Human Bone Marrow Mesenchymal Stem Cells Isolation, Expansion and Identification

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Abstract
The present study aimed to isolate and diagnose mesenchymal stem cells derived from human bone that is the source generating cells that are the best types of treatment for tissue diseases.

Cells were isolated from the back bone of the human pelvis, separated using density gradual sedimentation method and then the cells were grown on the culture media RPMI-1640 \ 20% FBS.

To detect the purity of cells that have been isolated and have been transplanted immune use the method using CD44 (mesenchymal stem cells marker) CD43, a specific marker for hematopoietic cells Nestin, (the neurons private marker).

The present study has shown that mesenchymal cells that have been isolated and expanded in this experiment has reached up 99.7% for the CD44 marker and only 1.1% for the hematopoietic cells marker CD34 and finally was 23.9% for the parameters for the neuronal marker Nestin.

Keywords: Human; BMSCs; Ficoll.

الخلايا اللحمية الوسيطة المشتقه من نخاع العظم البشري: عزلها، تنميتها و تشخيصها

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الخلاصه

هدفت الدراسه الحاليه لعزل وتنميه وتشخيص الخلايا اللحميه المشتقه من نخاع العظم البشري، الذي يعد مصدر للخلايا المولده التي تعتبر افضل انواع لعلاج الامراض النسيجيه. تم عزل الخلايا من الجزء الخلفي لعظم الحوض البشري وفصلها بطريقه الترسيب التدريجي ثم نميت الخلايا على الوسط الزرعي RPMI-1640\20%FBS. وللكشف عن نقاوه الخلايا التي تم عزلها وزرعها فقد تم استخدام الطريقه المناعيه باستخدام CD44(المعلم الخاص للخلايا اللحميه)،CD43(المعلم الخاص للخلايا المكونه للدم Nestin (المعلم الخاص للخلايا العصبيه). اثبتت الدراسه الحاليه ان نقاوه الخلايا اللحميه التي تم عزلها وتنميتها في هذه التجربه تصل ل 99.7% لمعلم ال CD44 و 1.1% فقط لمعلم الخلايا المكونه للدم CD34 واخيرا كانت 23.9% للمعلمات الخاصه بالخلايا العصبيه Nestin

Introduction

Stem cells is unspecialized cells with the ability to renew itself indefinitely, under appropriate conditions, stem cells can give rise to a wide range of mature cell types [1].

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Two types of stem cells can be distinguished according to their origin and potential of differentiation, embryonic stem cells (ESCs) and somatic stem cells (SSCs) which also called adult stem cells (ASCs) [2]. The ESCs are derived from the early blastocyst and the inner cell mass (ICM) of the embryo and are able to differentiate into three germ layer cell types (pluripotentiality) [3], while SSCs are isolated from fetal (after gastrulation) or adult tissues [1].

The stem cells trophic factors can act in synergy to: inhibit apoptosis and limit tissue injury; attenuate pathologic fibrotic remodeling; promote angiogenesis and vasculogenesis; activate resident tissue stem cells; and modulate host immune response and reduce inflammatory oxidative stress [4]. The hope has been for the clinical use of such versatile cells in a number of diseases [5-7]. Korbling and Estrov (2003) demonstrate that ASCS enter "normal differentiation pathways" where they divide and mature, eventually generating specialized cells specific to their type [8].

Numerous studies have revealed that population of ASCs and supporting cells reside within specific areas designated as niches in most of adult mammalian tissues / organs including BM [9], skin [10], eyes [11], gastrointestinal tract [12], liver [13], pancreas [14] and lungs [15]. ASCs can also be obtained from many tissues including synovium, deciduous teeth, adipose tissue, brain, blood vessels, blood and umbilical cord blood [4, 16-18].

The spinal cord is the largest nerve in the body. Nerves are "cordlike" structures made up of many nerve fibers. The spinal cord is made up of many nerve fibers that acts as a telephone cable connecting the brain with other parts of the body [19].

A spinal cord injury can occur either from trauma or from a disease. In most spinal cord injuries, the vertebrae pinch the spinal cord. The spinal cord may become bruised or swollen. The injury may actually tear the spinal cord and its nerve fibers. An infection or disease can produce the same results [20].

