Osteogenesis of Mesenchymal Stem Cells Derived from Bone Marrow *in vitro*

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Abstract

This study was designed to evaluate osteogenic potential of mesenchymal stem cells (MSCs) isolated from mouse bone marrow *in vitro*. MSCs were isolated through collecting the thigh bone (femur and tibia). Cells were flushed from bones and MSCs isolated based on the ability of adherence to plastic surfaces. Reactivity MSCs to CD105 and CD34 were tested by immunocytochemistry. Isolated MSCs exhibited positive reactivity to CD105 and negative for the haematopoietic surface marker CD34. Differentiation of isolated MSCs into osteoblast was induced by osteogenic medium consisting of high glucose- Dulbecco's Modified Eagle Medium (DMEM) supplemented with 50 µg /ml Ascorbic acid, 1nM dexamethasone and10 Mm of beta glycerophosphate disodium salt hydrate after 21 days. Osteoblast activity was monitored by evaluation alkaline phosphatase (ALP) activity in osteogenic medium by Reflotron at (0, 7, 14 and 21) days of differentiation. Results recorded that a significant increase in Alkaline phosphatase activity in osteogenic medium at 7 and 14 day culture in comparison with zero day and decreased at 21 day.

Keywords: Osteogenesis, Mesenchymal stem cells, Bone disease.

Introduction

Bone diseases are disorders and conditions that cause abnormal development and/or impairment in normal bone development such as Osteoporosis which characterized by an abnormal loss of bone mass and disintegration of bone structure in older adults, this will lead to bone fragility and increases the risk of fractures and breaks[1], Osteogenesis imperfecta (OI) a genetic disorder that is characterized by brittle bones that break or fracture easily. It is caused by a gene defect in the production of collagen [2] and intervertebral disc degeneration remains a pervasive and intractable disease arising from a combination of aging and stress on the bony and cartilaginous elements of the spinal column [3].

Mesenchymal stem cells (MSCs) have biologic properties that most uniquely identifies due to their capacity for tri-lineage mesenchymal differentiation and they are self-renewing. Cells must be shown to differentiate into osteoblasts, adipocytes, and chondroblasts[4]. The most successful application of MSCs has been site-directed administration for repair of bone and cartilage. It is estimated that 1,600,000 bone grafts are performed anually to regenerate bone lost to trauma and disease, 6% (96,000) of which are craniomaxillofacial in nature [5]. Regenerative medicine attempts to repair, regenerate, or replace tissues damaged by factors such as injury or disease [6].

Restoring tissue structure and/or function in the body has largely relied on transplants or grafts (autografts or allografts) and more recently on the use of tissue substitutes like cells, growth factors (GFs) and synthetic devices, in combination or individually, as a therapeutic alternative. A considerable effort has been made, for each specific tissue, to mimic the natural microenvironment in which cells can proliferate and differentiate [7].

Tissue engineering with the use and manipulation of MSCs is a novel treatment modality targeting applications in a great variety of pathologies. The advantages of this approach are numerous; they include a high quality repair with regeneration of the injured tissue but without fibrous tissue formation [8]. Thus, the aims of the current works are:

Investigate the osteogenic potentials of BM-MSCs in osteogenic medium through:

1. Isolation and characterization of mesenchymal stem cell from mice bone marrow.
2. Induction of mesenchymal stem cells differentiation into osteoblast medium.

Materials and Methods

Cell culture

A bone marrow cells were isolated from the femur of 4-8 week-old male albino mouse with weights ranging from 10-15g. Mice were killed by cervical dislocation, then isolated whole bone marrow cells and resuspended in growth culture medium M (USBiological, USA) supplemented with 15% Fetal Bovine Serum (FBS), 1% Ampicllin/ Streptomycin (Troge, Germany). The cultured cells were incubation at 37°C, 5% CO_2 and left to adhere 24 hours. Media were changed 3 times a week and the non-adherent cells were removed. The colonies grew quickly between day 5 and 9, and the cells were passaged when the cells density approached 80-90% confluence [9].

