Comparative Study of Expansion and Proliferation of Adult Mice Mesenchymal Stem Cells Derived from Bone Marrow and Adipose Tissue

دراسة مقارنة لتضاعف واكثار الخلايا الجذعية الوسيطة (المزنكيمية) للفئران البالغة المشتقة من نخاع العظم والنسيج الدهني

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Abstract

The importance of Mesenchymal stem cells (MSCs) represents a favorable tool for new clinical concepts in supporting tissue engineering and cellular therapy. Bone marrow (BM) was considered important source contain mesenchymal stem cells .Another promising source of MSCs is adipose tissue (AT). MSCs derived from these sources compared regarding morphology, the success rate of isolating MSCs, expansion potential by rate of colony forming and immune phenotype. The obtained results from this study showed no obvious considerable differences concerning the morphology and immune phenotype of the MSCs derived from these sources were obvious. Differences observed concerning to the success rate of isolating MSCs, which was approximately more than 90% for BM, while it reached about 70% for AT after seven days of culturing, as well as the rate of colony forming was lower in AT cells in comparison to that obtained in BM at the same period. However, AT-MSCs could be required longest time to complete monolayer confluence, whereas BM-MSCs had the shortest proliferation period. Cells from both sources determined according to immunohistochemistry by $CD105^{+}$ and $CD34.^{-}$ Conclusions revealed that MSCs can easily and successfully obtained from bone marrow and adipose tissues, and both tissues appears suitable sources of stem cells for potential use in regenerative medicine, repairing damaged tissue nevertheless the BM-MSCs more effectual in expansion and proliferation.

Key Words*:* MSCs, cell therapy, adult mesenchymal stem cells, bone marrow mesenchymal, stemcells, adipose-derived stem cells.

الملخص

تتمثل اهمية الخلايا الجذعية الوسيطة (المزنكيمية) بكونها وسيلة ملائمة في المجال الطبي تدعم تقنية هندسة الانسجة واعتمادها في العلاج الخلوي. يعد نخاع العظم مصدرا مهما للخلايا الجذعية الوسيطة (المزنكيمية) والمصدر الواعد الاخر هو خلايا النسيج الدهني. قورنت الخلايا الجذعية الوسيطة المشتقة من كلا المصدرين في هذه الدراسة من الناحية المظهرية، كفاءة عزل الخلايا الوسيطة، تكون المستعمرات وفاعلية اتساعها والتعبير المظهري المناعي للخلايا. أظهرت نتائج الدراسة عد م ظهور فروق تعنى بالشكل المظهري والتعبير المظهري المناعي للخلايا الجذعية الوسيطة (المزنكيمية)المشتقة من كلا المصدرين، ولوحظت فروق تعنى بكفاءة ونجاح عزل الخلايا واكثارها والتي بلغت قريبا ما يزيد عن الـ 90% من خلايا العظم بينما كانت 70% من خلايا النسيج الدهني بعد 7 أيام من الزرع، وانخفض معدل تكون المستعمرات من خلايا النسيج الدهني بينما كان هو الاعلى في خلايا نخاع العظم لنفس الفترة، اذ تطلب زرع الخلايا المشتقة من النسيج الدهني وقتا اطول للتضاعف والوصول لنفس المقدار من اكثار واتساع الخلايا قياسا بالفترة ذاتها لاكثار خلايا نخاع العظم. كما حددت فحوصات الكيمياء النسيجية المناعية للخلايا المستحصلة من المصدرين اعتمادا على المؤشرين المناعيين +CD105 , -CD 34 امكانية الحصول على الخلايا الجذعية الوسيطة بسهولة من نخاع العظم والنسيج الدهني، وان كلا النسيجين اظهرا ملائمتهما لانتاج الخلايا الجذعية الوسيطة لفاعلية استعمالها في الطب التجديدي للخلايا، انتج العديد من البروتينات بمفاعلات حيوية واصلاح الانسجة التالفة فضلا عن الفاعلية الاكثر للتضاعف واكثار الخلايا الجذعية المشتقة من نخاع العظم.

