International Journal of Endorsing
HEALTH SCIENCE RESEARCH

IJEHSR

Original Article

Isolation and identification of Murine Mesenchymal Stem Cells from Bone Marrow.

Sehrish Jabeen[1], **Muhammad Sufyan Vohra**[2] & **Yasir Raza**[1]

[1]Department of Microbiology, University of Karachi, Karachi-Pakistan.
[2]One Health Diagnostics, Karachi-Pakistan.

Doi: 10.29052/IJEHSR.v10.i2.2022.150-156

Corresponding Author Email:
djsehrishjabeen@yahoo.com

Received 27/02/2022
Accepted 27/04/2022
First Published 16/05/2022

OPEN ACCESS

Abstract

Background: Mesenchymal stem cells (MSCs) are beneficial for cell and gene therapy approaches. These cells are highly potent in producing various cells, including adipocytes, osteoblasts, hematopoietic cells, etc. High proliferation and regenerative capacity are important phenotypes of MSCs which contribute to the recovery process at the damaged sites. Various techniques have been used, which have inconvenient effects on stromal cell properties. The present study evaluated the stem cell markers in cells isolated from the Murine bone marrow.

Methodology: The murine mesenchymal stem cells (mMSCs) were isolated from the bone marrow of murine Balb/c mice using a simple in vitro culture protocol. Trypan blue exclusion assay was performed for viable cell count, and cellular morphology was checked using an inverted microscope. The immunofluorescence staining was done to analyze the immunophenotypic features of mMSCs by treating cells with CD44, CD90, and CD45 fluorescent antibodies.

Results: mMSCs with spindle-like cell appearance were attained within three weeks. Immunofluorescence staining of isolated mMSCs displayed a statistically significant ($p<0.001$) number of CD44 and CD90 positive cells. Alternatively, an insignificant count of CD45 labeled cells was found.

Conclusion: A uniform mMSC population was achieved with high propagation capability in lower passages through regular replacement of medium and reduced trypsinization time. In addition, mMSCs were strongly expressed MSCs positive markers, including CD44 and CD90.

Keywords

Mesenchymal Stem Cells, In Vitro Culture, Immunofluorescence Staining.

Check for updates

Introduction

Mesenchymal stem cells (MSCs) are pluripotent with high proliferation and multidimensional differentiation potential in vitro[1]. These cells frequently exist in fetal and adult tissues[2,3]. MSCs were successfully isolated from bone marrow and other sources like subcutaneous adipose tissue[1]. Cord blood, placenta, and bone marrow are familiar sources of MSCs used for tissue repairing and immune variation studies[4]. Various studies have shown that BM-MSCs are highly potent in producing different cells in synthetic environments, such as adipocytes, hepatocytes, osteoblasts, cartilage, and hematopoietic cells[1]. The greater proliferative and regeneration capacity is an essential phenotype of MSCs, and this is a potential capacity of MSCs to contribute to the recovery process at the damaged sites. MSCs have been extensively used in regenerative medicine[5] and studied for immunomodulatory characteristics[2,3].

It has been primarily believed that MSCs are progenitor stem cells, especially from the origin of bone marrow. These cells have the principal role in tissue renewal in undifferentiated conditions. In addition, MSCs found to be fibroblast-like and readily adhere to the plastic surface in standard culture conditions. They can survive in a xenogeneic environment free from immune rejection and circumvent ethical constraints[6]. MSCs are a remarkable option for clinical trials since their isolation, in vitro culturing, and expansion are simple and fast[7]. In bio-preservation, these cells minimally lose potential.

In addition, there is a growing focus on using regenerative stem cells as BM-MSCs have been applied to treat human diseases[8]. MSCs are essential tools for cell and gene therapies[9]. Various studies reported that MSCs are highly useful in hematopoietic recovery, regeneration of bones, joints, or heart diseases[9,10]. Therefore, further extensive preclinical studies are required on MSCs from diverse species. Furthermore, bone marrow is thought to be an ideal choice for MSCs. The present study aims to examine stem cell markers in cells isolated from the Murine bone marrow.

