Original Article

Stem Cell INVESTIGATION

Equine bone marrow-derived mesenchymal stem cells: optimization of cell density in primary culture

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Background: The primary cell seeding density of bone marrow-derived mononuclear cells (BM-MNCs) affects several cellular behaviors, including attachment to the culture dish, proliferation, and differentiation.

Methods: The aim of this study was to determine the best density of equine BM-MNCs in primary culture (P0) for obtaining the maximum bone marrow-derived mesenchymal stem cell (BM-MSC) yields at the end of P0. Bone marrow samples of two healthy mares were aspirated. The MNCs were isolated and cultured at different densities (1×10^5, 2×10^5, 4×10^5, 8×10^5, and 1×10^6 cells/cm^2). Within the 7th and 14th days after seeding, the colonies containing more than 15 cells were counted and the percentage of confluency and the number of cells were calculated on day 21.

Results: The lowest density of MNCs was associated with the least number of colonies, number of adherent cells, and confluency percentage, whereas the highest density was associated with the maximum number of colonies and confluency percentage ($P<0.05$). However, the maximum number of cells at the end of P0 was associated with the intermediate (4×10^5 cells/cm^2) and the highest concentration ($P<0.05$).

Conclusions: The maximum number of MSCs at the end of P0 was obtained at the densities of 1×10^6 and, especially, at 4×10^5 cells/cm^2.

Keywords: Cell count; cell yield; horses; mesenchymal stromal cells; mononuclear cells (MNCs)

Received: 11 June 2018; Accepted: 27 August 2018; Published: 09 October 2018.
doi: 10.21037/sci.2018.09.01
View this article at: http://dx.doi.org/10.21037/sci.2018.09.01

Introduction

Exciting achievements in the research area of stem cells have brought new hopes of curing the incurable diseases, although the existing methods in this research area have not yet provided good clinical results (1). Until today, adult stem cells have been derived from different human and animal sources, such as bone marrow (BM), peripheral blood, umbilical cord blood, and fat tissue (2,3). The parenchyma of red BM consists of numerous megakaryocytes, plasma cells, monocytes, and hematopoietic stem cells (HSCs), which produce all the three classes of blood cells that exist in the circulatory system (4). The BM stroma consists of fibroblasts, macrophages, adipocytes, osteoblasts, osteoclasts, and endothelial cells, which comprise a special microenvironment and indirectly play a role in hematopoiesis (5).

Another type of cells in the BM is the mesenchymal stem cells (BM-MSCs) (6), which can be used for the treatment of certain diseases. These multipotent stem cells have been

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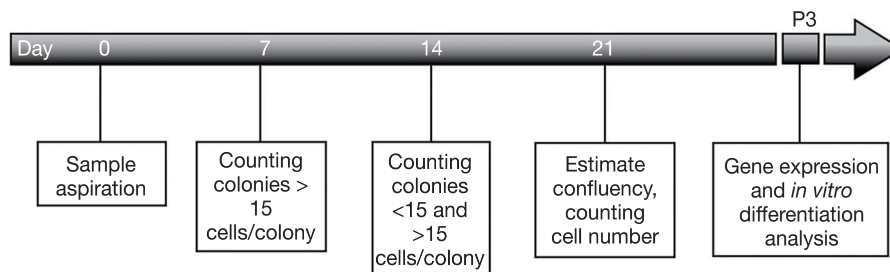

Figure 1 Experimental procedure timeline. P3: Cells at passage 3.

differentiated into several specific cells such as osteoblasts, chondrocytes, myoblasts, adipocytes, β-cells of pancreatic islets, and even neurons (7). Due to this ability, MSCs have been successfully used for the treatment of degenerative diseases such as musculoskeletal disorders, including osteoarthritis (8), tendon and ligament injuries (9,10), rheumatoid arthritis (11), spinal cord injury (12,13), liver disease (14), and bone defects (15). Therefore, stem cells, especially MSCs derived from different tissues, are a better promising strategy for curing common orthopedic diseases in horses (16).