الكلمات الدالة : الخلايا الجذعية الوسيطة (المزنكيمية) ، العلاج الخلوي ، الخلايا الجذعية الوسيطة البالغة ، الخلايا الجذعية الوسيطة لنخاع العظم، الخلايا الجذعية الوسيطة من الخلايا الدهنية

Introduction

Stem cell therapies can provide an alternative approach for repair and regeneration of tissues and organs. The availability of a dependable source of stem cells is important for the cell-based therapies which can provide a therapeutic approach in field of medicine.Tentatively, the rapid advance of

therapeutic trials of stem cells warrants an update of important scientific studies that are underway involve which source of stem cells consider more attractive for clinical use. Two major characteristics have been defined as criteria for the identification of stem cells: self-renewal ability and the potential for differentiation [1]. The MSCs can be isolated from several tissues such as bone marrow, skin, adipose tissue, tendon, synovial membrane, periodontal ligament and nervous system [2]. The use of adult stem cells (ASCs) has been less controversial than the use of embryonic stem cells (ESCs) in various applications because they are few ethical concerns, and low immunogenicity [3]. In addition to adult mesenchymal stem such as bone marrow mesenchymal stem cells (BMMSCs), adipose-derived stem cells (ADSCs) have attracted increasing attention because they are less easily to obtain, can be safely transplanted into an autologous or allogeneic host and exhibit higher cell activity [4-6]. According to the International Society for Cellular Therapy, MSCs are defined as being (i) plastic-adherent in the standard cell culture condition, (ii) multipotent, i.e., able to differentiate into osteoblasts, adipocytes and chondrocytes in vitro and (iii) positive for CD90 and CD105, and negative for CD34, CD45 in their cell surface immunophenotype [7]. The aim of this study was to compare the efficiency isolation of MSCs from the two sources, at identical conditions(growth medium, cells culturing and replacement medium) *in vitro* with respect to their morphology, rate of isolating of cells, expansion of colony forming characteristics and immunophenotype .

Materials and Methods

1. Collection, Isolation and separation of bone marrow mesenchymal stem cells (BM-MSCs)

Bone marrow cells were isolated from the femur of 4-8 week-old, mice male (albino mice) at weights ranging from 10-15g. The mouse was killed by cervical dislocation; a flushing method was used to flush the bone marrow cells from bones using an insulin syringe containing 1ml of PBS(phosphate buffer saline) [8]. The simplest method used implies the adherence properties of MSCs which were identified by Friedenstein, *et al.* [9]. Freshly isolated whole bone marrow cells were resuspended in growth culture medium (Minimal Essential Medium) MEM supplemented with 15 % FBS(Fetal bovine serum) , 1 % Ampicllin and Streptomycin, the collected BM cells were centrifuged at 1000 rpm for 10 min at 18°C , supernatant was aspirated and the pellet was washed twice with PBS according to Fortier ,*et al* [10]. The obtained cells were collected from one mouse 2 femurs and 2 tibias) , seeded in 6 ml of MEM in tissue culture flask, incubated at 37°C and left to adhere for 24 hours, the non-adherent cells were removed. The adherent cells considered as Mesenchymal stem cells selected by adherence after first 24 h. and maintained in growth culture media [11]. The media were changed 3 times /week until the culture reached to 90-100 % confluence.

2. Collection and isolation of adipose-derived stem cells (ADSCs)

Cells from adipose tissue were collected from mouse abdomen after it was killed by cervical dislocation, then the cells were disaggregated by enzymatic digestion. Briefly, 2 mI of tissue slurry was placed in a sterile Petri dish, chopped and washed vigorously with 5 ml of PBS. Cells in the wash fraction were retained to the collected tube, the enzymatic disaggregation done to treat the collected cells with an equal volume of 0.2% collagenase at 37°C for 15 min. This technique was done according to Freshney [12].Complete medium (MEM) supplemented with 20% FBS was added to disaggregated cells after it treated with RBC lysis buffer. The digested tissue was passed through a filter and centrifuged at 1500g for 5 min then the pellet cells were resuspended in MEM medium then transfer to tissue culture flask and incubated at 37 °C for 24 h., non-adherent cells were discarded with medium after 24 h., fresh growth media were added when it needed.