Methodology

Isolation and Culturing of mMSCs

mMSCs were isolated from the bone marrow of murine Balb/c mice (6-8 weeks) using the method described by Soleimani and Nadri[9]. Bone marrow cells were grown, in particular Dulbecco's modified Eagle medium (DMEM) (Gibco) in the specific culture plates (60-mm). Medium was added containing 10% fetal bovine serum (FBS, Gibco), penicillin 100 µg/ml/streptomycin 100 µg/ml (Gibco) and 2 mM L-glutamine (Gibco). Cell culture was incubated at 37ºC in a CO_2 (5%) incubator. Initially, the culture media was discarded slowly to remove the non-adherent cells after 3 hours. The medium was replaced after an additional 8 hours from the initial culture for up to 72 hours. Adherent cells were washed (considered passage 0) with PBS (Sigma-Aldrich), and fresh medium was added every 3–4 days. Cellular morphology was analyzed using an inverted microscope, including shapes and adherence capacity (Nikon ECLIPSE TS100, USA).

Culture Expansion

When cultures became confluent (~80%), cells were trypsinized within 2 weeks by treating 0.05% trypsin/1 mM EDTA (Gibco) for 2-3 minutes at room temperature. Trypsin was neutralized by adding the same culture medium, and then marrow cells were split into multiple flasks (25 cm^2). The medium was changed after 72 hours.

Cell viability assay

After isolating bone marrow cells, viable cells were determined on a hemocytometer using Trypan blue dye (Sigma-Aldrich).

Immunofluorescence staining to identify mMSCs

The immunofluorescence staining was performed to analyze the immunophenotypic characteristics of mMSCs by incubating the cells with specific fluorescent antibodies. Cells were grown on sterilized coverslips in culture plates (24-well). After 24hours, cells were cleaned using PBS and then fixed with paraformaldehyde (4%). 1% BSA (Invitrogen) was used for blocking for 30 minutes. Then, cells were treated with anti-mouse CD44,

CD90, and CD45 (1:200) antibodies (Santa Cruz Biotechnology) for 1 hour at room temperature in the dark. Cells were washed three times consuming PBS and treated with 1:500 diluted specific FITC-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) as secondary antibodies for 1hour. Cells were washed with PBS three times. Cell imaging was done using Nikon 90i Upright Microscope (Nikon, Japan). The average mean of positive fluorescent cells was calculated. Around 300 cells were counted for each antibody labeled sample.

Statistical analysis

Mean values were calculated by applying the Student t-test from three representative experiments. All analysis was completed using IBM SPSS version 20.0 software. Statistically, significant differences were determined, and p-values <0.05 were considered significant.

Results

Approximately $7x10^7$ cells were found from the femur and tibia with 98% viability in the present study. The spindle-shaped cells were observed on the third day under the microscope. Initially, numerous hematopoietic cells were found in the collected marrow cells. After three days of starting culture, we observed a considerable reduction in the numbers of hematopoietic stem cells. In the 3rd week, most isolated-cells were showed a spindle-shaped representation. Bone marrow culture became confluent (70-80%) in two weeks with small number of hematopoietic cells detection. An undistinguishable type of mMSCs with a spindle-like morphology was attained within three weeks of starting culture presented in figure 1 (iv). mMSCs culture was confluent within seven days after trypsinization. The images were captured at 10X magnification with a scale bar of ~100 µm.