Materials and Methods

The BMSCs were collected from 20 donor from the posterior iliac crest at 20-35 years of age. The bone marrow were isolated under aseptic conditions and transferred in a conical tube containing 500 units of heparin and 6 ml RPMI 1640, using a 10 G needle. The cell pellet was obtained and containing B-lymphocytes, granulocytes, monocytes, adipocyte, very small embryonic-like cells (VSEL) mesenchymal, hematopoietic, and endothelial stem cells all the mononuclear cells (MNC) were suspended in RPMI1640, 20% fetal bovine serum (FBS), 100 u/ml penicillin, 100 µg/ml streptomycin, 25 ng/ml amphotericin B and 2 mM L-glutamine. The harvested cells were seeded on a 25 cm^2 flask (Nunc, Denmark) at 37 °C, 5% CO_2 incubator for 24 hours. The flasks were washed with PBS in order to remove the haematopoietic cells. The cells were incubated for 2-3 days to reach the confluency. The cells were detached with 0.25% trypsin and 1 mM EDTA for 2 mins at 37°C in order to obtain a single-cell suspension. Nearly 6,000 cells/cm2 were replated in new gelatin-coated flasks. This cycle was repeated four times (passage 1, 2 and 3 respectively). At the 3th passage, the cells were checked for BMSCs purity using anti-nestin immunostaining as well as CD44 and CD 34.

At the end of the 3ed phase, the cell population of the flasks was harvested for evaluating the immunocytochemical analysis. The isolated and induced BMSCs were plated on a gelatin-coated glass coverslip, washed in PBS and fixed with 4% paraformaldehyde in phosphate buffer saline PBS for 15 minutes. The fixed cells were washed twice with PBS before staining. Permeabilization and blocking nonspecific antigen reaction were carried out in blocking buffer consisting of 0.1% Triton X-100 and 10% goat serum in PBS for 1 hour. The primary antibodies (mouse anti-nestin monoclonal antibody diluted at 1:100, mouse anti-CD44 polyclonal antibody diluted at, 1:100, mouse anti-CD34 monoclonal antibody diluted at 1:100) were incubated overnight at 4°C temperature and washed three times in PBS. The secondary antibodies (biotin (US-Biological, diluted at 1:150)) were used for 30 mins at room temperature. The mixture of streptavidin conjugated to horseradish peroxidase was incubated at room temperature for (30) minutes before use, then added to cover the cells, and then the cells were washed with PBS three times.

Each plate was received 1-2 drops of DAB solution and this step was made in darkness and then left for 10-20 minutes. The cells were washed one time with distilled water.

Two drops of Harris Haematoxylin and Eosin stain were added and left for 5-10 seconds respectively, and then washed with distilled water. Then left for five minutes to dry.

The cells were mounted with gelatin and viewed under a light microscope, and the images were captured with a CCD camera directly connected to the system. Nuclear counting was done for the

isolated and induced BMSCs. The numbers of immunoreactive cells were divided by the total cell number in order to estimate the percentage of immunoreactive cells.

Each experiment was replicated at least 7 times so that reproducibility could be insured.

Results and discussion

After the collection of human bone marrow, the bone marrow components separated by density gradient centrifugation into four layers (Figure -1 A, B). The lower layer represents (45-50%) of the entire volume of the blood and made up of erythrocytes, the layer immediately above which separated fluid (Ficoll-paque), while the layer immediately above which represents (1%) of the blood volume and it is white or grayish in color which is called the buffy coat and consists of MNCs, the upper layer, which is translucent, yellowish that is, represents the plasma.

Bone marrow isolations reached $6 \pm 2 \times 10^6$ cells per 6ml of BM with viability ranging from 98% to 100% (Figure-1C). The cell suspensions were seeded in tissue culture flasks $25cm^2$ at the density of 3×10^6 cell/ml using RPMI-1460 with 20% FBS. Non adherent cells were carefully removed after 24 hours by washing the adherent cells with complete media RPMI-1640 for three times and 5ml of fresh complete isolation media (CIM) RPMI-1640/20%FBS were replaced. Then adherent cells (passage 0) received fresh complete medium every 72 hours and the culture were observed via phase contrast microscopy for their morphology. The initial culture (time 1 hour) of the bone marrow suspension, cells were found to be round and glistening contained a heterogeneous cell populations, including erythrocyte, B-lymphocytes, granulocytes, monocytes, adipocyte, very small embryonic-like cells (VSEL) mesenchymal, hematopoietic, and endothelial stem cells etc. (Figure -2 A).