3. Immunocytochemistry tests for cell surface antigens of bone marrow and adipose tissue derived cells

Immunocytochemistry was evaluated by the phenotypic nature of MSCs according to the cell surface antigens. The following markers CD105+ and $CD34^{-}$ were examined by Immunocytochemistry staining technique, according to the manufacturer's instruction Kits. Briefly, two ml of newly cell suspension obtained from monolayer cultures of the cell were recultured in Labtek slide chambers 4 wells (Nunc, Denmark) for 28 hrs. to allow the cells forms a confluence monolayer. Then the cells were fixed in 4% neutral buffer formalin for 10 min and rinsed gently with PBS. The cells were

incubated in a humid chamber, first with blocking reagents for 10 min and then with different marker antibodies for 60 min. The antibodies (Santa Cruz biotechnology, USA) used were against some mesenchymal stem cells surface markers (CD105+ and CD34¯). The standard protocol for biotin-streptavidin technique was followed using a Universal HRP Immunostaining kit (Santa Cruz biotechnology, USA), including the substrate 3', 3' diaminobenzidine as the chromogen for antibody detection in cells that have been staining.

Results and disscusion

Growth characteristics and Morphological observations of bone marrow mesenchymal stem cells (BM- MSCs) in culture

The obtained feature of cells culture from BM during the first hours of culturing under the inverted microscope showed, most BM cells are floating in culture medium Figure (1) then its begin to adhere on culture flask progressively after 24 h. of culturing, most of BM cells seemed rounded and constituted the majority of the cells attached in the tissue culture flask Figure (2).The non-adhere cells were removed with the media and replacing it with a new fresh media. After three days of primary culture, the BM-MSCs were attached to the tissue culture flask sparsely, single adhered cells observed, many of these cells have begin forming colonies and still it seemed like rounded . At this time the cell culture of Bone marrow cells revealed at high density which was seemed enough to enable the cells to enhance each other's proliferation, resulting in rushed fibroblast-colony formation and cell proliferation. BM-MSCs displayed a spindle-like shaped after 5 days of growing ,at this period the cells looks fibroblast-like morphology with one nucleus, then these cells increased in proliferation in culture growth medium Figure (3). The observed number of cellular colonies showed differing in size and cell clustering , then they increased in large and growth on sixth days, at the end of the seventh day ,the primary culture reached nearly to 95% of confluence monolayer of adherent cells. This layer must expanded by subculture and consider as one passages Figure (4). The adhesion cell has been an important part of tissue culture researches, where it was shown how cells anchorage dependence is requirement for a solid substratum for attachment before growth to form monolayer in contact with the surface. Attachment, adhesion, and spreading belong to the first phase of cell interactions and the quality of this first phase will influence the cell's capacity to proliferate and differentiate in contact with the implant. In vitro studies demonstrated the role of adhesion molecules in cell shape, polarization, cytoskeletal organization, cell motility, and survival [13]. Loss of attachment to the matrix causes apoptosis in many cell types [14], MSCs adhere rapidly and can easily be separated from the non-adherent hematopoietic cells by repeated washing. Non-adherent hematopoietic cells (HCs) are removed with media changes, whereas adherent HC disappear from the cultures within several passages or are depleted using immunoselection techniques [15,16]. MSCs release various cytokines and growth factors that influence the microenvironment by either modulating the host immune response or stimulating resident cells to increase proliferation during culture progression [17].

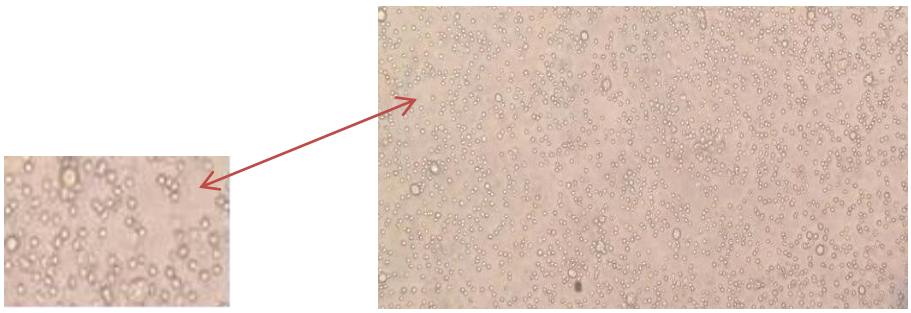


Fig. (1): Bone marrow cells during the first hours of culturing, most BM cells are floating in the media(10X)