Identification of BM-MSCs by Immunocy to chemistry analysis

After MSCs were dispersed with trypsin-versene, and suspended in MEM growth media, cells were re-cultured in multi-well tissue culture plates (8 well) in MEM supplemented with 15% FBS, the plates were incubated at 37˚C to allow the cells for development a monolayer of adherent cells within 1-3 days, after that the medium was aspirated and cells were fixed by 4% paraformaldehyde for 10 min. In this study we used the following CD markers (primary antibody) for detection of MSCs (mouse anti human-CD +105, Mouse anti human CD -34) (US-biological, USA). This method was done by primary antibody binds to the corresponding antigen in the tissue section, and the secondary antibody binds to determinants on the primary antibody. Then the avidin-biotin complex containing the horseradish peroxidase enzyme was allowed to bind to the biotin molecule attached to the secondary antibody and 1-2 drops of permanent mounting medium (Fluka, Germany) was added and examined by light microscopy (Scopetek, USA).

Cell differentiation *in vitro*

Osteoblastic differentiation was induced by culturing confluent BM-MSCs for 3 weeks in L-Dulbecco's modified eagle's medium (USBiological, USA) supplemented with 10% FBS, 10mM Beta glycerophosphate,50 µg /ml of Ascorbia acid and 1nM of Dexamethasone. All osteogenic supplements were obtained from US Biological Company. Cultures were incubated at 37^0C in humidified atmosphere of air with 5% CO_2. Culture medium was exchanged every 2-3 days for 3 weeks [10].

Evaluation of osteoblast activity

The alkaline phosphatse (ALP) activities were measured using test strip method [11]. In this method thirty two µl from osteogenic media at (0, 7, 14 and 21) days was added to test strip, and then the sample allowed to flow into the reaction zone. The result was displayed after 135 seconds in U/L by using Reflotron instrument (Roche, Germany). The ALP enzyme activity was calculated by measuring the dye formation is (determined kinetically at 37^oC after adding sample into reaction zone and the ALP hydrolyzes O-cresolphthalein phosphate to O-cresolphthalein and transfers the phosphate group to the acceptor molecule methylglucamine). The colored hydrolysis product O-cresolphthalein that is produced per unit of time under alkaline conditions is directly proportional to alkaline phosphatase activity.

Results and Discussions

Morphology of bone marrow mesenchymal stem cells (BM-MSCs)

The isolated adherent cells were observed as heterogeneous groups of cells. However they became homogeneous as the cells continued to proliferate, and were more homogeneous after subsequent passaging. The initial heterogeneous morphology may be due to presence of other types of adherent cells such as macrophages, lymphocytes, and endothelial cells[12]. MSCs isolated by adherent on plastic culture flasks during first 24 hr[13] and Non-adherent cells were removed by exchange media with a new fresh media as seen Fig.(1). At six days of primary culture the adherent cells were nearly 80-90% confluent and formed monolayer of adherent cells Fig.(2). Bone marrow cell seeding density was high enough to enable the cells to enhance

each other's proliferation, resulting in rushed fibroblast-colony formation and cell proliferation. At these periods the cells displayed a spindle-like shaped (fibroblast-like morphology) with one nucleus, and then these cells began to proliferate in culture growth medium. The best candidate cells for this purpose are MSCs, because they are multi-potent and have a high proliferative capacity. Because there is no definitive marker to identify MSCs, the gold standard procedure to prove their stem cell identify is their adherence on cell culture plates after isolation, their expression of specific marker, and differentiation potential to osteoblasts, adipocytes and chondrocytes *in vitro* [14]. Identify of MSCs by existing +105 CD markers on the surface (Fig. 3A) to distinguish them from -34 CD markers on the surface of HSCs Fig.(3B).

