observed after the induction of differentiation using BME. A) AMSC morphology was observed to be more spherical after differentiation than that observed before differentiation, after the first 24 h (early differentiation media) at 10X; B) the same cells were observed at 40X; C) Late differentiation media after 27 h, at 10X; D) at 40X; E) differentiated cells after 29 h, at 10X; F) differentiated cells at 40X. G) after undergoing differentiation for 34 h, cells were increased in size, and finally formed the branches of neuronal cells, as seen at 10X and H) at 40X. Phase contract pictures were obtained using an inverted light microscope.

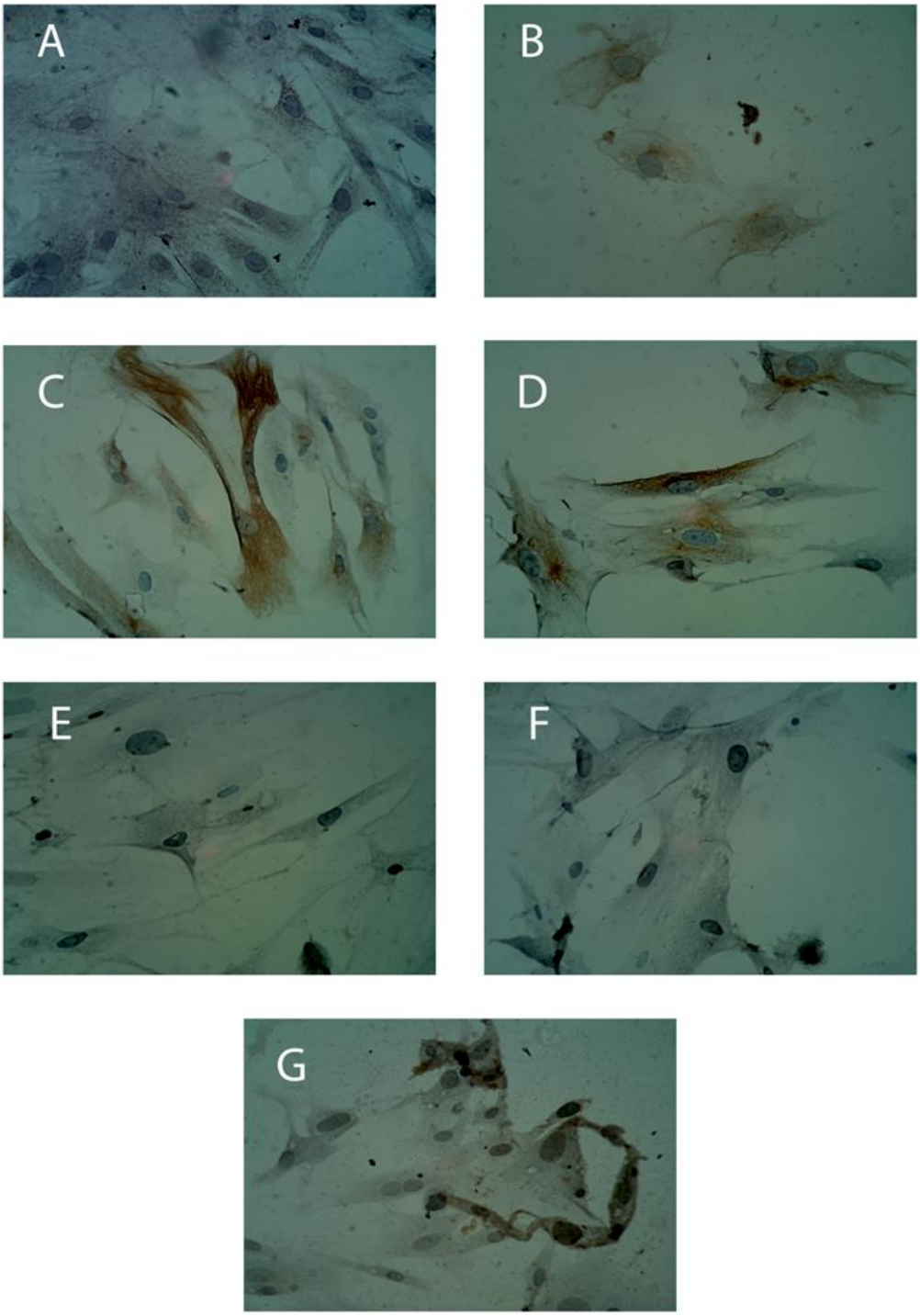

Figure 4. Immunocytochemistry assay for the differentiated AMSCs. (A) Non-induced AMSCs were not found to be positive for both nestin and NF-L, after cell staining; B) induced cells were mildly positive for NES after 25 h; C) differentiated cells were strongly positive for NES after 27h of induction; D) cells were similarly seen to be strongly positive after staining cells for nestin, 29 h after induction; E) induced cells were stained and found to be negative for the NF-L marker, after they underwent induction for 25 h; F) after undergoing induction for 27 h, the NF-L proteins were mild expressed. G) After being induced for 29 h, cells were strongly positive for the NF-L protein, which proves that the cells had started to transform into neurons. All figures have been obtained at a magnification of 40X.

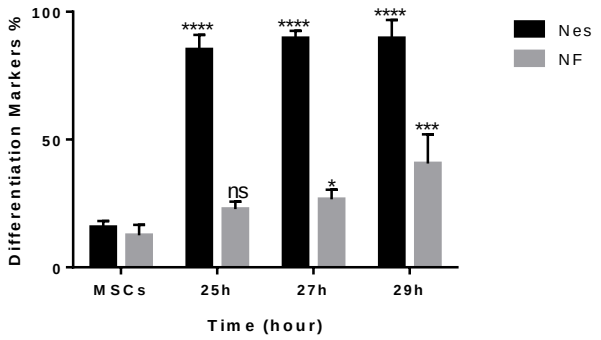

Figure 5. Percentage of cells expressed nestin and NF-L comparing to non-treated cells is shown at different time intervals, after the ICC assay. These results show the expression of the NES marker from the early exposure period onwards, along with their significant difference levels in compare to control MSCs cells; they also indicate the expression levels of the NF protein increased during the late exposure period. All time intervals measured compared to non-treated MSCs.