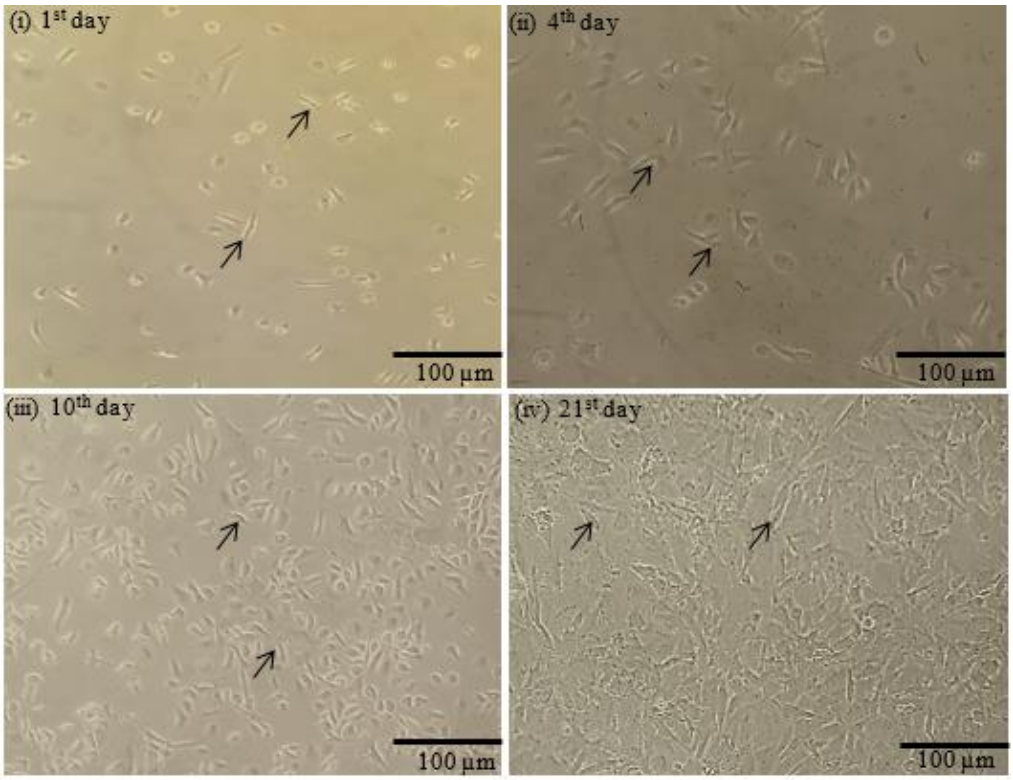

Figure 1: Morphological features of mMSCs (i) Spindle shapes morphology of mMSCs appeared on day 1. (ii) & (iii) The numbers of cells were gradually increased on days 4–10. (iv) Purified population of mMSCs at 21 days of culturing (10× objective).

Isolated mMSCs indicated strong expression of surface markers and higher growth potential. The phenotype analysis revealed that the mMSCs exhibited strong fluorescent staining for CD44 and CD90, while mMSCs labeled with CD45 displayed negative staining, as shown in figure 2 (i), (ii) and (iii), respectively. The number of CD44 and CD90 positive cells was significantly (<0.001) high in the pure culture of mMSCs in passage number[6-8]. On the other hand, an insignificant count of CD45 was observed (figure 3).

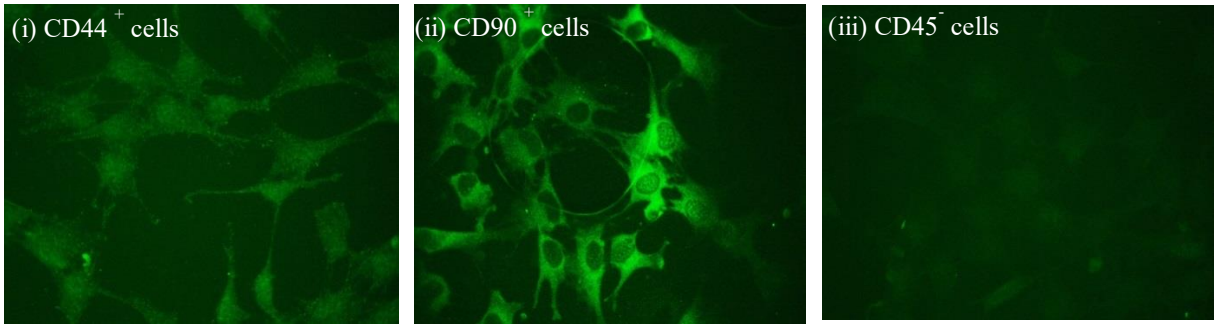

Figure 2: Identification of mMSCs by Immunofluorescence staining. Immunofluorescence staining showed that mMSCs positively express MSCs surface markers (i) CD44 and (ii) CD90. (iii) Isolated mMSCs displayed negative staining for CD45 at 20x magnification, Scale bar =100µm.

Table 1: Average Means of Identification Markers of mMSCs.

Experimental Groups	Number of cells observed	p-value
CD44+ cells	294±3	<0.001
CD44- cells	6±3	<0.001
CD90+ cells	290±5	<0.001
CD90- cells	10±5	<0.001
CD45+ cells	5±3	<0.001
CD45- cells	295±3	<0.001

Mean ± standard deviation (SD) was calculated by T-test, and p-values <0.05 considered statistically significant.