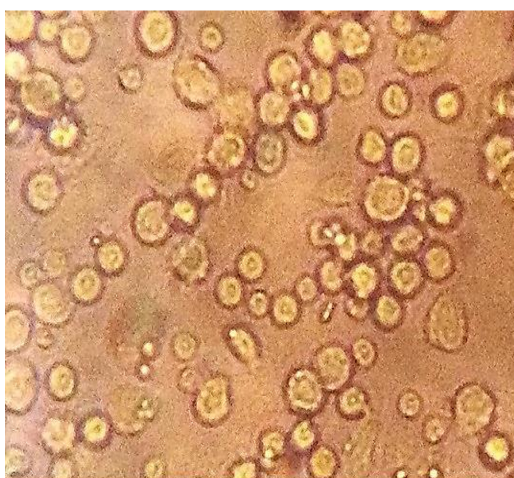


Fig.(1):Morphology of BM-MSCs after 24hrs under inverted microscope (40X).

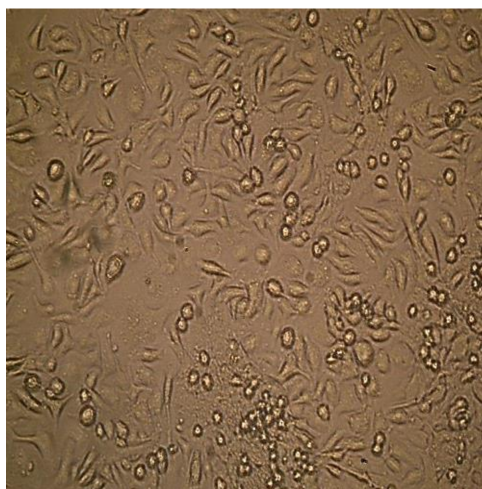


Fig.(2): Morphology of BM-MSCs at 6 day under inverted microscope (20X).

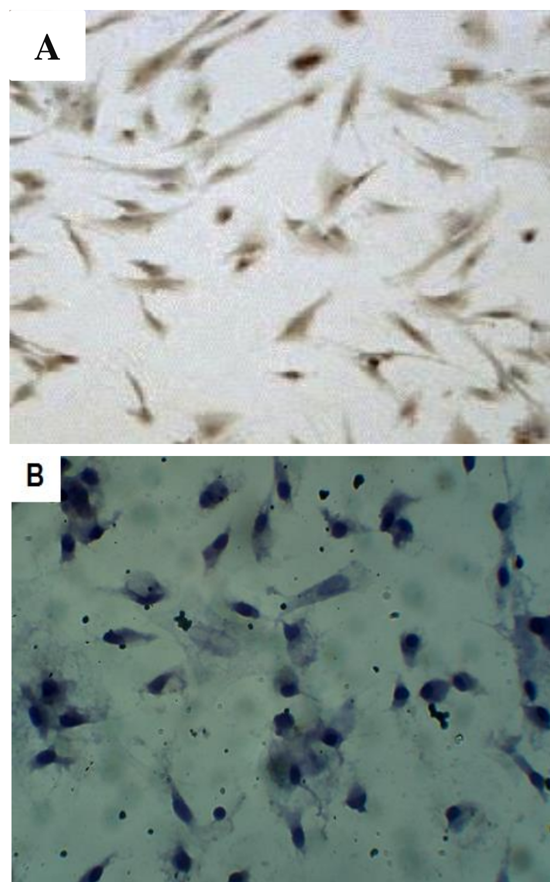


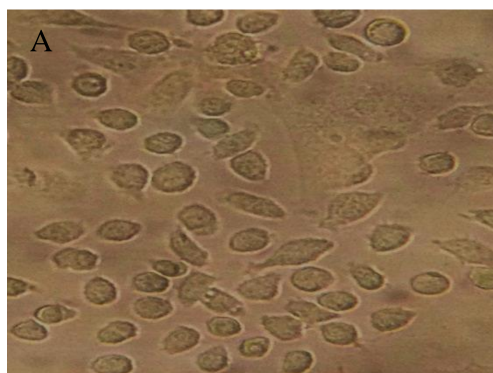
Fig.(3): Immunocytochemistry analysis of MSCs. (A) MSCs were positive for CD105 marker and stained with brown color DAB stain (X40).(B) MSCs were stained negative for CD34 marker (X40).