Mesenchymal stem cells were isolated based on their ability to form adherent monolayer in culture flask and the lack of adherence in other cells in the bone marrow stroma such as erythrocyte, adipocytes and macrophages. Hence, change media performed after 24 hours, were many of MSCs had attached to the base of the tissue culture flask (Figure- 2 B). These adherent cells were remained attached to the bottom of the flask, formed high numbers of scattered plastic adherent colonies (at 48 hours). Some of adherent cells assumed spindle morphology, a characteristic oval body with the flattened ends. When they slowly elongate, others appeared as individual small size spindle-shaped cells (Figure- 2 C). While the floating round cells remained suspended in the medium and were mostly eliminated from the culture with subsequent media changes. The adherent cells tend to be locally confluent, growing in distinct colonies at about 72 hours (Figure- 2 C). Selective removal of cells in the suspension allowed the growth of adherent uniform spindle morphology cells formed colony-forming units fibroblast (CFU-F) were a large number of cells grew in a radial manner in neat rows, and the density increased significantly which fused to form the confluent monolayer (Figure -2 D,E). Upon reaching 80-90 % confluence before day 10 of primary isolation, the MSCs were subjected to passaging. Because CFU-F adherence was sensitive to trypsinization, 80-90% were detached after treatment of adherent cells with trypsin (without affecting viability) [19], and re-seeded at the density of 10^6 cells/ml at the split ratio of 1:2 using complete expansion media (CEM) RPMI-1640/ 20% FBS, and previously used re-filtered media.

The MNCs viability and cell count were determined by trypan blue stain. The dye stain only dead cells that appeared as blue color while viable cells exclude the stain and remain unstained that appeared shine

The isolated MNCs were used for cryopreservation [20] and *in vitro* cultivation [21].

Density gradient centrifugation is the most widely used method for isolating MSCs from human BM and mononuclear cells from peripheral blood [22, 23]. This method was suitable to obtain MSCs from 6ml of bone marrow by DGC and yielded almost half of initial content it is a well-known problem that DGC causes a significant reduction of BM-MNCs to only 15–30% of the initial content [24, 25]. Could be up to 50% [26].

