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Growth Kinetics and in Vitro Aging of Mesenchymal Stem Cells Isolated From Rat Adipose Versus Bone Marrow Tissues

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Abstract

Objective- To investigate and compare growth potential as well as aging of mesenchymal stem cells (MSCs) derived from rat bone marrow tissue and adipose tissue (AT) occurred at epicardial and epididymal regions.
Design- Experimental study.
Animals- 10 Wistar Rats.
Procedures- Rat MSCs occurred at bone marrow and epicardial and epididymal AT were isolated and culture expanded through several successive passages. Differentiation potential along bone, cartilage and adipose cell lineages was used to verify MSC identity of the isolated cells, and then the cells were comparatively investigated in terms of their colonogenic ability, population doubling time and growth curve characteristics. Furthermore, the number of senescent cells at different passages was quantified using senescent- associated (SA) ß galactosidase staining method.
Results- MSCs from both AT appeared to have more proliferation capacity in culture than those from bone marrow since they exhibited significantly more colony number and shorter PDT value ($P<0.05$). Epicardial AT-MSCs indicated even more significant proliferation capacity than their epididymal counterparts. With respect to cell aging, marrow-MSCs cultures indicated higher percentages of senescent cells than AT-MSCs ($P<0.05$). Although the percentages of senescent cells in epididmal AT-MSCs were higher than epicardial AT-MSCs but the difference was not statistically significant.
Conclusion and Clinical Relevance- Taken together we concluded that rat epicardial AT-MSCs could be appropriate cells for experimental and preclinical settings since they possess more expansion rate and less percentages of senescent cells in culture.
Key Words- Mesenchymal stem cells, Adipose tissue, Bone marrow, Growth kinetics, Senescence.

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Introduction

Mesenchymal stem cells are recognized by two important properties of extensive self renewal ability and multilineage differentiation potential into mesenchymal and non mesenchymal cell lineages. These cells represent promising candidate for application in regenerative medicine.[1] The existence of MSCs was first described by Friedenstein et al.[2] These investigators have found that bone marrow tissue contains a population of highly colonogenic cells with fibroblastic morphology, capable to produce foci of bone and cartilage-like deposits in vitro.[2] Following investigations have confirmed and developed Fridenstein et al findings.[3,4]

Since the discovery of MSCs many investigation has been conducted using marrow tissue as the main source of MSCs but the limitation associated with marrow and marrow-derived MSCs is decreased their suitability for clinical applications. Marrow harvest is indeed a highly invasive procedure and the number; differentiation potential and maximal life span of MSCs from marrow have been reported to decline with increasing age.[5-7] For these reasons, investigators have attempted to isolate MSCs from multitude of alternative sources including peripheral blood, umbilical cord blood, bone, cartilage and amniotic fluid.[8-12]

One alternative source is adipose tissue, which is abundant, relatively accessible with minimal invasion and replenishable. Stem cell from adipose tissue was first isolated and described by Radble et al[13-15] who collected rodent adipose tissue fragments through an open surgery. Later several groups have reported the isolation of stem cells from human adipose tissue taking advantage of liposuction aspirates as a tissue source.[16-17]

Limited studies have so far been undertaken to investigate characteristics of MSCs from adipose tissue compared with those from bone marrow.[18-22] Even in those rare researches, there is controversy regarding in particular the growth kinetics of adipose-versus marrow-derived stem cells. While some studies have reported that marrow-derived MSCs possess a higher propagation rate than adipose-derived stem cells in cultures,[18-20] the others have indicated the higher significant comparative growth rate of adipose-derived stem cells.[21-22] In these studies adipose tissue from which MSCs were derived was of different localizations (for example some study have used adipose tissue harvested from knee joint region and other have utilized the tissue obtained from inguinal region) and this would be the source of different results that they have reported. The relationship between adipose tissue localization and their MSCs differentiation potential as well as surface epitopes profiles have previously been reported.[23] The comparative in vitro growth of MSCs derived from adipose tissue of different location has yet to be investigated. The present study designed to comparatively investigate the growth kinetics of MSCs derived from rat adipose tissues occurred in epicardial and epididymal regions as well as bone marrow. Furthermore, since no study has considered the frequency of senescent cells in adipose versus bone marrow-derived MSCs cultures, we intended to determine and compare the percentage of senescent cells occurring at the cultures of adipose versus bone marrow derived MSCs.