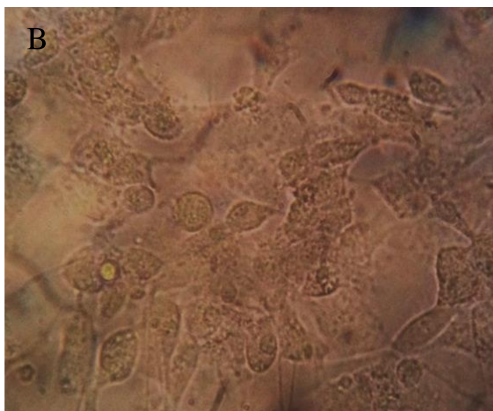
Cell differentiation

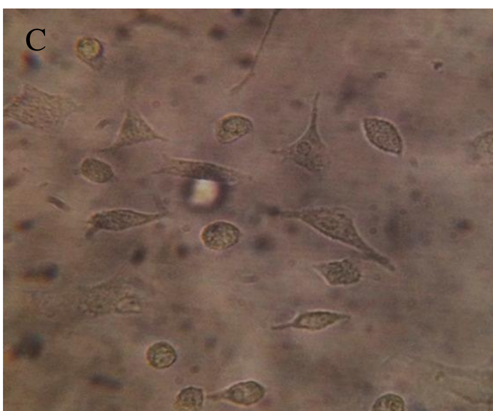
To confirm that expanded MSCs maintained the differentiation potential, MSCs at passage 2 were tested for the differentiation into osteoblast cells. The cells showed changes in cell morphology after 3 days of the supplementation of the culture media the cells under osteogenic conditions exhibited morphological changes typical as shown in Fig.(4A). Osteogenic lineage cells are a population of cells that include mesenchymal progenitors, preosteoblasts, osteoblasts, osteocytes, and bone- lining cells. The identities of cells at different stages of differentiation are not well defined. Fig.(4B) showed preosteoblasts are heterogeneous since they comprise all cells differentiating from progenitors to mature osteoblasts [15].

The osteogenic induction medium led to a morphological change of the MSCs from an elongated fibroblast-like cell type to shorter, cuboidal cells[16] Fig.(4C). After 21days from

inducing MSCs to differentiate into osteoblasts *in vitro*, the cells showed changes in cell morphology from spindle shape to cuboidal or polygonal Fig.(4D). Results revealed that a significant increase in ALP activity in osteogenic medium at 7 and 14 days of differentiation in comparison with zero day (32.13±0.46 and 23.33±0.88 *vs.* 5.22± 1.76 IU/L) and decreased at 21 day (15.33±1.76 IU/L). Moreover ALP activity was significantly enhanced in osteogenic induction compared with undifferentiated cells; ALP production followed by a subsequent decrease as the cells mature and lay down minerals [17] Fig.(5).







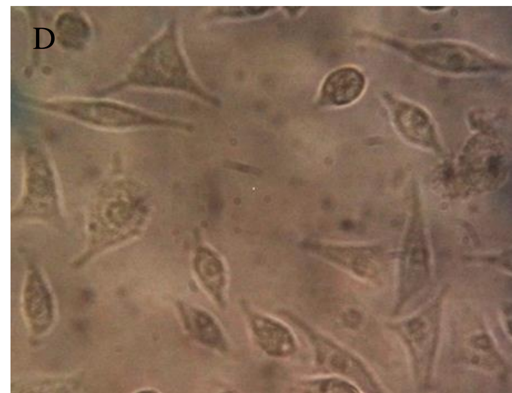


Fig.(5) : Morphology of MSCs differentiation in osteogenic medium (A-D) after 7, 14, and 21 days under inverted microscope (40X).

