

Original Article	Growth Kinetics of the Rat Mesenchymal Stem Cells from the Bone Marrow and Adipose Tissue. A Comparative Study
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ABSTRACT

Background: The bone marrow has been considered as a main source for isolation of multipotent mesenchymal stem cells (MSCs). They are still the most frequently investigated cell type and often identified as the gold standard. However, a similar cell population has been also isolated from other tissues such as adipose tissue. Unlike bone marrow, the adipose tissue is abundantly accessible source of stem cell that can give a good yield in culture.

Aim of the work: Was isolation of the rat bone marrow MSCs (BM-MSCs) and adipose tissue MSCs (AD-MSCs) and assessing their growth kinetics.

Material and Methods: The rat bone marrow and adipose tissue were isolated from 10 male adult albino rats and cultured and expanded through 6 passages. BM-MSCs and AD-MSCs biological characteristics evaluated for cell therapy (morphology, flow cytometric analysis, colony-forming unit-fibroblast assay, proliferation capacity at passages 2, 4 and 6, population doubling time (PDT) and cell growth curves).

Results: BM-MSCs and AD-MSCs attached to the culture flask and displayed spindle-shaped morphology, more evident in AD-MSCs. Proliferation rate of AD-MSCs in the analyzed passages was more than BM-MSCs. The increase in the PDT of both types of MSCs occurred with the increase in the number of passages.

Conclusion: The rat AD- MSCs have growth kinetic advantages in the proliferative capacity, colony-forming unite fibroblast and population doubling time more than that of BM-MSCs. these advantages should be considered when choosing a stem cell source for specific clinical application.

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INTRODUCTION

The bone marrow-derived MSCs (BM-MSCs) were first discovered by *Friedenstein et al. (1974)* as an adherent fibroblast-like population within the bone marrow stroma. They have the ability to differentiate into mesoderm derived lineages such as adipocytes, chondrocytes, osteoblasts, and myoblasts (*Ferrari et al., 1988; Woodbury et al., 2000; Scilling et al., 20007 and Satija, 2009*) as well as into neuro-ectodermal derived lineages (*Lee et al., 2013*). The biological characteristics,

experimental and clinical applications of them were described in many researches (*Wang et al., 2012*).

Although the bone marrow has been considered as a main source for isolation of multipotent MSCs for clinical use, it has raised several issues worthy of addressing, including the procedure of bone marrow harvest with related pain, side effects, and morbidity in addition to the low cell yield (*Anker et al., 2003*). Many researchers have investigated alternative sources of MSCs such as liver, lung, spleen, dental pulp, peripheral blood, umbilical

cord (Anasetti et al., 2012; Kisiel et al., 2012; Mahdiyar et al., 2014 and Ding et al., 2015), and adipose tissue (Mehrabani et al., 2015).

The adipose tissue, as well as bone marrow, originate from the mesenchyme and contain a supporting stroma that could be readily available for isolation. Unlike bone marrow, adipose tissue is abundantly accessible source of stem cell that can be collected in a large volume with minimal morbidity, which therefore reduces the time in culture and expense required to generate a therapeutic cell dose (Kretlow et al., 2008).

MSCs proliferation and senescence have been considered important issues by researchers aiming to use them for therapeutic purposes. In vitro expansion of MSCs is required to generate a pure cell population in a sufficient amount for the clinical application. During this expansion process, cells enter cellular senescence, which leads to a gradual reduction of their potency and significant changes in protein expression (Jo et al., 2008 and Kretlow et al., 2008).

Comparative studies of the growth kinetics of BM-MSCs and AD-MSCs are lacking. Therefore, aim of the current study was to isolate, culture and compare rat BM-MSCs and AD-MSCs for their growth characterization, proliferation capacity using growth curve analysis, colony-forming unit fibroblast assay (CFU-F) and population doubling time (PDT).

MATERIAL AND METHODS

Animals: Ten adult male albino rats were used as a source of bone marrow and adipose tissue. Their ages were 6 months and their weight ranged between 200 and 250 grams each. All procedures are in accordance with animal guideline care of Ethical Committee of the medical research center, Ain Shams University.