Mononuclear cell (MNC) population in the BM consists of a variety of cells such as MSCs, monocytes, lymphocytes, and HSCs (17). MSCs can be easily separated from HSCs through culture and adherence to plastic dishes (18). BM-derived nucleated cells show variations in morphology (19). During sequential subcultures, the heterogeneity decreases and spindle-shaped fibroblast-like cells become predominant (20). The proportion of BM-derived MSCs has been reported to be 1 to 3.8×10^5 in felines (21), 2.5×10^4 in canines (22), 10^4–10^5 in humans (23), and 1 to 4.2×10^3 in equines (19).

The number of MSCs obtained through the culture of equine BM samples has been reported to be influenced by the isolation methods (24). Several methods have been used to isolate and culture equine MSCs (24); however, due to the large volume of BM aspirated samples in most of the studies (generally more than 10 ml), the gradient density method is preferred to the classic method (culture of whole BM sample) (24). MNCs have been isolated by the gradient density protocol, except non- or poly-nucleated cells. The presence of these contaminant cells, including erythrocytes, platelets, granulocytes, and myeloid progenitor cells, in the classical method could potentially weaken the proliferation of MSCs (24).

Stanley *et al.* [1995] have demonstrated the importance of appropriate cell density for the expression of some cell adhesion molecules in the bladder and colonic cell lines. Cell–cell and cell–matrix interactions such as anchorage, migration, proliferation, and differentiation of the cells are being intervened using cell adhesion molecules (25). These interactions could also modify the gene expression and establishment of the cellular scaffold and lead to modification of the morphology of the cells (26). We speculated that attachment of cells to the culture dish surface can be affected by cell density, because it has been shown that the expression of β1 and β4 integrins of adhesion molecules would change with changes in cell density (27). On the other hand, De Schauwer *et al.* (28) proposed the minimal criteria for defining equine MSCs and demonstrated that β1 integrin (also termed as CD29) is one of their putative surface markers. This implies that β1 integrin is a primary surface marker expressed on MSCs and plays an important role in the adherence and function of these cells. Moreover, Piedimonte *et al.* (29) demonstrated that the transportation of small nutrients such as amino acids is altered at various cell densities. Another study suggested that this modulation of nutrient transport in normal cells is related to the cell–cell contact that is mediated by adhesion molecules (30).

Considering the above mentioned aspects, it appears that it is necessary to optimize the cell density in the primary culture of bone marrow-derived mononuclear cells (BM-MNCs) for the isolation of MSCs. Therefore, we conducted this study to investigate the effects of MNC number in the primary culture on the yield of MSCs.

Methods

All the media and solutions were purchased from Sigma-Aldrich Company (Germany). The experimental procedure is depicted in *Figure 1*.

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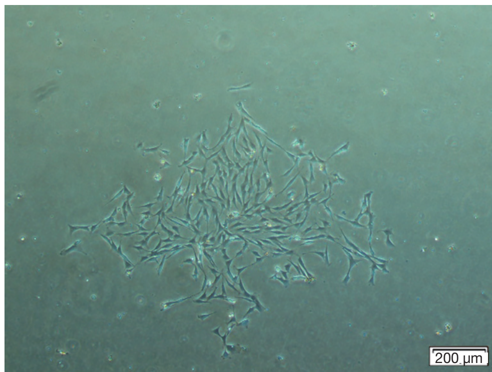

Figure 2 A colony with more than 15 cells.

Sample aspiration

BM samples were collected from two healthy 9-year-old mares. After restraining and using ultrasonography to determine the exact sampling place on the sternum, 0.5 mg/kg xylazine was injected intravenously as a sedative. Then, 10 mL of lidocaine (2%) with adrenaline was injected as a local anesthesia around the entrance of the biopsy needle. Using Jamshidi needle (gauge 13, 10-cm length), at least 10 mL of BM was aspirated into a 20-mL syringe containing 10,000 IU heparin (1,000 IU/mL). Within 4 h, all samples were carried on ice to the laboratory for further experiments.

