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Mesenchymal Stem Cells Cultivation and Characterization from the Bone Marrow of Adult Male Albino Rats

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Abstract

Background: **Stem cells are self-renewing, undifferentiated cells that can be differentiated into functional cells. Mesenchymal stem cells (MSCs) are a subpopulation of mesodermal stem cells population that can be found in numerous living tissues along with bone marrow. Numerous studies have begun to reveal the characteristics of MSCs, which go far beyond forming connective tissue and their simple derivation from various tissues, making them attractive for research.** ***Aim:*** **The present study aimed to isolate, culture, and characterize the MSCs from the bone marrow of long bones of adult male albino rats.** ***Materials and Methods:*** **both femoral bones and tibia were obtained from adult male albino rats. Their bone marrow was washed using complete media formed of DMEM + 10% FPS+ 1% Pen/strept. The mixture was seeded in the culture flask and incubated at 37° humidified atmosphere containing a 5% co_2 incubator. The culture flasks were examined under a light microscope to monitor the growth of cells. The cells were then harvested and subcultured using the same technique. Subcultured cells were identified using CD 34 & 44 surface markers.** ***Results:*** **the subcultured MSCs appeared as a sheet of adherent cells having a polygonal appearance with prominent nucleolus & a well-defined cell process. Most of the cells showed positive expression of the CD44 surface marker and negative expression of CD 34.** ***Conclusions:*** **cells that were isolated and cultured from the bone marrow of adult male albino rats had the morphological & immunophenotyping characteristics of MSCs.**

Keywords: stem cell, bone marrow, culture

Introduction

Stem cells are undifferentiated cells that can proliferate, regenerate, and convert to differentiated cells. Stem cells can be found throughout the human body, localized in niches that provide a cultivating microenvironment for the cells and control their proliferation and differentiation[1]. MSCs are multipotent stem cells that can differentiate into a range of specialized cell types such as adipocytes, chondrocytes, and osteoblasts[2]. MSCs have been isolated from bone marrow, adipose tissue, tooth pulp, skin, umbilical cord blood, and other adult and perinatal human tissues[3]. MSCs have been used either alone or in combination with other treatments to get significant benefits for every diseased tissue of the human body[4]. This is due to their trophic, paracrine, and immunomodulatory functions

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which may have the highest therapeutic impact in vivo[5]. Rat bone marrow contains two different types of stem cells, hematopoietic stem cells which are involved in blood cells formation, and non-hematopoietic bone marrow MSCs (BMSCs) which provide maintenance and support of the hematopoietic stem cells[6]. BMSCs are isolated and purified through their physical adherence to the cell culture plate[2]. Different protocols have been used to culture and harvest the BMSCs. These protocols depend largely on the adhesive property of the BMSCs. However, the morphology, growth rate, and phenotype was different in each protocol[7]. The current study aimed to find such an easy-to-practice and efficient method to isolate and culture MSCS from the rat bone marrow for further handling in regenerative medicine.

Materials and Methods

Animals

Six male Sprague Dawley albino rats aged 10-12 weeks were used. Animals were purchased from the animal house of the Faculty of Veterinary Medicine, Suez Canal University. The animals were kept in the animal house for two weeks for acclimatization to the new environment before utilization for experimental purposes. The animals received water and food ad libitum. Animal care before and during the experimental procedures was done in accordance with the guidelines of the Animal Ethical Committee, Faculty of Medicine, Suez Canal University. The study was approved by the Animal Research Ethics Committee of the college.

Chemicals

Dulbecco's modified Eagle's medium (DMEM) Low Glucose, w/L-Gluta-mine,w/ Sodium Pyruvate (Biowest), penicillin/ streptomycin, (p/s) (Sigma-Aldrich), Fetal bovine serum (FBS) (sigma-Al-drich), Dulbecco's Phosphate buffered saline (PBS) (Lonza-bel-gium), Trypsin (Lonza,Walkersville).