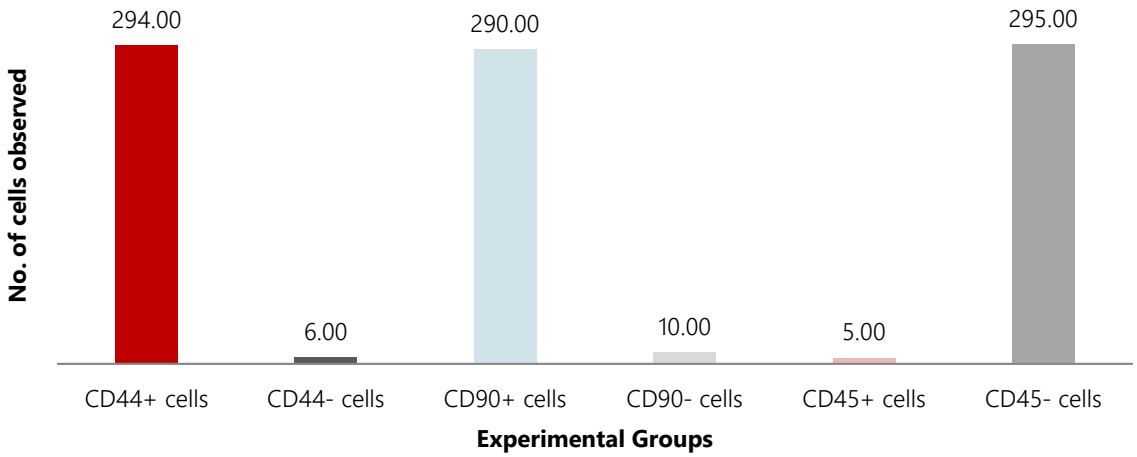

Figure 3: Immunofluorescence staining analysis of mMSCs.

Immunofluorescence staining results demonstrated a significantly high mean of CD44+ and CD90+cells in isolated mMSCs confirmed by T-test and p <0.001. In contrast, CD45+cells are remarkably low in number.

Discussion

MSCs have been thought to be a perfect source of stem cell treatment therapy or regenerative drugs. These cells can be cultured and expanded with multi-potential properties[1]. BM-MSCs were isolated and cultured in specific culture conditions to obtain a pure cell population in the present study. It has been observed that the initial culture contained mixed types of cells like the published protocol[9]. However, round-shaped cells were high that then slowly dropped after repeated media changes, and their proliferation speeded over time. These present results are consistent with previous studies[11]. It has been clarified that frequent medium replacement and decreased trypsinization time are advantageous for obtaining a net population of MSCs.

Similarly, repeat medium change reduced the attachment of non-specific cells on the plastic dishes[9]. Previously, hematopoietic cells have been reported to contaminate BM-MSC's starting culture and expansion[12]. Comparable to results[9], a pure mMSCs culture with spindle-like morphology was reached within three weeks. Another study presented relevant results as; BM-MSCs isolated by plastic adherence were mixed type and included hematopoietic cells[11,13]. This was due to the high number of hematopoietic cell counts in the primary and passage cultures[14]. In addition, previous studies described that mMSCs encourage the growth of the hematopoietic cells by secreting many growth factors, such as stem cell factor interleukins (IL) IL-6, IL-7, IL-8 IL-11, etc., macrophage-CSF, LIF, and FLt-3 ligand[15]. Repeated medium change possibly inhibits the adherence of non-specific cells on culture dishes[9]. Presently, mMSCs culture was confluent every 7 days over subculture. Alternatively, MSC populations promptly produced a monolayer with PD15 and achieved 70-80% confluency within 2-3 days[16].

Various researchers have examined identification markers CD44, CD90, and CD45 to isolate maximum pure MSCs culture[9,17,18]. Where positive detection of CD44 and negative expression of the CD45 presented in the mouse BM-MSCs[9,19]. Moreover, the immunofluorescence staining of BM-MSCs indicated positive results for MSCs markers CD90 and CD44[20,21]. The current immunophenotype results related to positive CD44 and negative CD45 surface markers were similar to those previously reported for marrow MSCs[22,23]. It was clearly shown that there was no contamination of hematopoietic cells. Likewise, it was tested that ≥ 95% of the MSC's population necessarily showed CD105, CD73, and CD90 expressions. However, these cells must indicate a lack of CD45 expression[6]. Moreover, the entire MSC population was positive for MSCs markers such as CD105, CD90, and CD73, indicating their origin in the mesenchymal cells. Besides, the negative expression of CD45 has been reported, which demonstrated successful separation from the hematopoietic stem cells that existed in large numbers[16].