MNC isolation

Samples were thoroughly mixed with twice the volume of basic culture medium (DMEM-high glucose supplemented with 10% FBS, 1% penicillin–streptomycin, and 0.1% amphotericin B) under a sterile laminar hood/cabinet in the laboratory. Two portions of the cell suspension were carefully loaded onto the top of one portion of density gradient media (Histopaque® 1077) in 15-mL polypropylene Falcon tubes. The samples were centrifugated in a swinging bucket rotor at 400 g for 30 min at 4 ℃ (with the brake off). Then, the layer of MNCs on the top of the density gradient media was collected gently using a Pasteur pipette. These MNCs were rinsed twice with Dulbecco's phosphate-buffered solution (without Ca^{2+} or Mg^{2+}) at 600 g for 5 min at 4 ℃.

Various seeding densities and culture

The final obtained pellet was resuspended with 4 mL of fresh culture medium, and the cells were counted on a Neubauer hemocytometer counting chamber. Then, cells at different densities (1×10^5, 2×10^5, 4×10^5, 8×10^5, and 1×10^6 cells/cm^2) were cultured in the usual cell culture flasks (T75 filter-capped flasks, SPL, Korea). These cells, considered as primary (P0), were cultured for 21 days, and their medium was changed every 3 days to eliminate non-adherent or dead cells.

Observation procedures

On the 7th and 14th days after seeding, the colonies containing more than 15 cells (*Figure 2*) were counted under an inverted microscope (Labomed TCM400 California, USA). On day 21, the approximate percentage of confluency and the exact number of adherent cells were calculated after trypsinization using a Neubauer counting chamber.

Mesenchymal stem cell characterization

The harvested cells at P0 were seeded at the usual density of 5,000 cells/cm^2 in new T75 flasks as a first passage (P1). The cell culture was continued until P3, and finally, the P3 cells were subjected to analyses of specific marker expression and *in vitro* differentiation.

For gene expression analysis, total RNA was isolated using Total RNA Isolation Kit (DENAzist Asia, Iran) according to the manufacturer's instruction, and cDNA was synthesized using the AccuPower® RT Premix kit (Bioneer, USA). PCR was performed using specific primer sets for analyzing the expressions of CD29 (F: 5'aatcgggacaagttacctca3', R: 5'cttccaaatcagcagcaat3'), CD44 (F: 5'aacctcgggtcccatac3', R: 5'tccattgagcccacttgc3'), CD90 (F: 5'agaataccaccgccaca3', R: 5'ggataagtagaggaccttgatg3'), CD34 (F: 5'tgatgaatcgccgtagt3', R: 5'cgggttgtctcgctga3'), and MHC-II (F: 5'ggaacgggcagcaggacat3', R: 5'aagccattcacagagcagacca3'). GAPDH was used as an internal control (F: 5'tgtcatcaacggaaaggc3', R: 5'gcatcagcagaaggagca3'). Thermal cycling was carried out under the following conditions: initial denaturation at 95 ℃ for 5 min, followed by 30 cycles of 95 ℃ for 30 s, 51–61 ℃ for 45 s, and 72 ℃ for 1 min. The final elongation was performed at 72 ℃ for 10 min. The amplified PCR products were electrophoresed with ethidium bromide on a 1.5% agarose gel.

For *in vitro* differentiation, P3 cells were induced to undergo tri-lineage differentiation. For osteogenic and adipogenic differentiations, 3×10^5 cells were cultured under defined conditions (29). In addition, 5×10^5 cells were cultured as a micropellet in a 15-mL Falcon tube for

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chondrogenic differentiation and were treated as previously described by Alipour *et al.* (31). In all assays, the control group was cultured with the basic growth medium.

Statistical analysis

Statistical analysis was performed using SPSS statistical software version 17.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as mean ± standard deviation. ANOVA, followed by Tukey's *post hoc* test, was conducted to investigate the effects of different cell densities on the colony number, the confluency percentage, and the cell yield. A probability of $P<0.05$ was considered as statistically significant.

Results

After culturing the BM-derived MNCs at the various densities in P0 (*Figure 3*) and changing the medium during the culture, the non-adherent and dead cells were discarded. The first colonies were observed at day 4 after seeding.