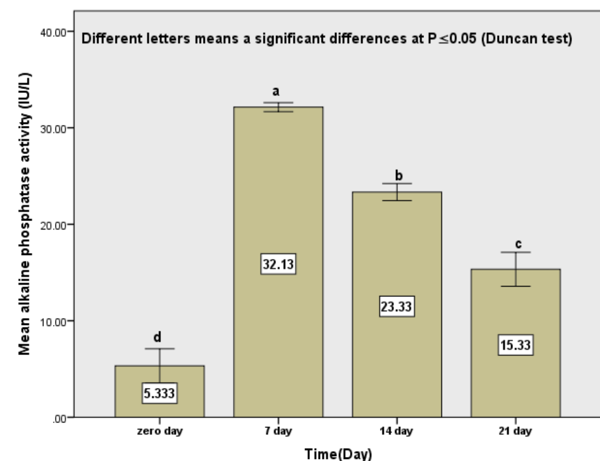


Fig.(5) :Alkaline phosphatase activity (IU/L) in osteogenic medium after 0, 7, 14 and 21 days Differentiation period.

In conclusions, bone marrow derived mesenchymal stem cells had osteogenic potential and could be a suitable option for cell-based tissue engineering therapies. Osteogenic medium of dexamethasone, beta-glycerophophate and ascorbic acid induced MSCs osteogenesis *in vitro* after 21 days. The alkaline phosphatase activity in osteogenic medium found to reach a peak at 14 days of differentiation.

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الخلاصة

صممت الدراسة الحالية لتقييم إمكانية تحول الخلايا الجذعية الوسيطة المعزولة من نخاع عظم الفأر الى خلايا مولدة لخلايا العظم في المختبر. عزلت الخلايا من عظم فخذ ٥٠ فأرة على أساس قدرة التصاقها بالسطوح البلاستيكية. اختبرت الخلايا الجذعية الوسيطة للمعلمات CD105 الخاص بالخلايا الجذعية الوسيطة و CD34 الخاص بالخلايا الجذعية المولدة لخلايا الدم بواسطة كيمياء الخلوية المناعية. أظهرت الخلايا الجذعية الوسيطة المعزولة تفاعلا ايجابيا أتجاه CD105 سلبيا للمعلم على سطح الخلايا الجذعية المولدة لخلايا الدم CD34. حفزت الخلايا الجذعية الوسيطة المعزولة على التمايز لخلايا مولدة لخلايا العظم بواسطة وسط مكون يتألف من DMEM مع ٥٠ ميكروغرام/ مل حمض الأسكوربيك، ١٠نانو مولاري

ديكساميثازون و10 ملي مولاري بيتا كليسروفوسفيت هيدرات الصوديوم بعد ٢١ يوما. درست فعالية الخلايا المولدة لخلايا العظم عن طريق تقييم فعالية انزيم الفوسفاتيزالقاعدي (ALP) في الوسط المكون لخلايا العظم بواسطة Reflotron بعد فترات مختلفة من التمايز (٠، ٧، ١٤، ٢١) يوما. سجلت النتائج زيادة معنوية P≤0.05 في فعالية ALP في وسط التمايز في اليوم ٧ و ١٤ مقارنة مع اليوم صفر (٣٢,١٣ ± ٠,٤٦ و ٢٣,٣٣ ± ٠,٨٨ مقابل ٥,٢٢ ± ١,٧٦ وحدة دولية/ لتر) وانخفضت في اليوم ٢١(١٥,٣٣±١,٧٦ وحدة دولية/لتر. تم التأكد من وجود جينات بيتا الأكتين وأوستيوكالسين في الخلايا المتمايزة من قبل (RT-PCR) بعد ١٤ يوما من التمايز. اكدت النتائج ظهور حزمتين بحجم الجزيئي (٢٠٠) زوج قاعدي لبيتا الأكتين و (١٦٩) زوج قاعدي لأوستيوكالسين.