Materials and Methods

Cell culture

The use of 10 male Wistar rats with 2-4 weeks old was approved by ethic committee of Royan Institute. MSCs isolation from the epicardial, epididymal and marrow tissue was almost performed according to the procedure that has previously been published.[24] In brief, the animals

were sacrificed by cervical dislocations; the epicardial and epididymal adipose tissue was dissected off and collected in 15-ml sterile tubes. Bone marrow tissue was flushed off from tibia and femur and collected in 15-ml tubes. Under sterile conditions, the adipose tissues were minced into small pieces and then subjected to enzymatic digestion using 0.075% collagenase type I (Sigma, Germany)at 37 °C for 2 hours at the end of which the floating cells were separated from the vascular stromal fraction by centrifugation (1200 rpm) for 5 min. The pellet (stromal vascular fraction) was then filtered through a 200 µm nylon mesh to remove undigested tissue. Bone marrow specimen was added with 2 ml phosphate buffer solution (PBS) and washed twice by centrifugation at 1200 rpm for 5 min. Both adipose digest and marrow cells were suspended in 1 ml proliferation medium including DMEM (Dulbecoo Modified Eagle Medium, Sigma, Germany) containing 10% FBS (Fetal bovine serum, Gibco, Germany) and 10 IU/ml penicillin/streptomycin (Gibco, Gemany) and plated at 10^6 cells/ ml in 25 cm^2-culture flasks. The cultures were incubated in an atmosphere of 5% CO2 and 37 °C. The first medium substitution was done about 3 days after culture initiation and the subsequent medium exchange was performed two times weekly till the cultures reached confluence. At this time they subcultured at 1:3 ratios and allowed to reach confluent. The cells were propagated by three successive subcultures.

Differentiation potential

Osteogenesis: The passage 3 cells from all studied tissues were plated at 2×10^5 cells in 25-cm^2 culture flask in proliferation medium and allowed to attain confluency. The medium was then replaced by differentiating medium composing of DMEM medium supplemented with 50 mg/ml ascorbic 2-phosphate (Sigma, USA), 10 nM dexamethazone (Sigma, USA) and 10 mM β glycerol phosphate (Sigma, USA). The cells were kept in differentiating culture for 21 days with medium changes of twice weekly. At the end of this period, osteogenic differentiation was examined by alizarin red staining method.

Adipogenesis: Passaged-3 cells were plated at 2×10^5 cells in 25-cm^2 culture flasks in proliferation medium and allowed to become confluent. The proliferation medium was then replaced with adipogenic differentiating medium consisting of DMEM supplemented with 50 µg/ml ascorbic acid 3-phosphate, 100 nM dexamethazone and 50 µg/ml indomethcine (Sigma, Germany). The cultures were kept for 21 days during which the medium was changed twice weekly. At the end adipogenic differentiation was determined using Oil red staining method.

Chondrogenesis: For chondrogenic differentiation, 2.5×10^5 passaged-3 cells from all studied cells were pelleted under 300 g for 5 minutes and provided with differentiating medium which was composed of DMEM supplemented by 10 ng/ml TGF- ß3 (transforming growth factor-ß3)(Sigma, Germany), 10 ng/ml BMP6 (bone morphogenetic protein-6)(Sigma, Germany), 50mg/ml insulin transferin selenium+ premix(Sigma, Germany), 1.25 mg bovine serum albumin(Sigma, Germany) and 1% fetal bovine serum. The cultures were incubated at 37 °C and 5% CO2 for three weeks with medium changes of twice weekly. To prepare the cultures for assessment of cartilage differentiation, the pellets were fixed with 10% formalin, dehydrated in ascending concentrations of ethanol, cleared in xylene and embedded in paraffin. Five µm-thick sections were then made and stained by alcian blue.

Colonogenic assays

Epicardial, epididymal and marrow derived passaged-3 mesenchymal stem cells were counted using hematocytometer, plated at 10^3 cells in 10-cm Petri dish and allowed to grow for 7 days. The cultures were then stained with crystal violet and observed with a light microscope to count the number of produced colonies. We also measured the size of colony in either group.