Various conflicting outcomes have been documented in the literature related to the MSCs phenotype marker because their expression becomes changed upon subculture[18,24]. Presently, high numbers of CD44+, CD90+, and CD45- cells were observed in the initial 6-8 passages. Whereas, in BM-MSCs passages 8 and 13 and next, the positive expressions of CD29, CD44, and Sca-1, while negative of CD45, CD19, and CD11b were noted isolated from C57BL/6 mice[24]. Differently, in the initial culture of mMSC, more than 50% of CD45+ cells were present, then significantly reduced after sub-culturing. In contrast, CD44 expression was found to be elevated. In addition to this, after three passages, slightly diminished expression of CD90 was seen[18].

Furthermore, the negative and positive selection methods can be used to improve the purity of MSCs[24-26]. Based on the observation, it has been seen that mouse bone marrow is an ideal choice for separating MSCs. On the other hand, the study revealed that adipose tissue and compact culture bone MSCs were easier than MSCs from the marrow source[11]. The used protocol is cost-effective, and mMSC can be isolated on a large scale for in vitro experiments. Although numerous techniques have been established for MSCs separation from bone marrow[9,27]. For example,

some protocols require additional materials, which are costly and produce inconvenient cytotoxic effects on stromal cells[28,29].

The outcomes of the current study suggested that murine Balb/c mice are the best source of MSCs, especially from the bone marrow source. Because of the limited resources, we have not been able to characterize mMSCs through additional techniques; further studies are required to explore their features. Furthermore, this study prompted us to check the potential of human MSCs, which may provide life-changing treatments for various life-threatening illnesses.

Conclusion

Finally, a purified population of mMSCs was achieved in lower passage through repetitive medium change and decreasing trypsinization time. It was a simple and cost-effective protocol for isolating BM-MSCs without adding growth factors. Isolated mMSCs indicated high proliferation potential up to high passage. Moreover, mMSCs were strongly expressed MSCs markers.

Conflicts of Interest

The authors have declared that no competing interests exist.

Acknowledgment

We are thankful to Dr. Ayaz Ahmed (PCMD-UoK) for helping in immunofluorescence imaging. We also acknowledge the cooperation of Dr. Ismail Vohra (CEO-One Health Diagnostics, Karachi) in the provision of bench space and necessary consumables to carry out this research.

Funding

The Indigenous Dean's grant for Faculty of Science for 2017-18, University of Karachi, was obtained.

References

1. Lu T, Pei W, Wang K, Zhang S, Chen F, Wu Y, Guan W. In vitro culture and biological properties of broiler adipose-derived stem cells. Exp Ther Med. 2018;16(3):2399-2407.
2. Matsuda S, Nakagawa Y, Kitagishi Y, Nakanishi A, Murai T. Reactive oxygen species, superoxide dimutases, and PTEN-p53-AKT-MDM2 signaling loop network in mesenchymal stem/stromal cells regulation. Cells. 2018;7(5):36.
3. Denu RA, Hematti P. Effects of oxidative stress on mesenchymal stem cell biology. Oxid Med Cell Longev. 2016:2989076.
4. Vadalà G, Russo F, Ambrosio L, Loppini M, Denaro V. Stem cells sources for intervertebral disc regeneration. WJSC. 2016;8(5):185.
5. Wu J, Niu J, Li X, Wang X, Guo Z, Zhang F. TGF-β1 induces senescence of bone marrow mesenchymal stem cells via increase of mitochondrial ROS production. BMC Dev Biol. 2014;14(1):1-9.
6. Zhuang X, Hu X, Zhang S, Li X, Yuan X, Wu Y. Mesenchymal Stem Cell–Based Therapy as a New Approach for the Treatment of Systemic Sclerosis. Clin Rev Allergy Immunol. 2022:1-37.
7. Al-Dhamin Z, Liu LD, Li DD, Zhang SY, Dong SM, Nan YM. Therapeutic efficiency of bone marrow-derived mesenchymal stem cells for liver fibrosis: A systematic review of in vivo studies. WJG. 2020;26(47):7444.
8. Parekkadan B, Milwid JM. Mesenchymal stem cells as therapeutics. Annu Rev Biomed Eng. 2010;12:87-117.
9. Soleimani M, Nadri S. A protocol for isolation and culture of mesenchymal stem cells from mouse bone marrow. Nat Protoc. 2009;4(1):102-106.
10. Baksh D, Song L, Tuan RS. Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. JCMM. 2004;8(3):301-316.
11. Sung JH, Yang HM, Park JB, Choi GS, Joh JW, Kwon CH, Chun JM, Lee SK, Kim SJ. Isolation and characterization of mouse mesenchymal stem cells. Transplant Proc. 2008;40(8):2649-2654.
12. Baddoo M, Hill K, Wilkinson R, Gaupp D, Hughes C, Kopen GC, Phinney DG. Characterization of mesenchymal stem cells isolated from murine bone marrow by negative selection. J Cell Biochem. 2003;89(6):1235-1249.
13. Sun S, Guo Z, Xiao X, Liu B, Liu X, Tang PH, Mao N. Isolation of mouse marrow mesenchymal progenitors by a novel and reliable method. Stem cells. 2003;21(5):527-535.
14. Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science. 1997;276(5309):71-74.
15. Deans RJ, Moseley AB. Mesenchymal stem cells: biology and potential clinical uses. Exp Hematol. 2000;28(8):875-884.
16. Yusop N, Battersby P, Alraies A, Sloan AJ, Moseley R, Waddington RJ. Isolation and characterisation of mesenchymal stem cells from rat bone marrow and