The efficacy and functionality of BM-MNCs are significantly influenced by red blood cell contamination [27]. The content of apoptotic cells [28]. Different washing steps [29]. The centrifugation speed [24]. Even the choice of the density gradient medium [26].
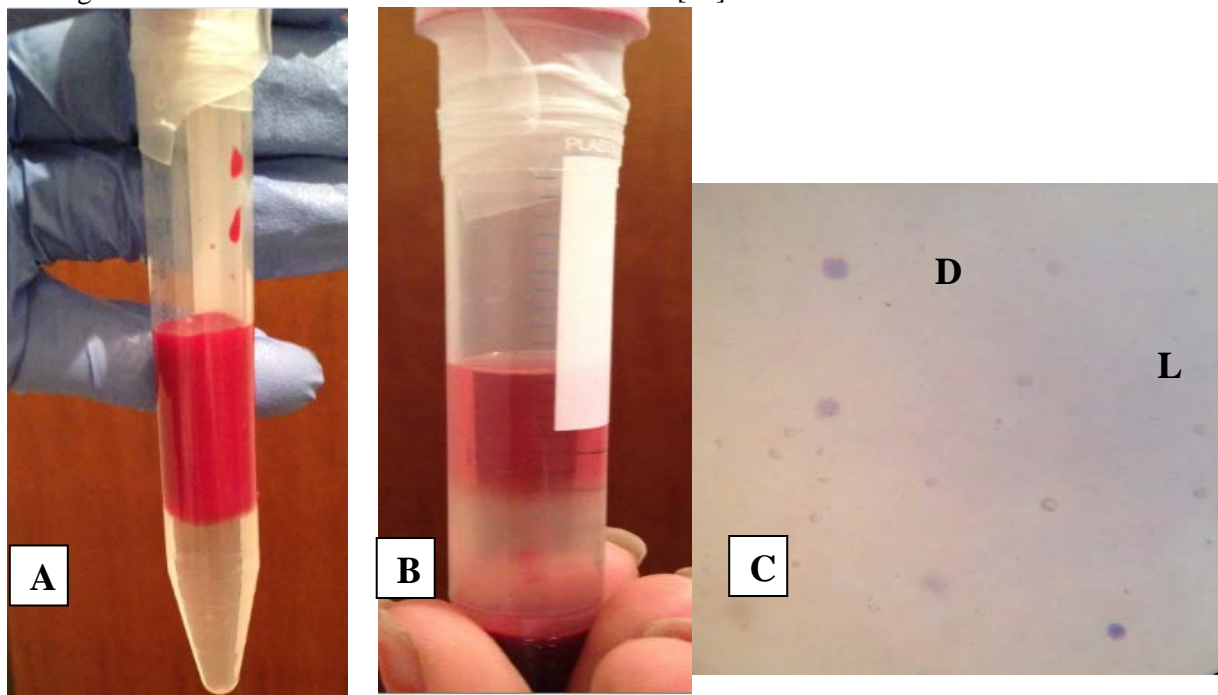
Density gradient centrifugation treatment removes erythrocytes form BM- crud suspension. Since, contamination with RBCs influences the functionality of BMCs used for cell therapy, impair the mitochondrial function of BM-MNCs [27]. Moreover, it is well established that mitochondrial function and metabolism are important determinants of stem cell self-renewal and differentiation. Reduced the viability, invasion, and CFU capacity of the applied BMCs [30, 31].

Hemolysis of RBCs results in high levels of free heme were leads to cellular injury [32]. Moreover, free hemoglobin was shown not only to directly induce apoptosis in cultured endothelial cells [33] but also to scavenge nitric oxide (NO) that plays a crucial role for stem cell maintenance, differentiation, and neovascularization capacity [34] and increases mitochondrial function in endothelial cells [35]. Thus, reduced NO levels in the BM-MNCs suspension might have contributed to impaired benefit of BMCs associated with mitochondrial dysfunction [27].

After centrifugation on Ficoll, human bone marrow was separated into several fractions such as plasma, mononuclear cells, granulocytes, and erythrocytes (figure -1 B). Since BM-MSCs belong to the mononuclear cells fraction in the bone marrow.

Recently, it was described that Ficoll DGC even depleted cells with a high regenerative potential, such as MSC [36] and very small embryonic-like cells VSEL [37], and irreversibly impaired cell function by decreasing expression of chemokines receptors [38, 39]. Excessive cell loss during DGC is a consequence of density medium-related cytotoxicity [40].

The DGC method is a time-consuming process by nature of the excessive handling involving at least three washing steps that make the system open (exposure to the environment) and more likely to result in bacterial contamination [41]. With many risks: toxicity, osmotic pressure changes and penetration into particles [42]. Which explain that after centrifugation, many suspended cells were found in the medium at 6 hours. This could be due to the density of cells, which was changed slightly in RPMI-1640. DGC using Ficoll and Percoll are associated with potential cell injury, as demonstrated by a significant reduction in tritiated thymidine incorporation and loss of lymphocyte viability following culture with Ficoll-Hypaque [43]. As apoptotic cells could be exerting influence on numerous biological processes through release of microparticles to induce apoptosis in neighboring cells [44-46] MSC from young donors exhibit the spindle-type morphology in very early cultivation and a gradual loss of these features over cultivation time [47].