Calculation of population doubling time (PDT)

To compare the in vitro expansion rate, the PDT value was determined for each studied cells. PDT, in this study was calculated according to the equation PDT=culture time (CT)/ population doubling number (PDN). To determine PDN, the formulae PDN= log N/N0×3.31 was used.[25] In this equation N stands for the cell number at culture end and N0 the number of the cells at culture initiation. To determine the culture time and value of N and N0, passaged-3 cells were plated at 10^4 cells/cm^2 in 25-cm^2 culture flasks for a period when one of the cultures reach confluent (which in this study it was the epicardial cells that achieved confluency in about 6 days). At this time all the cultures were terminated by trypsinization in order to determine the cell number (N).

Growth curve

The cultured cells are usually grown with characteristic pattern in which three phases including lag, log and plateau phase can be recognized. Growth curve could be used to compare the growth characteristics of interest cells under study. In the present investigation, growth curve was plotted for each MSCs derived from epicardial adipose tissue, epididymal adipose tissue and marrow tissues in order to better compare growth kinetics of the cells. For this purpose, the passaged-3 cells derived from each tissue were plated at 5×10^4 cells/well in 12-well culture plates and allowed to become confluent. In a regular daily basis, some wells were trypsinized and the cell number was determined by hemocytometer count. Using the data growth curves was plotted.

Senescence-associated ß-galactosidase (SA- ß-gal) staining

The amount of senescent cells in the culture of MSCs from all studied tissues was investigated by SA-ß-galactosidase staining method according to previously-published procedure.[26] In this study, the percentages of senescent cells were determined for passage 1, 2, 3, 5 and 7. For this purpose, the cells growing on 4-wells culture plates were washed with PBS for 2 times, fixed with 3% formaldehyde for 4 min and then incubated with ß galactosidase substrate staining solution at 37 °C for 14 hours. The solution was composed of 150 mM NaCL, 2 mM MgCL2, 5mM potassium ferricyanide, 5 mM potassium Ferro cyanide, 40 mM citric acid, and 40mM sodium phosphate, pH 6, 1 mg/ml 5-bromo-4-chloro-3-indolyl-D-ß-galactosidase. At the end of this procedure, the senescent cells were stained blue which was observed by standard light microscope and a minimum of 100 cells was counted in 10 random fields to determine the percentage of SA-ß-gal-positive cells.

Statistical analysis

Each experiment described in here was replicated for 10 rats. All values stated as means ± standard deviations. The results were analyzed by ANOVA. A P value of <0.05 was considered to be statistically significant.

Results

Cell culture

At the primary cultures, majority of the cells from marrow (Fig. 1A), epicardial adipose tissue (Fig. 1C) and epididymal adipose tissue (Fig. 1E) were observed to be small spindly-shaped cells with a few small clear cells present among them. All primary cultures reached confluence in about 8-10 days. At passages the cell growth apparently assumed rapid rate, such that the cultures reached confluence a few days earlier than primary cultures. In this regards epicardial adipose-derived MSCs was the first to reach confluence and the marrow MSCs was the last cell culture which achieved confluence. Passaged-3 cultures from all the studied tissues i.e. marrow (Fig.1 B), epicardial adipose tissue (Fig. 1D) and epididymal adipose tissue (Fig. 1F) tended to be composed of homogenously fibroblastic cells in monolayer (Fig. 1).

Differentiation

All the cells that were derived from marrow tissue, epicardial and epididymal adipose tissues appeared to be successfully differentiated into adipose (Fig. 2A), bone (Fig. 2B) and cartilage (Fig. 2C) cell lineages (Fig. 2A-I). These differentiation abilities were indeed indicative of MSC nature of the isolated cells. The differentiations were confirmed by specific staining carried out for each culture. At adipogenic cultures, lipid droplet produced in cell cytoplasm was stained red upon oil red staining. Mineralized foci in osteogenic cultures were stained red following alizarin red staining method. The sections prepared from the chondrogenic pellet cultures was positively stained with alcian blue.