All applicable institutional and national guidelines for the care and use of laboratory animals were followed. They were housed under standard conditions for a week prior to use.

Isolation and culture of AD-MSCs (Mehmet et al. 2012)

The animals were sacrificed by cervical dislocation. Omental and pre-renal adipose tissue was isolated and collected in 15 ml sterile tubes. Under sterile conditions, the excised adipose tissue was rinsed with phosphate-buffered saline (PBS, Sigma-Aldrich), minced into small pieces and digested with collagenase type I (0.1%) (Sigma-Aldrich) at 37°C for 30 minutes. The

samples were neutralized with an equal volume of Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco) and centrifuged at 1800 rpm (rate per minute) for 10 minutes. The pellet was then filtered through a 200 µm stainless steel mesh (Sigma) to remove undigested tissue.

Adipose digested cells were suspended in DMEM supplemented with 10% FBS, 1% penicillin and streptomycin (Sigma-Aldrich) and cultured in 75-cm² flasks. The cultures were incubated at 37°C with 5% CO₂ and saturated humidity. The first culture media was changed after 24 hours to remove non-adherent cells and the adherent cells were cultured and passaged to expand the MSCs population. The subsequent medium exchange was performed every 3~5 days till the cultures approximately 80~90% confluence.

The adherent cells were washed twice with PBS and the cells were harvested using with 0.25% trypsin (Gibco) for 5-10 minutes and the enzyme was inactivated with same amount of complete culture media. AD-MSCs were passaged up to 6 times. At each passage the cells were counted using hemocytometer and analyzed for cellular growth.

BM-MSCs isolation and culture (Yoshimatsu et al. 2015)

Under sterile condition both femur and tibia from the rats were excised and carefully cleaned of adherent flesh. The ends of the bones were cut and the bone marrow was harvested by flushing with 10 ml syringe with DMEM. After washing and centrifugation at 1800 RPM for 10 minutes, cell pellet was collected and cultured in a 75-cm² flask in a DMEM medium supplemented by 10% FBS and antibiotics. The cultures were incubated at 37°C in a 5% CO₂ environment and saturated humidity. After incubation, the culture media was changed and passaged as described for AD-MSC.

Characterization of the cultured cells:

Flow cytometry analyses of harvested cells at P3 on a BD FACS Caliber flow cytometer (BD Biosciences), for CD34, CD44 was performed for both AD-MSCs and BM-MSCs (Pawitan et al., 2013).

Cell counting and growth curves

Growth curves were plotted for each BM-MSCs and AD-MSCs in order to compare growth

kinetics of the cells (Mehrabani et al., 2014). Cellular Growth rate and expansion during the passaging from P2 to p6 was performed to both types of stem cells. The 2nd passage started with 1.5×10^5 cells then the cells were counted every passaging till the 6th one. For the assessment of growth characteristics, BM-MSCs and AD-MSCs at passage 2, 4 and 6 were seeded in a 24-well plates at a density of approximately 5×10^4 cells per well. Cells were collected from each well 1 to 7 days after seeding and counted microscopically using hemocytometer to produce cell growth curves.

Colony forming unite fibroblast assay (CFU-F assay):

Ten tissue culture flasks of both AD-MSCs and BM-MSCs containing 1×10^6 cells/each, of 1st passage, were cultured for 14 days then fixed with acetone/methanol 1:1 and stained with Giemsa stain. Cell colonies of more than 50 cells were counted then the mean \pm SD was estimated to compare the colonogenic potential of the two types of stem cells (Franceschini et al., 2014).

Population doubling time (PDT)

For doubling time experiments, both cell types were counted at the beginning and at the end of passage 2, 4 and 6 using hemocytometer and the population doubling time was calculated using this formula $PD = (\log_{10}(N_h) - \log_{10}(N_p)) / (\log_{10}(2))$ where N_h is the collected cell number and N_p is the plated cell number. The PD for each passage was added to the PD of the previous passages to give cumulative population doubling (CPD) (Li et al., 2015).