Figure 1-Human BM-MNCs isolation by DGC using Ficoll- Hypaque with density= 1.077g/ml. (A): The whole BM suspension cells was layered carefully on top of the Ficoll without disturbing the solutions. (B): After gradient centrifugation, cell suspension component will start to sediment into separate zones. Our goal of this process was the light density white cells (black arrow), which represent the BM-MNCs. Those MSCs are belong. (C): The viability test of BM-MNCs isolated by DGC method using Ficoll (40X) Revealed under light microscope.
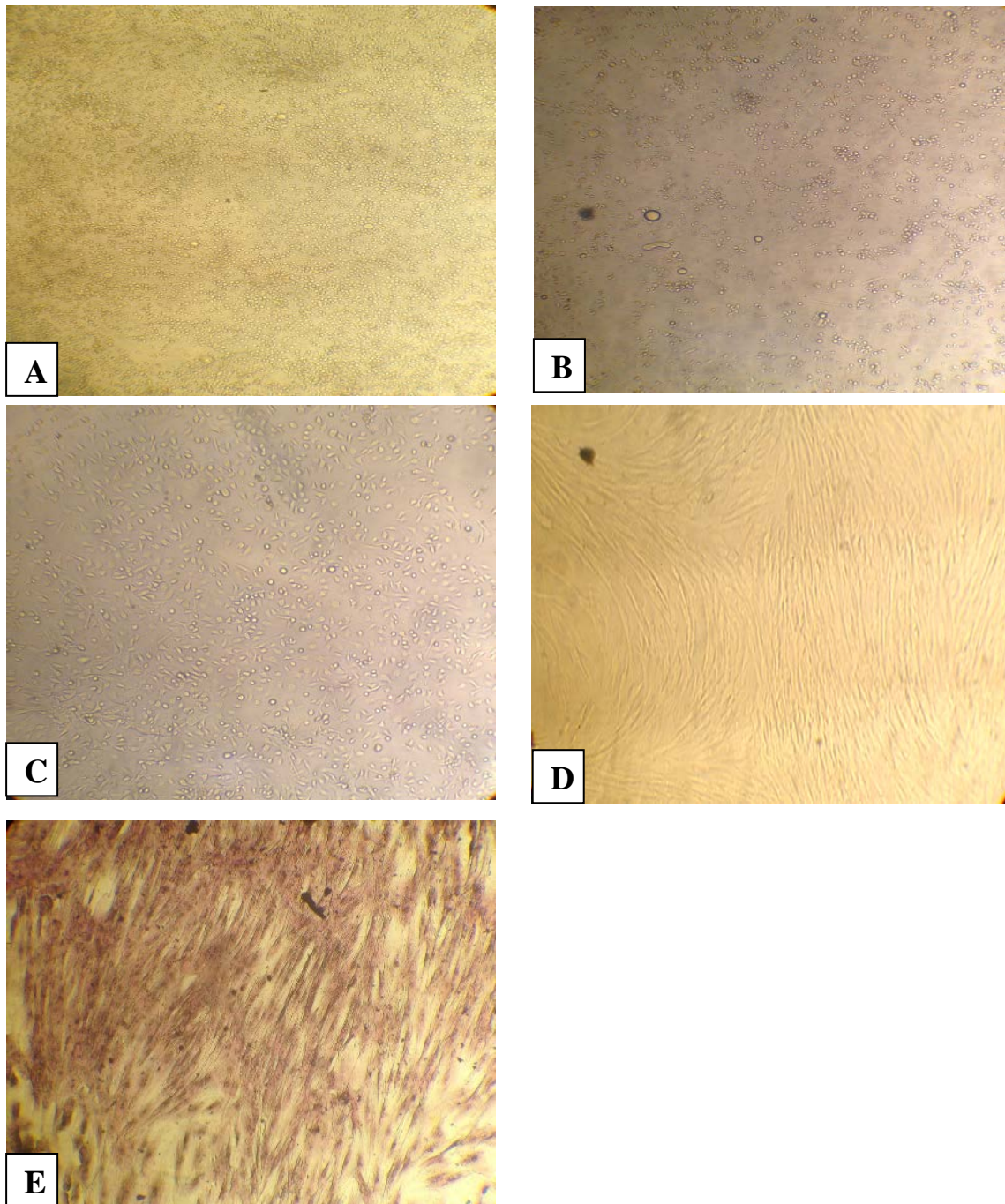
Figure 2- The morphology of human BM-MSCs primary culture, which cultured in RPMI-1640+20% FBS revealed under inverted microscope. (A): Cultured buffy coat (10X) at time (1 hour). (B) Cultured cells after 6 hours –many of MSCs were adhered firstly and others remain floated (20X). (C) BM-MSCs forms many colonies (CFU) after 5 days (10X). (D) Displayed the confluent monolayer of BM-MSCs, the density of spread cells increased significantly which fused to form the monolayer (10X). (E): Displayed the confluent monolayer of BM-MSCs, the density of spread cells increased significantly which fused to form the monolayer (10X), (D): H&E staining monolayer of BM-MSCs primary culture (10X).