Note: The symbol "*" indicates that the differences were significant if p> 0.0001.

Discussion:

In the current study, we successfully isolated mesenchymal stem cells from subcutaneous adipose tissue that exhibited the same potential of differentiation as that of bone marrow-derived mesenchymal stem cells. Many researchers sourced mesenchymal stem cells from adipose tissues and bone marrow, as they can be used in regenerative medicine (3, 22, 23). The obtained results of ICC showed that cultured adipose MSCs were positive for mesenchymal cell markers CD44 and CD105. Mesenchymal stem cells derived from adipose tissues contain a fraction of cells that express certain mesenchymal stem cell-specific antigens and do not express hematopoietic stem cell antigens (24). Adipose MSCs are reported to be utilized in various applications more frequently than stem cells from embryonic origins, because of the fewer ethical concerns and low immunogenicity associated with AMSCs (25). Furthermore, AMSCs have attracted attention because they are easy to obtain and can be transplanted safely into an allogeneic or autologous host, and show a higher cellular activity (26). The results of differentiation experiments showed that BME is a good inducer of neurogenesis in AMSCs. Several supplementary trophic factors enhanced cell growth and neuronal differentiation in AMSCs in vitro, including melatonin, BME, CHIR99021, neurotrophin-3, and lithium chloride (27-32). BME, which is an anti-oxidant reagent, was most commonly used at low concentrations for cell differentiation and therapy (33). Zinc was also suggested to enhance AMSC proliferation and induce neuronal differentiation (34).