Colonogenic assays

According to our results epicardial AT-MSCs produced 38.9 ± 7.5 colonies (Fig. 4A) with average size of 2.78+ 0.6 mm^2 (Fig. 4B) while epididymal AT-MSCs generated 32±9.4 colonies (Fig. 4A) with average size of 2.47+0.09 mm^2 (Fig. 4B) and marrow-MSCs 19.9±7.9 1colonies with 1.13±0.09 mm size. All the differences was statistically significant ($P<0.05$).

Population doubling time (PDT)

The PDN values for epididymal AT-MSCs, epicardial AT-MSCs and marrow MSCs tended to be 2.71±0.5, 3.42±0.3 and 1.87±0.2 respectively. According to our results, PDT was 70±15.18 hour for epididymal AT-MSCs, 42±8.9 for epicardial AT-MSCs and 77.33± 27 hours for marrow MSCs (Fig. 3C). The differences were significant ($P<0.05$).

Growth curve

According to the plotted curve, the culture of both epicardial and epididymal AT- MSCs had almost no lag time indicating that either cell was very adaptive to culture conditions so that they immediately after plating started to proliferate (Fig. 4A). Marrow-derived cells exhibit a short lag time in their growth curve.

SA- ß-gal staining

Senescent cells were not present at the passages 1 and 2 of MSCs derived from all the studied tissues. Such cells were first observed at passage 3 of all cultures and increased with advancing the passage number (Fig. 4B). The percentages of the cells were indicated at table 1. According

to the table, epicardial AT-MSCs had less senescent cells than both epididymal and marrow derived MSCs. All these differences were statistically significant ($P<0.05$).

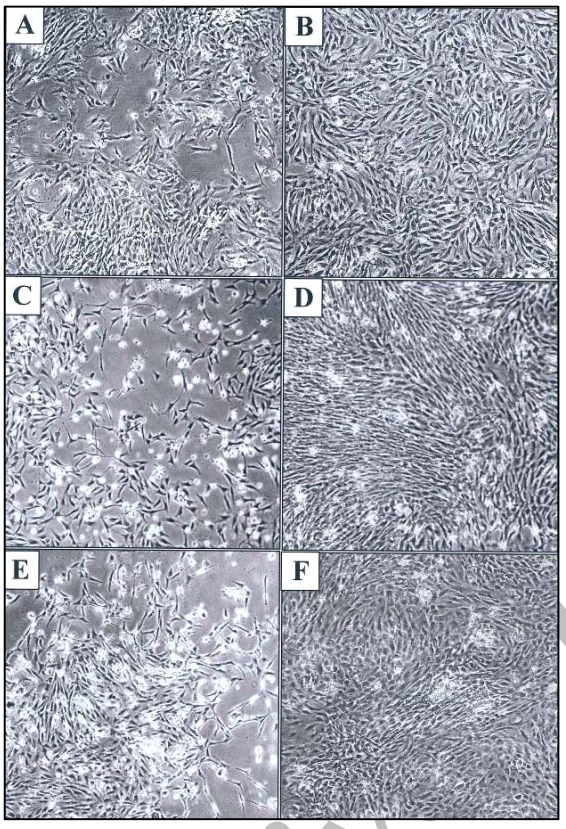

Figure1. Photomicrographs of the cultures prepared from the cells derived from bone marrow and adipose tissues. The cells from marrow (A), epicardial adipos tissue (C) and epididymal adipose tissue (E) were observed mainly as spindle-shaped cells at the primary cultures. After three successive subcultures, fibroblastic cells dominated the cultures (B: marrow cells, D: epicardial adipose tissue cells and F: epididymal adipose cells). Magnification of all images = ×150

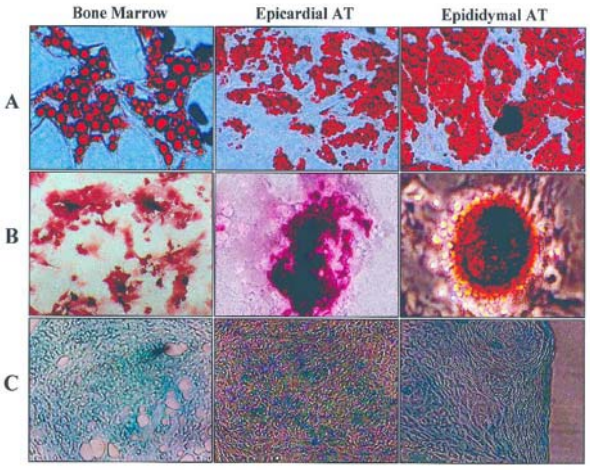

Figure 2. Photomicrographs of the differentiated cultures of the MSCs from bone marrow and adipose tissues. A: lipid droplets developed following adipogenic differetiation of the cells was stained red with oil red staining method. B: osteogenic foci appeared as red area following alizarin red staining method. C: the sections prepared from the chondrogenic nodules were positively stained by alcian blue staining method. Magnification of all images = ×100