Statistical analysis

The mean and SD of counted cells in growth curve analysis were compared using one-way ANOVA (SPSS for Windows, version 12 and Tukey post-hoc test. Values of $p \leq 0.05$ were considered significant.

RESULTS

Morphological assessment:

BM-MSCs and Ad-MSCs were isolated from rats. The first attached cells to the substratum of the tissue culture flask were observed 3 and 6 hours after seeding regarding the BM-MSCs and AD-MSCs respectively. After 48 hours, the number of the attached cells increased while the number of the floating round-shaped cells gradually decreased and they were discarded by the 3rd day with the first media exchange. The attached cells of both types showed heterogeneous

morphology with various shapes, including flat, star shape, sperm shape triangular and elongated and reached confluency around the 9th or 10th day after seeding. The cytoplasmic processes of AD-MSCs were longer than that of BM-MSCs (Fig.1).

After passage 2, both types of cells became relatively homogeneous showing a similar morphology with abundant cytoplasm and large nuclei with multiple nucleoli the fibroblast-like appearance was clearly seen in AD-MSCs while the BM-MSCs tended to be polygonal (Fig. 2). The colony formation was evident on both types of cells from the 7th day of 1ry culture and through all passages. The colonies of AD-MSCs showed over growth of cells on each other while cell to cell growth inhibition was seen in the colonies of BM-MSCs (Fig. 2).

Fibroblast-like cells were observed in all passages (Fig. 3).

Phenotypic characterization of MB-MSCs and AD-MSCs:

Flow cytometry analysis during the 3rd passage of BM-MSCs and AD-MSCs showed that both types of cells expanded in FBS-supplemented medium. BM-MSCs exhibited specific MSC marker (CD44) at a higher level than that of Ad-MSCs (80% and 65% respectively), and hematopoietic cell markers (CD34) at a lower level than AD-MSCs (9% and 22% respectively). Moreover the homogeneity of the phenotype was observed in both types of cells (Fig. 4).

Cellular growth curve:

Both AD-MSCs and BM-MSCs showed an exponential growth from P2 to P6, shown by trend lines in Fig. 5, with a maximum population density of BM-MSCs at the 5th passage followed by recognizable stability from P5 to P6. The number of cultured attached AD-MSCs exceeds that of BM-MSCs at almost all passages but this increase was statically non-significant in passage 2 ($P < 0.5$) and highly significant through the subsequent passages ($P < 0.05$).

CFU-F assay:

Regarding the tendency of colony formation, both AD-MSCs and BM-MSCs showed tendency to form colonies both in primary culture and subcultures. There was a significant difference of AD-MSCs colonogenic potential ($9.5 \pm 3.7\%$) in comparison to that of BM-MSCs ($4.7 \pm 1.6\%$) during the 1st passage, $P < 0.05$ (Fig. 6).

Population doubling time (PDT):

PDT is the time by which cell population doubles in number and considered to be an indicator to assess the rate of cellular growth. According to our result AD-MSCs proliferative capacity is significantly more than that of BM-MSCs as the PDT of AD-MSCs during the 2nd, 4th and 6th passages were 52.3 h, 61.2 h and 82.5 h, respectively. Also PDT was 63.6 h, 69.6 h and 93.5 h at the same passages for BM-MSCs (Fig. 6 and Table).

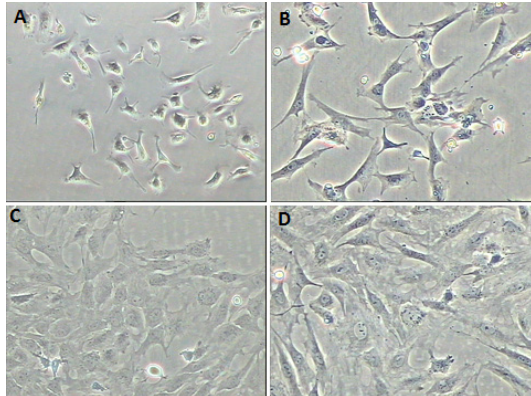


Fig. 1: A phase contrast of rat BM-MSCs (A, C) and AD-MSCs (B, D) in primary culture 3 days after seeding (A, B) and 10 days after seeding (C, D). The cells were attached to the substratum and took different morphological appearance. The AD-MSCs possess longer cytoplasmic process (B) and reached about 95% confluency by the 10th day post seeding (D).