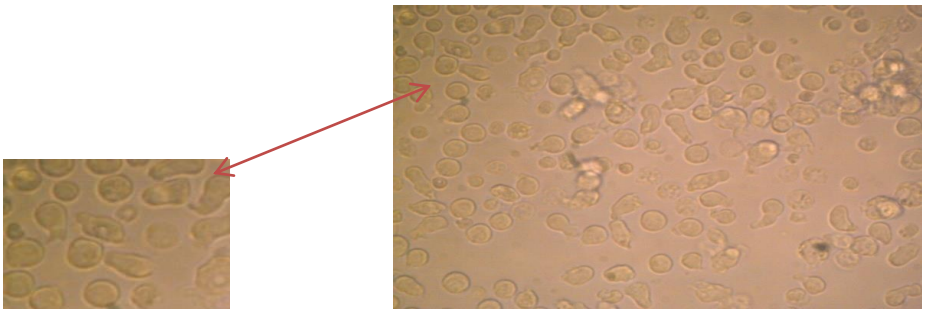


Fig. (2): Bone marrow cells after 24 hrs. of culturing, most of BM- cells seemed rounded and constituted the majority of the cells be attached in the tissue culture flask (40 X).

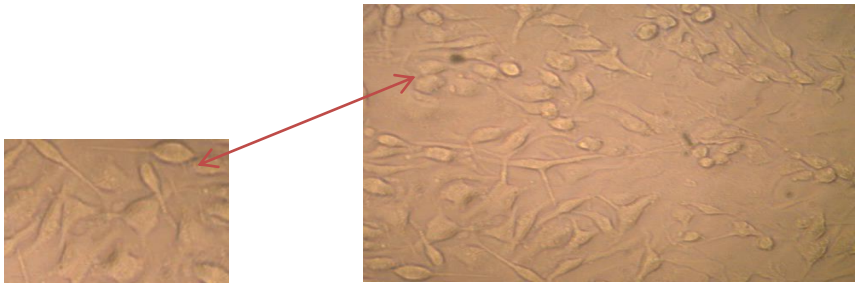


Fig. (3): Bone marrow cells at fifth day displayed a spindle-like shaped (fibroblast-like morphology) with one nucleus (40 X).

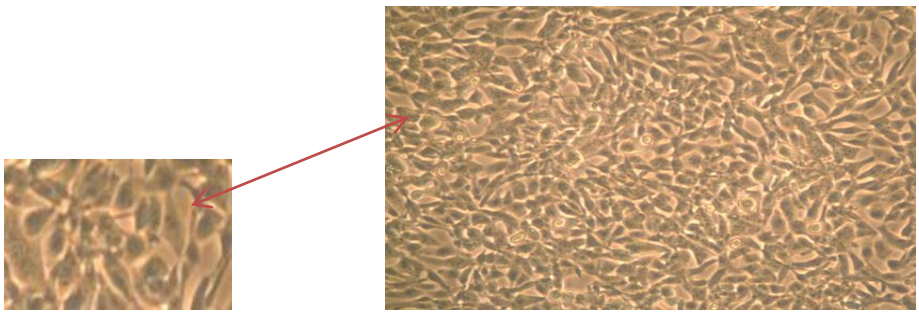


Fig. (4): Bone marrow cells after seven day of primary culture, the adherent cell reached nearly to 95% confluence monolayer of adherent cells (20 X).