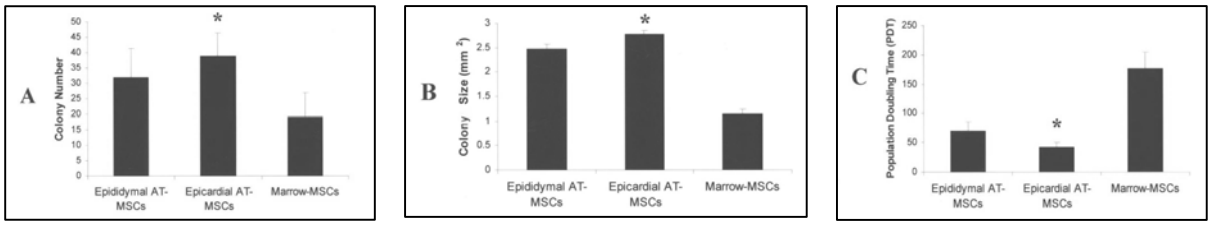

Figure 3. Graphs indicating the adipose tissue (AT) and marrow-derived MSCs proliferative capacity in culture. A: colonogenic assay, * indicates the comparative significant high colonogenic capacity of epicardial adipose tissue (AT)-MSCs (P<0.05). B: Colony size produced at each cell culture; * indicates significant difference of epicardial AT-MSCs compared with marrow-MSCs (P<0.05). C: population doubling time, * indicates significant short PDT of epicardial AT-MSCs compared with that of either epididymal AT-MSCs or marrow-MSCs (P<0.05).

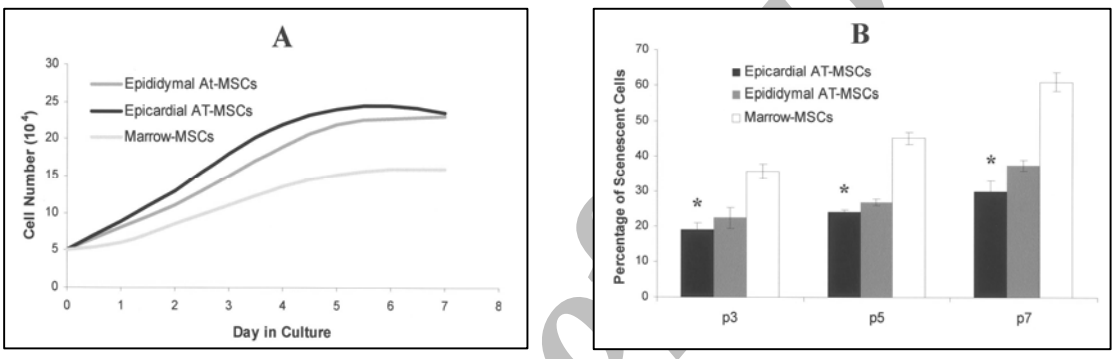

Figure 4. A: the growth curve plotted for the MSCs derived from adipose and marrow tissues. Almost no lag phase occurred at either adipose-derived MSCs. B: the graph indicating the percentages of senescent cells at different passages (P3-P7) of the MSCs derived from adipose and marrow tissues. * indicates the relative low percentage of senescent cells at epicardial AT-MSC culture compared with that of marrow-MSCs cultures (P<0.05).