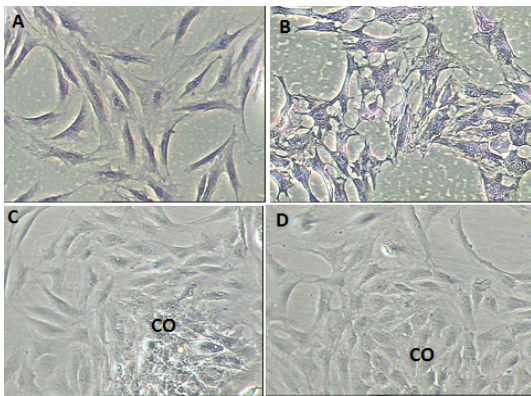


Fig. 2: (A and B): Two photomicrographs of AD-MSCs (A) and BM-MSCs (B) during the 1st passage stained with Giemsa, almost of the cells showed homogenous appearance with abundant cytoplasm and large nuclei. The AD-MSCs took a fibroblast-like shape while the BM-MSCs tend to be polygonal in shape. (C and D): Phase contrasts of a rat AD-MSCs and BM-MSCs respectively showed large colonies (CO) with over growth of cells in layer in AD-MSCs (C) while BM-MSCs showed cell to cell contact growth inhibition (D).

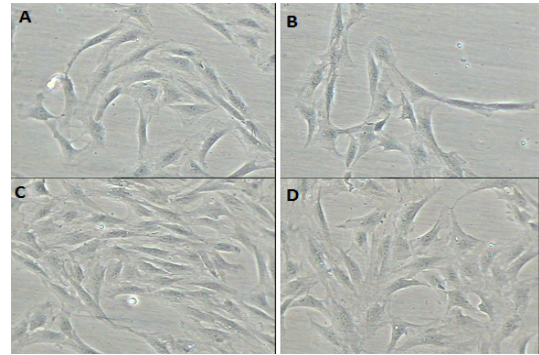


Fig. 3: A phase contrast of AD-MSCs (A, C) and BM-MSCs (B, D) during the 1st passage (A, B) and the 3rd one (C, D) showing the cells showed homogenous appearance with an abundant cytoplasm and large nuclei. Most of the cells took the fibroblast-like shape.

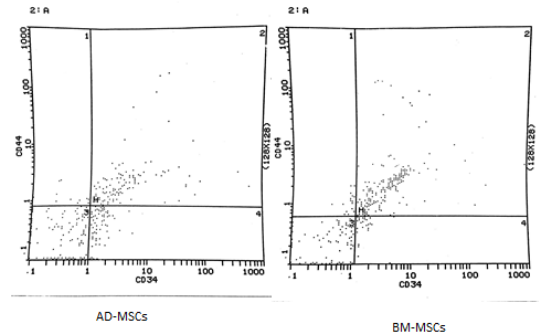


Fig. 4: A chart showing flow cytometry of a rat AD-MSCs and BM-MSCs during the 3rd passage. BM-MSCs exhibited specific MSC marker (CD44) at a higher level than that of Ad-MSCs (80% and 65% respectively), and hematopoietic cell markers (CD34) at a lower level than AD-MSCs (9% and 22% respectively).