Immunophenotypic characterization of BM-MSCs

The morphological homogeneity of the monolayer cultures at the third passage on falcon was also apparent by immunocytochemistry staining analysis, for the BM-MSCs isolated by direct plating of whole BM and ficoll methods.

For standard method we should use many MSCs markers, but because of the lack of availability of these markers we just used only 2 confirmative markers (CD44 and CD 34).

The antigenic phenotype was carefully checked by using two groups of cell surface molecules (CD44 and CD34). The immunocytochemical staining results were shown in (Figures-3). All immunoreactivity staining was performed on the same sample, and cells were counted by inverted microscope under (X10) and (20x) power.

Immunophenotypic evaluation demonstrated that human and rat BM-MSCs uniformly positive for CD44 (Figure- 3 A) and negative for hematopoietic specific markers CD34 (Figure- 3 B) and for nestin (Figure- 3 C). Cells immunostained with positive marker stained strongly brown. Negative control and cells immunostained with negative one showed no staining.

The immunoreactivity percentage of antigenic phenotypes for BM-MSCs at the third passages was shown in table -1. The results showed that MSCs are present in the human and rat BM, by using DGC direct plating culture techniques, can be successfully isolated and expanded *in vitro*. Although the initial cell culture consisted of fibroblastoid, spindle-shaped morphology and another with epithelioid, polygonal morphology, only the fibroblastoid population remained after enzymatic digestion and passaging until the third passage it appear pure homogenous population of MSCs. In their undifferentiated state MSCs are spindle-shaped and resemble fibroblasts. Based on the expression profile of these markers, BM- MSC behave as one sole cell population, as all the studied markers were homogeneously expressed compatible with agreed MSC markers. Indicating that the cells used in present study had the characteristics of MSCs [48-50].

In addition, the isolated MSCs of high purity at third passage were not contaminated by heamatopoietic or endothelial cells as immunocytochemical staining demonstrated that cell preparations were always negative for CD34 and always positive for CD44.

Identifying the MSC fraction in the BM will be a more formidable challenge, since there are no markers that specifically and uniquely identify MSCs and therefore they are defined by their Immunophenotypic profile.

Table 1- Types of CD markers used for antigenic phenotypes analysis of mouse BM-MSCs and their percentage at third passage.

Cell Surface Character	BM-MSCs- detection Percentage %
CD44$^+$	99.7%
CD34$^-$	1.1%
Nestin	23.9%

There has been a common opinion that CD105, CD90, and CD44 are highly specific for MSCs [51]. In the BM, where the over whelming majority of cells are heamatopoietic, these markers may indeed be useful to explain that the phenotyped cells are non-heamatopoietic and stromal in origin. CD44, CD105 and CD90 were also reported to react with undifferentiated MSCs and thus seem suitable for isolation of more pure MSC population [52-54].

More recently, several studies showed that these markers were expressed on stromal cells from many locations as well as on skin fibroblasts [55, 56]. Since, the contamination of MSC cultures with fibroblasts lead to run-down of the stem cell differentiation potential because fibroblasts undergo senescence and eventually die.