Discussion

In this study, MSCs from adipose tissue of different localizations (epicardial and epididymal regions) as well as bone marrow were isolated and compared in terms of their in vitro growth kinetics and the percentages of senescent cells in their cultures. Several studies have so far compared MSCs from adipose tissue with those from bone marrow but the results concerning comparative growth kinetics of the cells remained as the subject of controversy.[18-22] While some studies have reported that adipose tissue derived MSCs possess significantly more expansion rate than those from bone marrow, the others have failed to come this conclusion. We hypothesized that this discrepancy would be the result from different localization from which adipose tissue has obtained in different studies. Therefore, in the present study adipose tissues used for MSCs isolation were selected from two different localizations (rat epicardial and epididymal region) and the in vitro growth of MSCs isolated from the tissues was compared with those from marrow. Our results indicated that the both adipose derived MSCs possessed a higher expansion rate than marrow derived MSCs. In addition the growth kinetics of MSCs derived from different adipose tissue was varying. This result is in accordance with the report by Prunet-Marcassus et al who have indicated that stem cells derived from adipose tissue of different localizations possess varying differentiation as well as surface epitopes.[23]

Table1. The percentages of the senescent cells at the cultures of mesenchymal stem cells (MSCs) from adipose tissues (AT) and marrow. Epicardial AT-MSCs tended to have less senescent cells than both epididymal and marrow derived MSCs. All these differences were statistically significant (P<0.05).

	Epicardial AT-MSCs	Epididymal AT-MSCs	Marrow -MSCs
Senescent cell percentage at passage 3	20± 1.77	21.9± 3.01	35.7± 2
Senescent cell percentage at passage 5	23.9± 0.79	28.5± 0.73	45.2± 1.75
Senescent cell percentage at passage 7	30± 3.1	37.45± 1.5	61± 2.71

Former investigations have indicated that stromal vascular fraction (SVF) being produced after collagenase digestion of adipose tissue contains varying cell types including MSCs, hematopoietic stem cells and non-stem cells such as endothelial cells, fibroblast, blood cells, preadipocyte and pericytes. To separate mesenchymal stem cells from other cell types, one method is to plate the cells on plastic culture surfaces for an extended period. Majority of the related studies have been used this strategy to culture expand and purify MSCs from other cell populations.[13-17] During plastic culture, all anchorage- dependent cells adhere on the surfaces and therefore survive while suspension-growing cells such as hemotopoietic cells fail to grow and eventually eliminated from the cultures. As the culture period is extended with performing a several round of passages, the non-stem cells as endothelial cells which possess a limited proliferation and life span are also not be able to resist the culture conditions hence disappearing from culture while stem cells having an extensive self-renewal capacity dominate the cultures.[27-29] In this study such strategy was used to purify MSCs cells from adipose and marrow tissues.

One possible best method by which the cell under study can be recognized is to characterize the cells by their specific molecular markers. No specific surface marker has so far been reported to express on marrow-derived MSCs surfaces, instead several non specific surface antigens have been demonstrated on human MSC surfaces. Furthermore former research works have indicated that the surface immunophenotype of adipose-derived stem cells are similar to that of bone marrow-derived mesenchymal stem cells.[35] Direct comparison made by Zuk et al have indicated that adipose and marrow derived MSCs are phenotypically >90% identical.[36] On the other side, it has been demonstrated that MSCs from human marrow-MSCs exhibit several markers including CD133, LNGFR (low affinity nerve growth factor receptor) and STRO-1 on their surfaces.[37-39] No such data is available for MSCs from animal models. To solve this problem, however, MSCs committee of international society for cell therapy has proposed two criteria by which animal MSCs can be recognized: being plastic-adherent and being able to give rise to three classical cell types as bone, cartilage and adipose cell lineages.[40] In this study, we used these criteria to recognize our isolated cells as MSCs.

In the present study, the isolated cells were characterized in terms of their in vitro proliferation. Colonogenic assays are among those assays that frequently being used to determine the cell proliferation potential,[27-29] but this assay measures only the colony number and the colony size is

ignored. For this reason, in addition to colonogenic assay that was performed in this study, the size of colony was also measured. The other indices indicating the cell proliferation rate is PDT (Population doubling time) which defines as the time by which the given cell population double their numbers by undergoing proliferation. The other method that could well indicate the proliferative property of the isolated cells is to plot a growth curve which was done in this study. According to the all these evaluation MSCs derived from epicardial adipose tissue appeared to be much better than those from both epididymal adipose and marrow tissues. Our data is in accordance with that of Kern et al who compared the population doubling values of the stem cells derived from human adipose aspirates with those of marrow derived stem cells.[19] Rapid in vitro proliferation of the mesenchymal stem cells would be of interest, in particular for those who are involved in stem cell applications in regenerative medicine where the huge number of cells is required to fulfill the repair of large tissue defects.