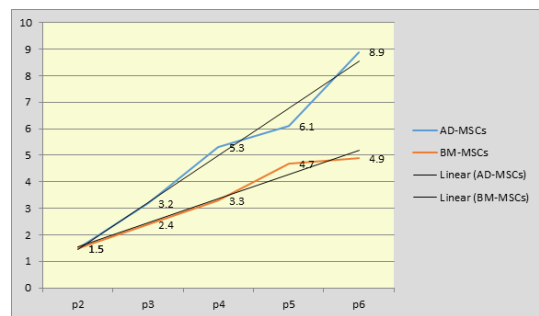


Fig. 5: A bar chart showing comparison of mean ± SD of cell counts(X105) in growth curves of rat bone marrow-derived mesenchymal stem cells (BM-MSCs) and adipose tissue-derived mesenchymal stem cells (AD-MSCs) between passages 2 and 6. The proliferation rate of Ad-MSCs and BM-MSCs increased gradually from passage 2 to passage 6 (trend lines). The number of AD-MSCs significantly exceeds that of BM-MSCs at almost all passages ($p < 0.05$).

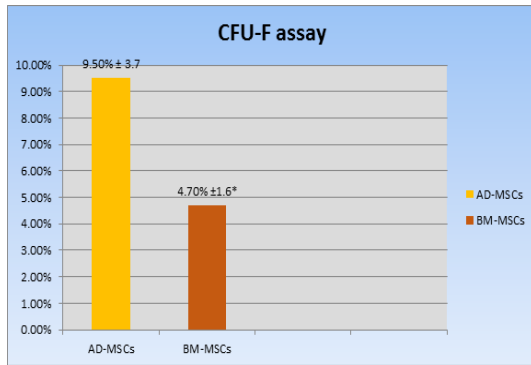


Fig. 6: A bar chart showing colony forming unit fibroblast assay (CFU-F assay) of rat AD-MSCs and BM-MSCs .AD-MSCs show a statistically significant increase of CFU-F assay in comparison to BM-MSCs during the 1st passage. *: $P < 0.05$

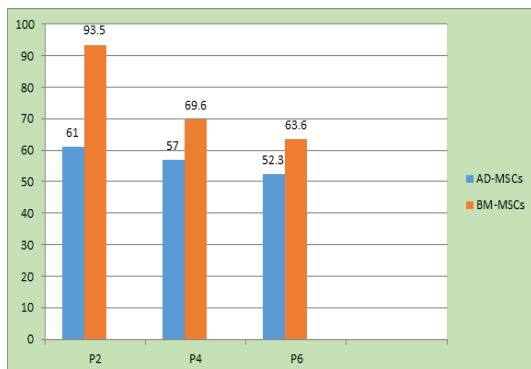


Fig. 7: A chart showing the population doubling time of rat AD-MSCs and BN-MSCs during the 2nd, 4th and 6th passages (P2, P4, P6). There was a significant increase of AD-MSCs proliferative capacity in all these passages ($P < 0.05$).

Table: Number of the rat AD-MSCs and BM-MSCs at the beginning and at the end of the 2nd, 4th and 6th passages and their population doubling time through these passages. The PDT decreased in both types of cells with the increase of the passage number and there was a significant increase in AD-MSCs proliferative capacity $P < 0.05$.

AD-MSCs	Cell N. at the beginning of passaging	Cell N. at the end of passaging	PDT
Passage 2	20 000	53000	82.5 h*
Passage 4	20 000	57000	61.2h*
Passage 6	20 000	61000	52.3 h*
BM-MSCs	Cell N. at the beginning of passaging	Cell N. at the end of passaging	PDT
Passage 2	20 000	41000	93.5h
Passage 4	20 000	44000	69.6 h
Passage 6	20 000	49000	63.6 h

DISCUSSION

Stem cells have greater plasticity and can differentiate into multiple cell lineages (Terai et al., 2003). Some researches had used the bone marrow cells, in addition to bone marrow, the adipose tissue has been known as a new interesting source of MSCs (Peng et al., 2008). Moreover, abdominal adipose tissue is abundantly accessible and often applied for the isolation of MSCs and tissue engineering (Peng et al., 2008 and Kakudo et al., 2014). Therefore, comparison between MSCs from adipose tissue and those from bone marrow but the results concerning comparative growth properties and differentiation potential of the cells remained as a subject of controversy (Yoshimura et al., 2007 and Peng et al., 2008).