Growth characteristics and Morphological observations of adipose tissue mesenchymal stem cells

Isolation of adipose tissue mesenchymal stem cells (AT-MSCs) was not successful from the first primary culture, in first trails and first days it showed lower rate of proliferation and expansion of cell culture, although it was done at the same condition of isolation and cultivation of BM-MSCs. Collection and culturing AT-cells appeared very similar to BM - MSCs at first hours of culturing, at this period they seems difficult to distinguish between two source of cells because both cells are small and flat Figure (5), after 24h of culturing, these cells appeared to be adherent to the tissue culture flask and proliferated with a considerable rate. The shape of these cells at 72 hrs. is fibroblast-like and had spindle shape that is consistent with MSC morphology Figure (6). Attachment of adherent cells such as MSCs depending on the property of substratum, fibronectin which provide a surface coating conducive to cell attachment .Conditioning factors are released by cells into the medium and help in forming a bound between cell surface glycoproteins and the substratum, all of them increased the attachment cells during first 24 h. [9]. In general the regularity of MSCs obtained from BM aspirates is about 0.01% or lower in first time of plating [18,19], while MSCs obtained from Adipose tissue is another alternative source that can be obtained by a less invasive method and in larger quantities than BM [20]. Vieira,*et.al* [21] and Fraser,*et.al* [22] established that Millions of MSCs can be obtained easily from a single individual

because large adipose samples can be obtained from multiple harvest sites. Adipose tissue has 500-fold more stem cells than bone marrow. The proliferation of AT cells seemed lower rapidly in expansion the culture during 3-5 days in comparison to BM cells Figure (7) at this period the initial clone appears in largamente and expanded into round-shaped colonies composed of fibroblastic cells developed to colonies .Then The cells expansion giving rise to distinct colonies that are illustrated in Figure (8) at the end of day seven to eight.

These colonies, derived from a single precursor cell, can differentiate into all the different cell lineages of the limb mesoderm (osteoblasts, chondrocytes, adipocytes etc.) [23]. The initial number of MSCs can be increased as much as 36.6% by simple collection and replanting of the initially non-adherent cell population which is washed out during the first feeding [24]. Adipose tissue is a convenient, abundant and readily available source of stem cells, and the harvest procedure is less invasive than bone marrow aspiration and is associated with little discomfort for the individual [20]. It has been demonstrated that AT contains stem cells (ASCs) similar to BM-MSCs in morphology and possess the properties of MSCs traditionally isolated from the bone marrow [25,26].

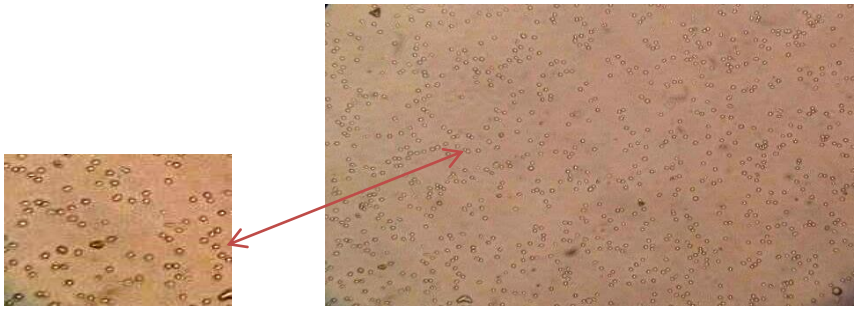


Fig. (5): Disaggregation cells from adipose tissue, (AT- cells) at first hours of primary culture, almost cells floating in culture media (10 X).

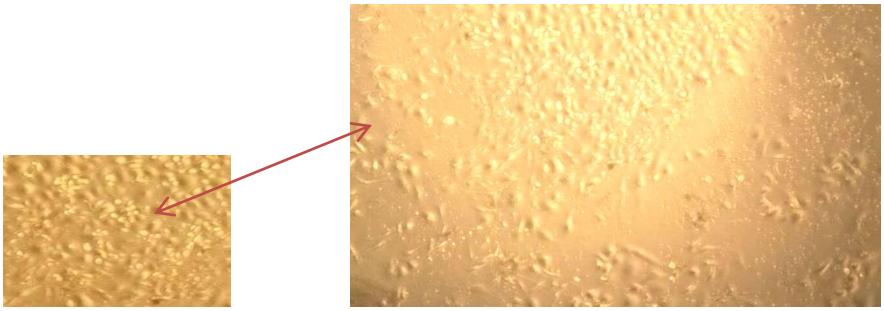


Fig. (6): AT cells after 72h, these cells are seemed as fibroblast-like and some had spindle shape that is consistent with MSC morphology (10 X).