the endosteal niche: a comparative study. Stem cells Int. 2018; Article ID 6869128.
17. Han Y, Li X, Zhang Y, Han Y, Chang F, Ding J. Mesenchymal stem cells for regenerative medicine. Cells. 2019;8(8):886.
18. Baustian C, Hanley S, Ceredig R. Isolation, selection and culture methods to enhance clonogenicity of mouse bone marrow derived mesenchymal stromal cell precursors. Stem Cell Res Ther. 2015;6(1):1-3.
19. Ge T, Yu Q, Liu W, Cong L, Liu L, Wang Y, Zhou L, Lin D. Characterization of bone marrow-derived mesenchymal stem cells from dimethyloxallyl glycine-preconditioned mice: evaluation of the feasibility of dimethyloxallyl glycine as a mobilization agent. Mol Med Rep. 2016;13(4):3498-3506.
20. Zhang F, Hong Y, Liang W, Ren T, Jing S, Lin J. Co-culture with Sertoli cells promotes proliferation and migration of umbilical cord mesenchymal stem cells. Biochem Biophys Res Commun. 2012;427(1):86-90.
21. Lee DH, Joo SD, Han SB, Im J, Lee SH, Sonn CH, Lee KM. Isolation and expansion of synovial CD34–CD44+ CD90+ mesenchymal stem cells: comparison of an enzymatic method and a direct explant technique. Connect Tissue Res. 2011;52(3):226-234.
22. Atashi F, Modarressi A, Pepper MS. The role of reactive oxygen species in mesenchymal stem cell adipogenic and osteogenic differentiation: a review. Stem Cells Dev. 2015;24(10):1150-1163.
23. Guzik TJ, Harrison DG. Vascular NADPH oxidases as drug targets for novel antioxidant strategies. Drug Discov Today. 2006;11(11-12):524-533.
24. Hu Y, Lou B, Wu X, Wu R, Wang H, Gao L, Pi J, Xu Y. Comparative study on in vitro culture of mouse bone marrow mesenchymal stem cells. Stem Cells Int. 2018; Article ID 6704583.
25. Nadri S, Soleimani M. Isolation murine mesenchymal stem cells by positive selection. In Vitro Cell Dev Biol Anim. 2007;43(8):276-282.
26. Xu S, De Becker A, Van Camp B, Vanderkerken K, Van Riet I. An improved harvest and in vitro expansion protocol for murine bone marrow-derived mesenchymal stem cells. J biotechnol biomed. 2010; 105940.
27. Eslaminejad MB, Nikmahzar A, Taghiyar L, Nadri S, Massumi M. Murine mesenchymal stem cells isolated by low density primary culture system. Dev Growth Differ. 2006;48(6):361-370.
28. Modderman WE, Vrijheid-Lammers T, Löwik CW, Nijweide PJ. Removal of hematopoietic cells and macrophages from mouse bone marrow cultures: isolation of fibroblastlike stromal cells. Exp Hematol. 1994;22(2):194-201.
29. Falla N, Van V, Bierkens J, Borremans B, Schoeters G, Van Gorp U. Characterization of a 5-fluorouracil-enriched osteoprogenitor population of the murine bone marrow. Blood. 1993;82(12):3580-3591.