Acid ß-D-galactosidase as a hydrolase is located in eukaryotic cell lysosomes.[34] The activity of this enzyme can be detected in situ in most mammalian cells by means of a cytochemical assay normally carried out at pH 4 [34]. Recently it has been described a pH 6 ß galactosidase activity, which was found specifically in senescent fibroblastic cultures[26] and referred to as senescence associated (SA) - ß-galactosidase. Since then, SA- ß-galactosidase assay was used to examine the senescence of variety of cells in culture.[41-43] Using this marker, in the present study, the percentages of the senescent cells in different cell passages were determined and then compared. According to our findings, comparatively more percentage of senescent cells was present at the cultures derived from marrow tissue. To the best of our knowledge, such comparison was not done in related similar studies. Moreover such data would be of interest for investigators who are seeking an appropriate source for MSCs for use in regenerative medicine.

Taken together, mesenchymal stem cells derived from epicardial adipose tissue of rat seemed to have more proliferation rate in culture and less percentage of senescent cells during the passages than the same cells derived from epididymal adipose and marrow tissue. Furthermore, proliferation capacity and senescent cell percentages of adipose derived stem cells are varying according to localization from which the cell was derived. Considering these data, rat epicardial AT-MSCs seemed to be an appropriate cellular material for use in experimental and preclinical settings.

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چکیده

مقایسه رشد و پیری سلول های بنیادی مزانشیمی مشتق از بافت چربی و مغز استخوان

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هدف - مقایسه پتانسیل تکثیر و پیری سلول های بنیادی مزانشیمی مشتق از مغز استخوان و بافت های چربی ناحیه اپیدیدیم و اپی کاردیوم موش صحرایی.

طرح مطالعه - مطالعه تجربی.

حیوانات - ۱۰ موش صحرایی با نژاد ویستار.

روش کار - سلول های بنیادی از بافت مغز استخوان و بافت چربی واقع در نواحی اپیکاردیوم و اپیدیدیم موش صحرایی جدا سازی شد و با انجام چند پاساژ سلولی تکثیر گردید. برای تایید ماهیت بنیادی مزانشیمی سلول های جدا شده، از تمایز به سه رده استخوان، غضروف و چربی استفاده شد و سپس سلول ها از لحاظ فعالیت کلون زایی، مدت زمان دوبله شدن جمعیت سلولی و ویژگی های منحنی رشد مورد مقایسه قرار گرفتند. به علاوه، تعداد سلول های پیر با روش رنگ آمیزی بتا گالاکتوزیداز تعیین و گروه ها از این نظر با همدیگر مقایسه شدند.

نتایج – سلول های بنیادی مزانشیمی حاصل از دو بافت چربی بطور معنی داری بیش از سلول های بنیاددی مغز استخوان تکثیر شدند ($P<0.05$). سلول های بافت چربی اپیکاردیوم در مقایسه با سلول های اپیدیدیم از توان تکثیری بالایی برخوردار بودند. در رابطه با پیر شدن سلول ها در محیط کشت، درصد سلول های پیر در کشت سلول های بنیادی مغز استخوان بیش از دو گروه دیگر بود($P<0.05$). اگرچه در صد سلول های پیر در کشت سلول های بنیادی مزانشیمی مشتق از بافت چربی اپیدیدیم بیش از در صد آنها در کشت سلول های اپیکاردیوم بود ولی تفاوت از لحاظ آماری معنی دار نبود.

نتیجه گیری و کاربرد بالینی- روی هم رفته می توان نتیجه گرفت که سلول های بنیادی مزانشیمی مشتق از بافت چربی اپیکاردیوم موش صحرایی سلول های مناسبی برای مطالعات تجربی و پیش کلینیکی می باشند زیرا از توان تکثیر بالایی برخورددار هستند و در صد سلول های پیر در کشت آنها پایین است.

کلید واژگان- سلول بنیادی مزانشیمی، بافت چربی، مغز استخوان، رشد سلول، پیری.