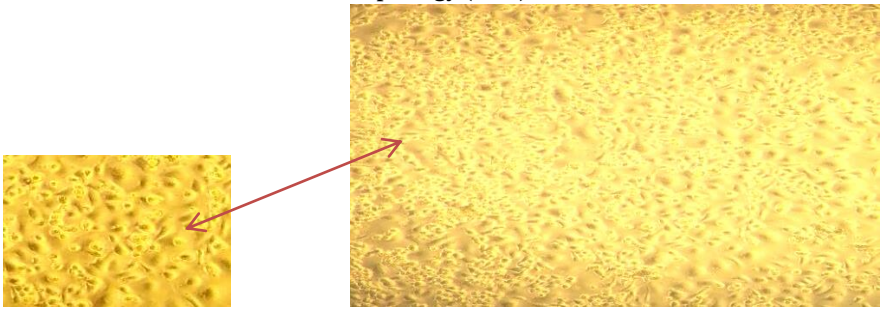


Fig. (7): AT-cells between at 3-5 days displayed a colony expansion with spindle-like shape cells (fibroblast-like morphology) to round shaped colonies with one nucleus (10 X).

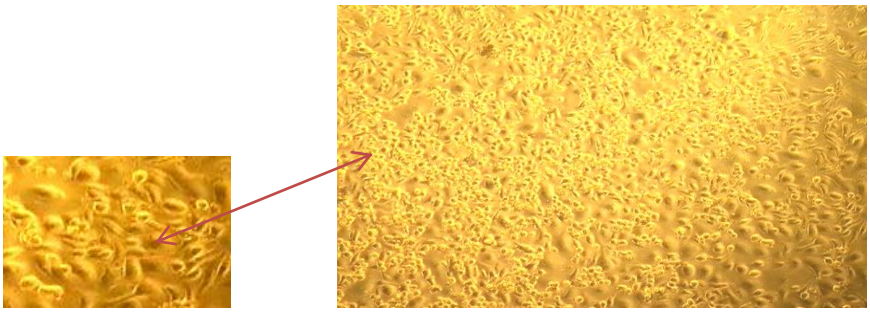
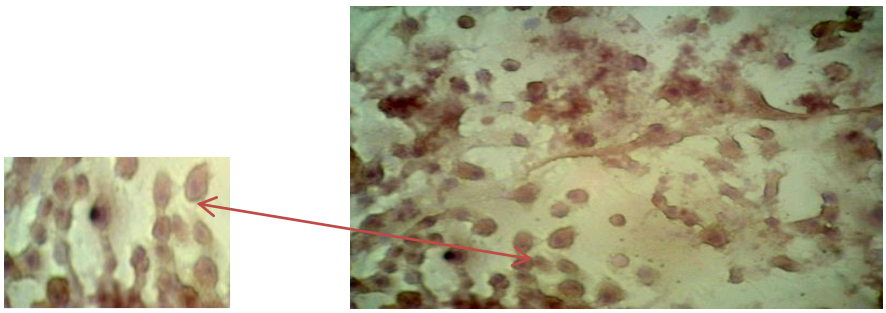


Fig. (8): AT cells expansion at end day seven of primary culture, the adherent cells reached to nearly 70-80 % confluence monolayer of adherent cells (10 X).

Immunocytochemistry of MSCs

To determine the phenotypic nature of MSCs, the surface antigens $CD105^{+}$ and $CD34^{-}$were examined by immunocytochemistry staining technique. The cultured cells were examined under light microscope, the results of immunocytochemistry examination for $CD105^{+}$ showed that more than 90% of adherent BM-MSCs Figures (9) and AT-MSCs Figures (10) were clearly stained with brown stain in the cytoplasm of the cells, this color developed by DAB reaction which is considered positive result for protein expression at surface antigen CD105 ,such results were confirmed by Harvanova,*et.al* [27],whom they reported that MSCs were positive for CD105. In contrast, a majority of adherent cells from both sources of MSCs (BM and AT) revealed negative result for $CD34^{-}$, these cells stained with blue color of counter stain (hematoxylin) Figures (11,12) respectively. Commonly this result means no expression for $CD34^{-}$, similar results were illustrated and obtained by [28,29] whom reported that MSCs express cell markers CD105, CD73 & CD90 while it revealed negative results for CD11b, CD14, CD34 and CD45. MSCs Cultured have been extensively analyzed both morphologically and with respect to surface and molecular markers, there results can confirmed by immunocytochemistry analysis which indicate that these cells certainly are MSCs, and might be used for application of cellular therapy and for tissue engineering according to their enhancers and growth factors for proliferation and differentiation, then these cells check for therapeutic usage and the concept of proteins expression. The differences in the surface markers demonstrated by reported studies that appeared and explained by variations in culture methods and/or differentiation stages of the cells [30].