preparation of BMSCs

Hind limbs were harvested, and skin, muscles, and connective tissue were removed using a scalpel with the maintenance of bone ends to ensure the sterility of the bone marrow. In the tissue culture hood, the ends of each bone were removed by scissors, the syringe was filled with pre-warmed complete conditioned media (DMEM+10% FPS+ 1% Pen/strept) was forced by syringe through the bone shaft to extract all red marrow into 15 ml falcon tube using 10 ml of media for each bone[8]. The cell mixture was centrifuged at 1200 rounds per minute for 10 min. The supernatant was aspirated and discarded. The cell pellet was suspended in 6 ml culture media and mixed by pipetting. The mixture was seeded in the culture flask of 25 cm^2 at a density of ($1x10^6$ mononuclear cells/cm^2 and incubated at a 37°C humidified atmosphere containing a 5% Co_2 incubator in 10 ml of media. The media were changed every 3 days and the culture flasks were examined each time under the light microscope to monitor the growth of cells and to notice any sign of infection if there. On the 12^{th} day, when the BMSCs reached 90-100% confluence in each flask, the flasks were prepared for trypsinization to harvest the adherent cells as follows: All media were removed from the flask. Two ml of trypsin EDTA was added to the flask to form a thin film over the flask surface for 5 minutes. Then the action of trypsin was blocked by adding 5 ml of media to the flask. The mixture was put in a Falcon tube to be centrifuged at 1200 rpm for ten minutes. The supernatant was removed.

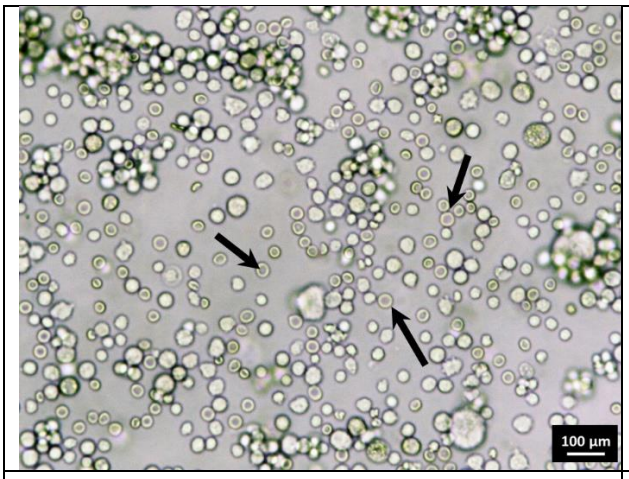

Figure.1: A photomicrograph of cultured BMSCs at the 1st day of incubation; they appeared rounded and floating with a central nucleus (arrows). (X 100).

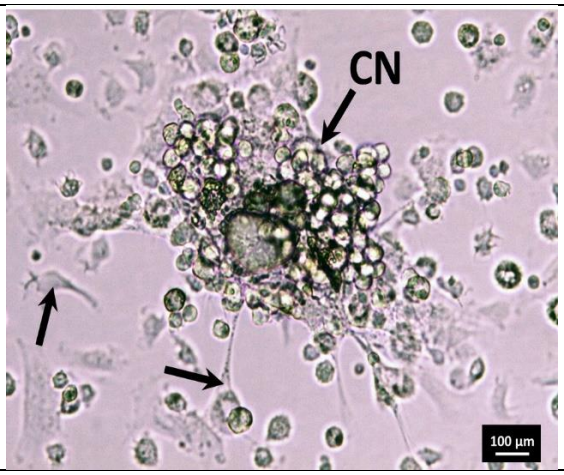

Figure.2: A photomicrograph of cultured BMSCs at the 4th day of incubation; a cell nest of BMSCs was formed (CN). Some cells began to lose their rounded contour with appearance of cell processes (arrows). (X 100).