Immunocytochemistry results confirmed that cells were differentiated into mature neurons, as the differentiated cells were positive to the neuronal markers NES and NF-L. NES, NF-L, and MAP-2 were found to be the most important markers for the evaluation of the neurogenesis process (16). Moreover, this research found out that there was an association between the marker expressed cells percentage and the observed morphological changes, such as the spherical shape of cells and branches of neural cells.

Another study showed that cells undergoing the neural differentiation process display elevated expression levels of neural progenitor markers, such as NES, along with low expression levels of SSEA-4. In addition, these cells were found to be elongated and express chain neurofilament (NF-M) and post-neuronal markers, such as βIII-tubulin, NeuN, and MAP2 (35). Furthermore, it was reported that a population of cells in adipose tissues have neuronal characteristics that enable them to participate in the repair of damaged neural tissues. In vivo experiments showed that adipose-derived stem cells could act as a possible source of brain progenitor cells and that they are clinically important in neurological applications (36). Moreover, their efficiency can be improved by the addition of growth factors to media formulations, as this would enhance the efficiency of differentiation of adipose tissue into neural stem cells (37).

Conclusions:

The protocol described in this study using BME can be used to induce neurogenesis in adipose tissue-derived MSCs in a manner similar to that observed for bone marrow derived MSCs. This is highly advantageous, as the isolation of MSCs from adipose tissues is easier and less invasive than the extraction of bone marrow MSCs. This advantage should encourage the use of adipose tissue MSCs in future applications for the treatment of central and peripheral nervous system injuries.

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Conflicts of Interest: None.

The author has signed on animal welfare statement.

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تمايز الخلايا المزنكيمية المشتقه من النسيج الدهني الى خلايا عصبية باستعمال البيتا ميركابتوايثانول

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الخلاصة:

تم استخدام الخلايا الجذعية المشتقة من Adipose cells كبديل لخلايا نخاع العظام في هذه الدراسة. الهدف: تم عزل الخلايا في المختبر، وتحديد هوية الخلايا الناتجة، وبعدها تحفيز تمايزها من خلايا جذعية الى خلايا عصبية من أجل إنتاج الخلايا العصبية والتي من شأنها أن تكون مفيدة في علاج إصابات الأعصاب. طرائق العمل: تم عزل الخلايا الجذعية المزنكيمية من الخلايا تحت الجلد لجذع الفئران المختبرية. بعدها تم تحفيز تمايز الخلايا الناتجة الى خلايا عصبية باستخدام مادة تحفيزية هي β-mercaptoethanol . ولغرض اثبات تمايز الخلايا الناتجة تم استخدام نوعين من الماركرات لمرحلتين متتاليتين من مراحل انتاج الخلايا العصبية وهما nestin وهو ماركر موجود في المراحل الاولية للانتاج (أي الخلايا العصبية في المراحل الاولية للتمايز او غير الناضجة) و neurofilament light-chain وهو ماركر موجود في المرحلة المتقدمة (أي الخلايا العصبية المكتملة التمايز او الناضجة). النتائج: أظهرت نتائج الكيمياء المناعية ICC نجاح استخدام مادة الـ β-mercaptoethanol في إنتاج الخلايا العصبية. ويعزى ذلك إلى الزيادة التصاعدية والإفراط الكبير في التعبير عن بروتين nestin خلال فترات التعرض المختلفة لمادة التمايز العصبي. وبالمقارنة كان مستوى التعبير عن بروتين neurofilament light-chain أقل خلال فترات التعرض لمادة التمايز العصبي بالمقارنة مع الخلايا الجذعية المزنكيمية قبل التمايز والتي استخدمت للمقارنة والتي أظهرت مستويات تعبير منخفضة جدا لكلا الماركرات. الاستنتاج: تشير نتائج هذه الدراسة إلى أن خلايا الـ Adipose تمثل مصدراً جيداً لانتاج الخلايا العصبية ويمكن الحصول عليها بسهولة مقارنة مع خلايا نخاع العظام المستخدمة عادة في الانتاج.

الكلمات المفتاحية: الخلايا الدهنية، التمايز، Nestin، Neurofilament، Neurogenesis.