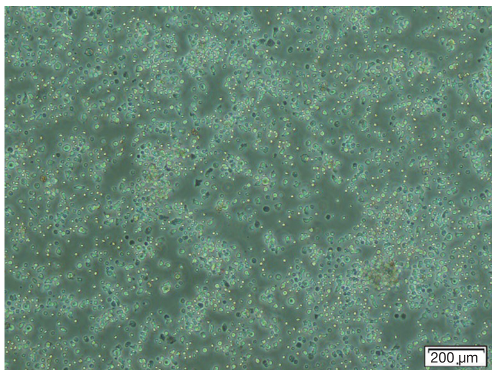

Figure 3 Initial seeding of bone marrow-derived mononuclear cells in flasks. Density: 4×10^5 cells/cm^2. Magnification, ×40.

The cells in the BM-derived colonies had a heterogeneous morphology, ranging from star-shaped to triangular and spindle-like cells.

The number of colonies, adherent cells, and the confluency percentage are shown in *Table 1*. On day 7 after seeding, there was no significant difference in the colony numbers between the groups. Fourteen and 21 days after seeding, the minimum number of colonies, number of adherent cells, and the approximate percentage of confluency were found to be related to the lowest cell density (1×10^5 cells/cm^2), whereas the maximum number of colonies and the approximate confluency percentage were associated with the highest density of cells (1×10^6 cells/cm^2) ($P<0.05$). However, the maximum number of cells at the end of P0 (day 21), which was utilized for the next subcultures and the expansion of cells, belonged to the intermediate cell density culture (4×10^5 cells/cm^2) ($P<0.05$). This number was found to be non-significantly higher than that of the group with the highest cell density (1×10^6 cells/cm^2) ($P>0.05$). Despite the lower number of colonies observed with the intermediate density, these colonies had a large number of cells. We postulated that the cells in these colonies are more dense and exhibit growth overlap (*Figure 4*). In the culture flasks with the highest cell density, due to the large number of colonies, the cells expanded rapidly and covered a higher percentage of the culture dishes.

Cells at P3 expressed CD29, CD44, and CD90 surface marker, but not CD34 (hematopoietic progenitor cell marker) and MHC-II (*Figure 5*). Moreover, the cells differentiated into osteogenic, adipogenic, and chondrogenic lineages (*Figure 6*).

Discussion

Based on our findings, it can be concluded that a high

Table 1 The number of colonies on days 7 and 14 after seeding; approximate confluency and cell numbers on day 21

Density (cells/cm^2)	7th day colonies (>15 cells)	14th day colonies (>15 cells)	14th day colonies (<15 cells)	21st day approximate confluency (%)	21st days adherent cell numbers
1×10^5	1.5±0.5^a	4.5±0.5^a	8.5±3.5^a	6±1^a	80,000±10,000^a
2×10^5	2.5±0.5^a	12.5±0.5^a	6.5±2.5^a	11.5±1.5^a	165,000±45,000^a
4×10^5	4.5±0.5^a	68.5±25.5^a	76±18^a	16±1^a	1,600,000±300,000^b
8×10^5	3.5±1.5^a	51±15^a	45±5^a	22.5±2.5^b	350,000±50,000^a
1×10^6	10±3^a	130±28.5^b	135±21^b	37.5±7.5^b	1,100,000±400,000^{a,b}

Data are presented as mean ± SD. Values with different alphabets (a, b) within columns are significantly different ($P<0.05$).