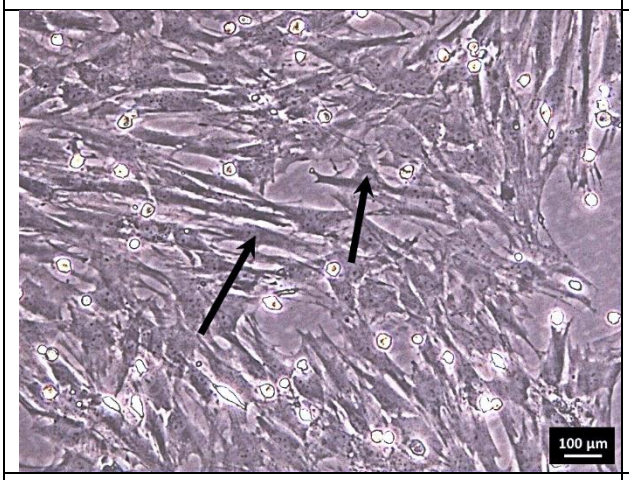

Figure.3: A photomicrograph of cultured BMSCs at the 8th day of incubation; spindle-shaped BMSCs were formed (arrows) that reached about 80% confluence. (X 100).

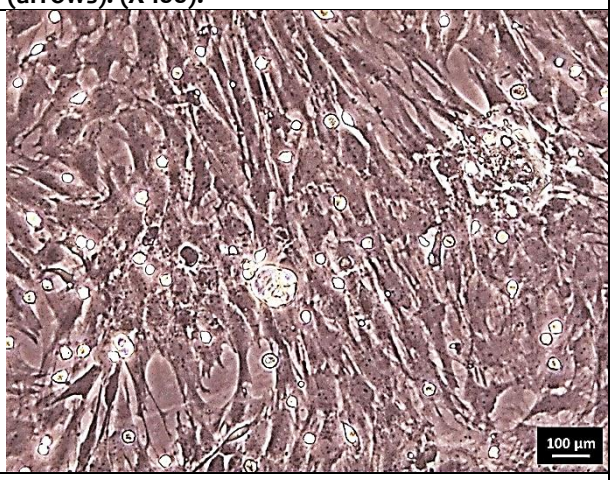

Figure.4: A photomicrograph of cultured BMSCs at the 12th day of incubation; a cell sheet of spindle-shaped BMSCs was formed (arrows) which reached about 100% confluence. (X 100).

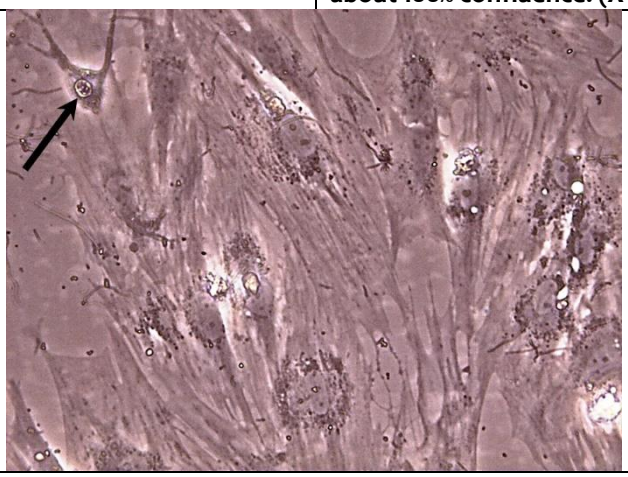

Figure.5: A photomicrograph of the BMSCs at the 3rd day of incubation after subculture; well-defined BMSCs which has polygonal appearance, prominent nucleolus & cell processes (arrow). (X 100).

A complete medium was added to the cell pellet & mixed by successive pipetting. The cell mixture was subcultured in a 75 ml culture flask in 22-25 ml of media and incubated at 37 °C humidified atmosphere containing a 5% Co_2 incubator.

Flow cytometry analysis

Flow cytometry were applied to study the immunophenotype of the cultured cells. Approximately $1X10^6$ BMSCs at the second passage were harvested and resuspended in the growth medium to prepare a single-cell suspension. Cell preparations were treated with the monoclonal antibody against CD34 labeled with fluorescein isothiocyanate (FITC), and against CD44 labeled with phycoerythrin (PE) for 30 min at 4°C. Fix/Lyse Solution was added for lyses the red blood cells and incubated for 10 minutes. Then the sample is washed and resuspended with 300 µl of PBS. All cell preparations were analyzed by FACSLyric TM flow cytometer (BD Life Sciences, San Jose, CA, 95131, USA)[9].