Since, in the present research, the rat AD-MSCs of abdominal regions as well as BM-MSCs were isolated and compared in terms of their in-vitro morphology, phenotypic characterization and growth characteristics.

Isolation of MSCs from bone marrow is more challenging in rats than other species. We used in this study the same species, age and sex. Young male rats were chosen because experimentally, MSCs were found in larger number in the young adult (six months) more than old adult (more than two years) and in the male more than in the female (Asumda & Chase, 2011 and Katsara et al., 2011). We compared several reported isolation strategies and selected a protocol for standardized, reliable and easy-to-perform isolation of mouse MSCs from the bone marrow and the adipose tissue.

Although we isolate both AD-MSCs and BM-MSCs on the basis of adherence to the plastic of the tissue culture flask and we expanded them in FBS supplemented media, the isolated cells showed morphological difference in primary culture. AD-MSCs appeared fibroblast like with longer cytoplasmic processes than that of AD-MSCs which tended to be polygonal in shape. While in late culture and subcultures both type of cells took the fibroblast-like appearance. These findings are consistent with the results of Nadri and Soleimani (2007) on the mice, Lotfy et al. (2014) on rats and that of Li et al. (2015) on the human MSCs.

Regarding the morphology of the cell colonies, ADSCs had ability to form multilayer colonies on the other hand colonies of BM-MSCs always occurred in monolayer; this was likely due to low contact inhibition consistent with results of Zhu et al. (2008). Hyper-density of cell popula-

tion displayed a negative role against cell proliferation because of contact inhibition and reciprocal effects of cells on each other (Peng *et al.*, 2008 and Suchanek *et al.*, 2009).

To further characterize of the isolated cells, cell surface markers were examined using flow cytometry. MSCs from both sources displayed, presence of mesenchymal cell surface markers (CD44) and the lack of hematopoietic cell surface marker (CD34) but with a dissimilarity. BM-MSCs express more CD44 and less CD34 than AD-MSCs. Li *et al.* (2015) stated in his research on human MSCs that there was a great similarity of expression of these markers on both types of cells. This difference may be caused by the difference in species difference or may be caused by the change of expression of these markers through a different passage; we characterized the cells through the 3rd passage while Li *et al.* (2015) characterized them during the 5th one.

Colony forming unit-fibroblast (CFU-F) assays were performed to measure the proliferation and colonogenic capacity the cells. The isolated AD-MSCs and BM-MSCs had a great tendency to proliferate and form colonies but AD-MSCs had better such tendency compared to BM-MSCs; these results are concordant with those of Peng *et al.* (2008).

According to the growth curve analysis, growth and proliferation rate of AD-MSCs and BM-MSCs increased gradually from passage 1 to passage 6. Other researcher proved that by increasing passage number of the human MSC, proliferation, growth rate and the number of cells in culture were decreased (Yoshimura *et al.*, 2007 and Rebelatto *et al.*, 2008). Aliborzi *et al.* (2016) stated the same results in their research on guinea pigs MSCs from passage 1 to passage 8. The dissimilarity in the results may be due to difference in the animal species or the change of culture conditions. Also the cells of this study may have different behavior if continuously expanded in more and more passages

By comparing of growth curves and PDT of both types of cells, it was evident in this study that the growth and proliferation rate of AD-MSCs was more than BM-MSCs. Also, in other researches on rat, guinea pigs and human, it was proved that AD-MSCs seemed to have less percentage of senescent cells, more proliferation rate and higher expansion rate during the passages in culture media than the BM-MSCs (Eslaminejad *et al.*, 2008; Li *et al.*, 2015 and Aliborzi *et al.*, 2016). The higher proliferation capacity in AT-

MSCs could be due to significant differences in the gene expression patterns and higher expressed of cell division cycle associated 8 (CDCA8), and cyclin B2 (CCNB2) genes in AT-MSC than in BM-MSCs (Alipour *et al.*, 2015). However, the species of the animal, the source of the samples, the cultivation conditions and various medium supplements may have an effect on PDT and proliferative rat of MSCs (Schipper *et al.*, 2008).