Figure(9): Immunocytochemistry analysis of BM- MSCs ,the culturing revealed most of adherent cells were positive result for CD105 marker and stained with brown color by DAB stain, light microscope (40 X).

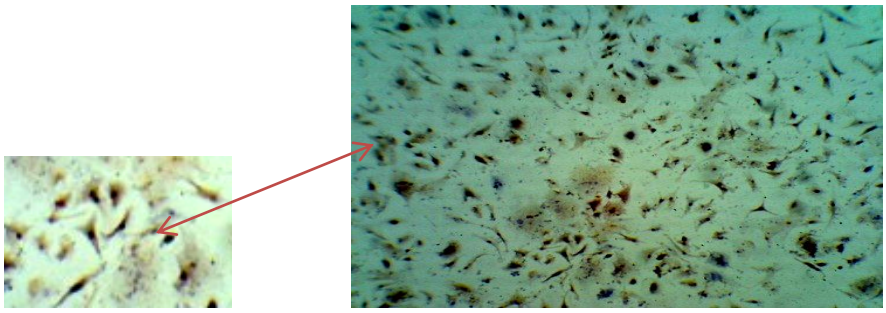


Fig. (10): Immunocytochemistry analysis of AT- MSCs, the culturing revealed most of adherent were positive result for CD105 marker and stained with brown color by DAB stain by light microscope, (10 X).

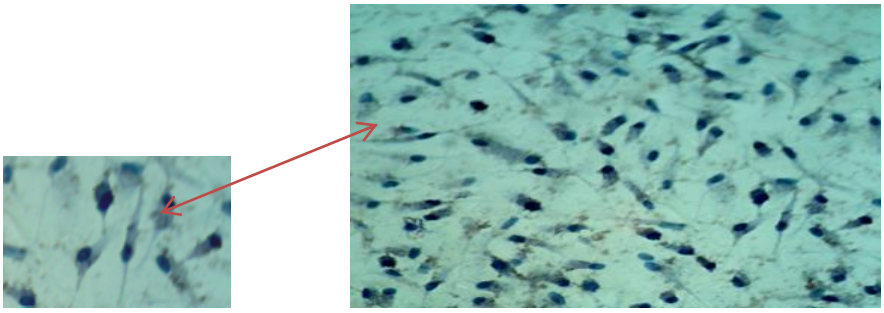


Fig. (11): The most of adherent cells of BM-MSCs were negative stained for CD34 marker, the majority nucleus cells stained with blue color (hematoxylin) (40 X).

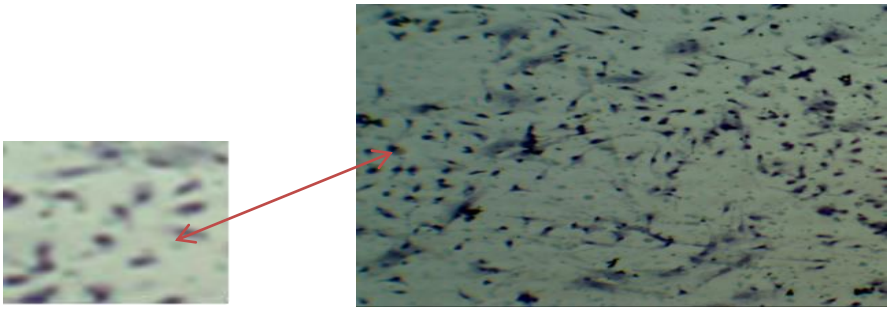


Fig. (12):The most of adherent cells of AT-MSCs were negative stained for CD34 marker, the nucleus cell stained with blue stain (hematoxylin),(10 X).

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