Results

On the 1st day of incubation, the bone marrow cells were rounded in shape with a central nucleus and floating on the surface of the tissue culture flask (Fig.1). On the 4th day, the cells became adherent to the culture flask and divided rapidly showing cell nests of cells. Some cells began to lose their rounded contour and cell processes started to appear (Fig.2). On the 8th day of incubation, adherent spindle-shaped BMSCs were formed with about 80% confluence (Fig.3). On the 12th day, a cell sheet of BMSCs was formed that reached about 100% confluence (Fig.4). Then cells were harvested and subcultured at 37°C with 5% Co_2. On the 3rd day of the subculture, well-defined BMSCs were seen having a polygonal appearance with prominent nucleolus & a well-defined cell process (Fig.5)

Flowcytometry

Most subcultured BMSCs showed positive expression of CD 44 surface marker (about 95%) which is one of the positive markers of MSCs (Figure.6). On the other hand, most of the cells showed negative expression of CD 34 (only about 4% positive cells) which is a marker of the hematopoietic cells (Figure.7).

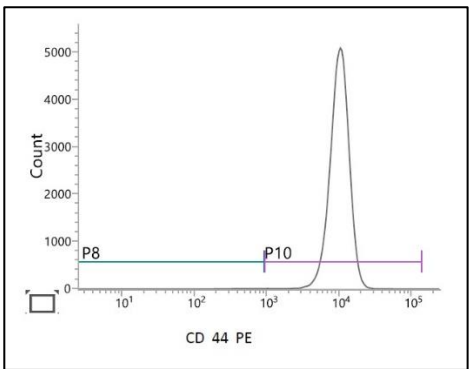

Figure.6: CD 44 positive cells.

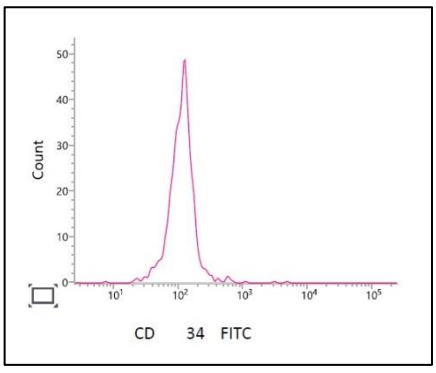

Figure.7: CD 34 negative cells.

Discussion

In the present study, the cell suspension of rat bone marrow containing both mesenchymal and hematopoietic cells was seeded in tissue culture flasks. At the end of the first two days, many of the rounded

as well as spindle-shaped cells had been found to be attached to the base of the tissue culture flask which is characteristic for MSCs, while the rounded hematopoietic cells remained suspended in the media and were mostly removed with further media changes. These findings are consistent with other studies that reported the varying adhesive characters of different cell types in bone marrow cell preparation[10,11]. On the 4th day of cultivation, cell nests were seen to be formed of MSCs aggregates. It was demonstrated that this MSCs aggregate is formed due to integrin binding to extracellular matrix proteins which facilitate cell-cell contacts to form cellular aggregates[12]. On the 8th day of cultivation, most of the cells had a spindle shape with many cytoplasmic cell processes and reached 80% confluence, then they reached about 100% of confluence on day 12. The subcultured cells had a well-defined polygonal appearance with many cytoplasmic processes. It was noticed that the morphology of MSCs is heterogeneous containing both small, elongated bipolar cells and polygonal cells. They added that the smaller fibroblastic-like cells between 60–100 μm are actively growing cells while the larger polygonal cells proliferate slowly[9,11,13]. In the current study, the immunophenotype of BMSCs was studied by flow cytometric analysis of surface molecular markers. The subcultured BMSCs showed positive expression of the CD 44 marker in most of the adherent cells which is a marker of the mesenchymal cells[14,15]. On the other hand, most adherent cells displayed negative expression for CD 34 which is considered a hematopoietic progenitor cell marker[15]. Therefore, the BMSCs used in this study met the standard criteria of the ISCT which include adherence to the culture flask and positive expression of stromal CD markers[16].

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