CONCLUSION

Comparison between the rat AD-SCs with BM-SCs as two different sources for MSCs was done. Although, many similarities between both types of cells regarding their morphology and phenotypic characterization, there are growth kinetic differences existing which can offer assistance on choosing the type of cells to be used in a specific disease.

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حركية نمو خلايا الفئران الجذعية الوسيطة من نخاع العظام والنسيج الدهنى. دراسة مقارنة

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ملخص البحث

الخلفية: الخلايا الجذعية هي الخلايا البدائية المكونة للجنين وتستمر معه حتى البلوغ ولكن في اماكن معينة في الجسم مثل نخاع العظام والخلايا الدهنية والحبل السري واجزاء اخري في الجسم، تتميز هذه الخلايا بقدرتها علي الانقسام والتجدد والتنوع والتحول الي خلايا اخري في الجسم اذا وضعت في وسط ملائم لها أو اذا تعرضت لهرمونات معينة تساعد علي تجدد ونمو هذه الخلايا. ويعد نخاع العظام من أهم المصادر الغنية لفصل الخلايا الجذعية الوسيطة التي تعد الأكثر شيوعا ومع ذلك فهناك العديد من الخلايا الجذعية المشابهة التي يمكن فصلها من بعض الأنسجة الأخرى مثل الأنسجة الدهنية التي تتميز بسهولة فصلها بالإضافة الي سهولة الحصول علي أعداد هائلة من الخلايا الجذعية عند فصلها من جزء بسيط من النسيج الدهني وسهولة الحصول علي الجرعة العلاجية المطلوبة..

الهدف من الدراسة: فصل الخلايا الجذعية الوسيطة من كل من نخاع العظام من الفئران وكذلك من الأنسجة الدهنية من الفئران أيضا وذلك لإعادة حقنها في فئران التجارب للأغراض العلاجية المطلوبة. كما تهدف الدراسة الي المقارنة بين فصل وبين خصائص كل من الخلايا الجذعية الوسيطة المشتقة من كل من نخاع العظام والأنسجة الدهنية للفئران..

المواد وطرق البحث: تم فصل الخلايا من عدد 10 فئران بيضاء بالغة تم زراعتها في وسط مناسب مع السماح بتمددتها وتزايدها في العدد ثم مقارنة معدل نموها وحساب الوقت الازم لتضاعف عددها. خصائص. الخلايا الجذعية الوسيطة التي تم فصلها من نخاع العظام ومن النسيج الدهني تم ايضاحها بالشكل الظاهري و باستخدام الصبغة الهستولوجية. كما تم توصيف الخلايا الجذعية الوسيطة عن طريق التدفق الخلوي لكل من سي دي 34 و سي دي 44 كما تم تحديد المنحني النموي لكل من النوعين من الخلايا لتحديد معدل النمو الخلوي خلال التمرير الثاني والرابع والسادس للخلايا ويتم حسابها مجهريا باستخدام الهيموسيتوميتر لانتاج منحني نمو الخلايا. وقد تم تشكيل المستعمرات التي تنتجها الخلايا الليفية وتم صبغتها بصبغة جيمسا للمقارنة بين الخلايا الجذعية من نخاع العظام ومن الانسجة الدهنية....

النتائج البحث: أثبتت الدراسة ان معدل نمو الخلايا الجذعية الدهنية أكبر من مثيله في الخلايا الجزعية المنفصلة من نخاع العظام حيث ان الوقت اللازم لتضاعف عددها اقل من الخلايا الجذعية المشتقة من نخاع العظام.

الاستنتاج: بالرغم من وجود تشابهات كثيرة بين النوعين من الخلايا من حيث الشكل المورفولوجي بعد الفصل من نخاع العظام ومن النسيج الدهني الا ان هناك بعض الاختلافات التي يمكن أن تساهم في اختيار نوع الخلايا لاستخدامها في الاغراض العلاجية المختلفة.