This study confirms the fact that CD44 marker expressed only on MSCs but not on fibroblasts. Hence, added to the proteomic analysis list, identification and elimination of fibroblasts from MSC cultures could improve the MSC yield and differentiation potential and prevent tumor formation after MSC transplantation.
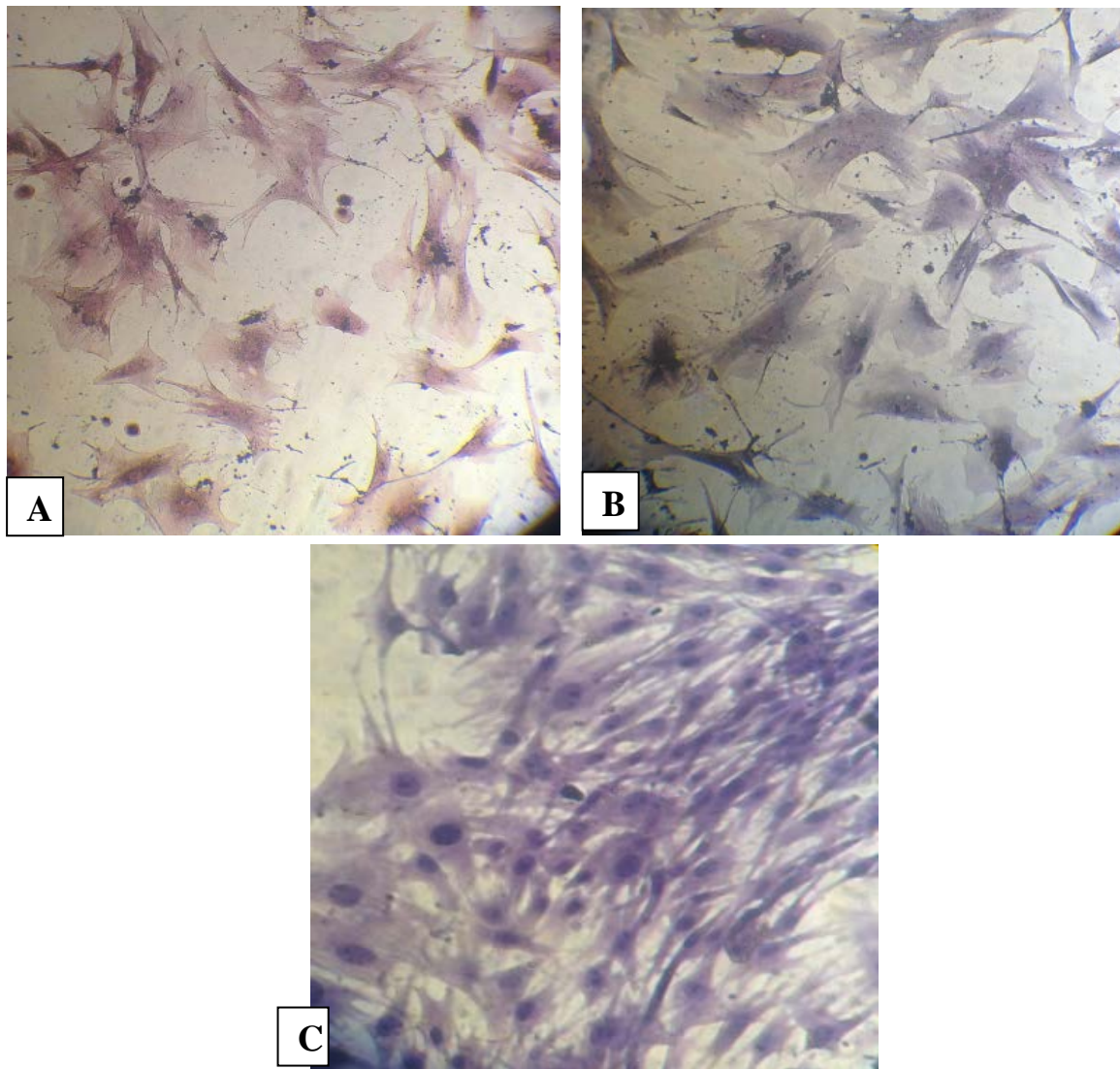
Figure 3- Immunophenotypic analysis revealed by light microscope. (A): BM-MSCs Most of adherent MSCs were positive response forCD44 marker were stained with brown color DAB stain and Hematoxylin stain (20X). (B): BM-MSCs most of adherent MSCs were negative response for CD 34 marker respectively were stained with brown color DAB stain and Hematoxylin stain respectively (20X). (C): BM-MSCs most of adherent MSCs were negative response for nestin marker were stained with brown color DAB stain and Hematoxylin (20X).

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