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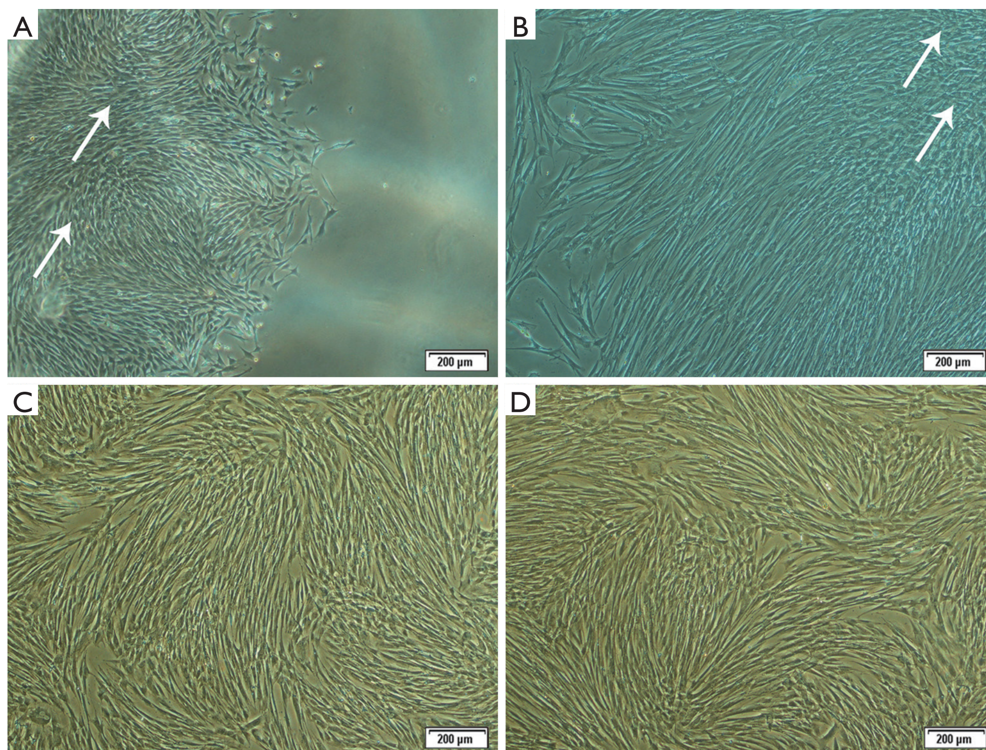

Figure 4 Overlapping and almost multilayer cell proliferation in the colonies of MSCs in the flask seeded with 4×10^5 cells/cm^2 at passage 0. The arrows represent the overlapping zones (A and B). Monolayer growth of MSCs in passage 1 (C) and in passage 3 (D).

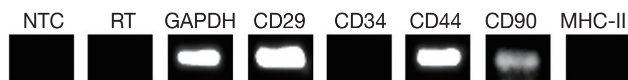

Figure 5 Gene expression analysis for GAPDH, CD29, CD34, CD44, CD90 and MHC-II, of BM-MSCs at passage 3. Gel electrophoresis of PCR products showed the expression of GAPDH (183 bp), CD29 (234 bp), CD44 (193 bp) and CD90 (155 bp). No expression of CD34 and MHC-II was detected. RT had mRNA instead of cDNA template in PCR to exclude DNA contamination. RT: RNA template; NTC: non-template (negative) control.

primary cell density does not have a negative impact on the adherence of MSCs to the flasks, although the intermediate seeding density of MNCs (4×10^5) resulted in more numbers of MSCs at the end of P0 (*Table 1*). Obtaining high enough cell number at the end of P0 is very important, because at the next passage (P1), more flasks can be subcultured with a proper density (3×10^3 cells/cm^2), and finally, sufficient cell numbers can be achieved at P3 or P4. In morphology assessment, heterogeneous cell population was observed which is similar to that reported by Vidal *et al.* (19), however, Giovannini *et al.* (32) reported that the fibroblastic morphology of all the BM-derived MSCs was similar throughout different passages. Increasing the passage number results in the homogeneity of cells (19), which can be used for further analysis or for treatment purposes.

Despite the lower confluency in the flasks seeded with 4×10^5 cells/cm^2, these flasks had a greater number of cells in comparison with the higher ones (8×10^5 and 1×10^6 cells/cm^2) because of the more dense colonies and the proliferation of cells in an overlapping pattern or almost as a bilayer. As reported by Stanley *et al.* [1995] indicating that low and excessive cell density leads to a decrease in expression of β1 and β4 integrins, it is probable that the density of 4×10^5 cells/cm^2 would result in an optimum expression of these adhesion molecules and, consequently to an optimum cell–cell and cell–matrix interaction (25). Nonetheless, it is possible to isolate MSCs from a lower cell density such as 1×10^5 cells/cm^2, which is applicable in cases where only a small amount of aspirated BM is available.

As the minimal criteria of equine mesenchymal stem cells (28), cultured P3 cells expressed the related specific

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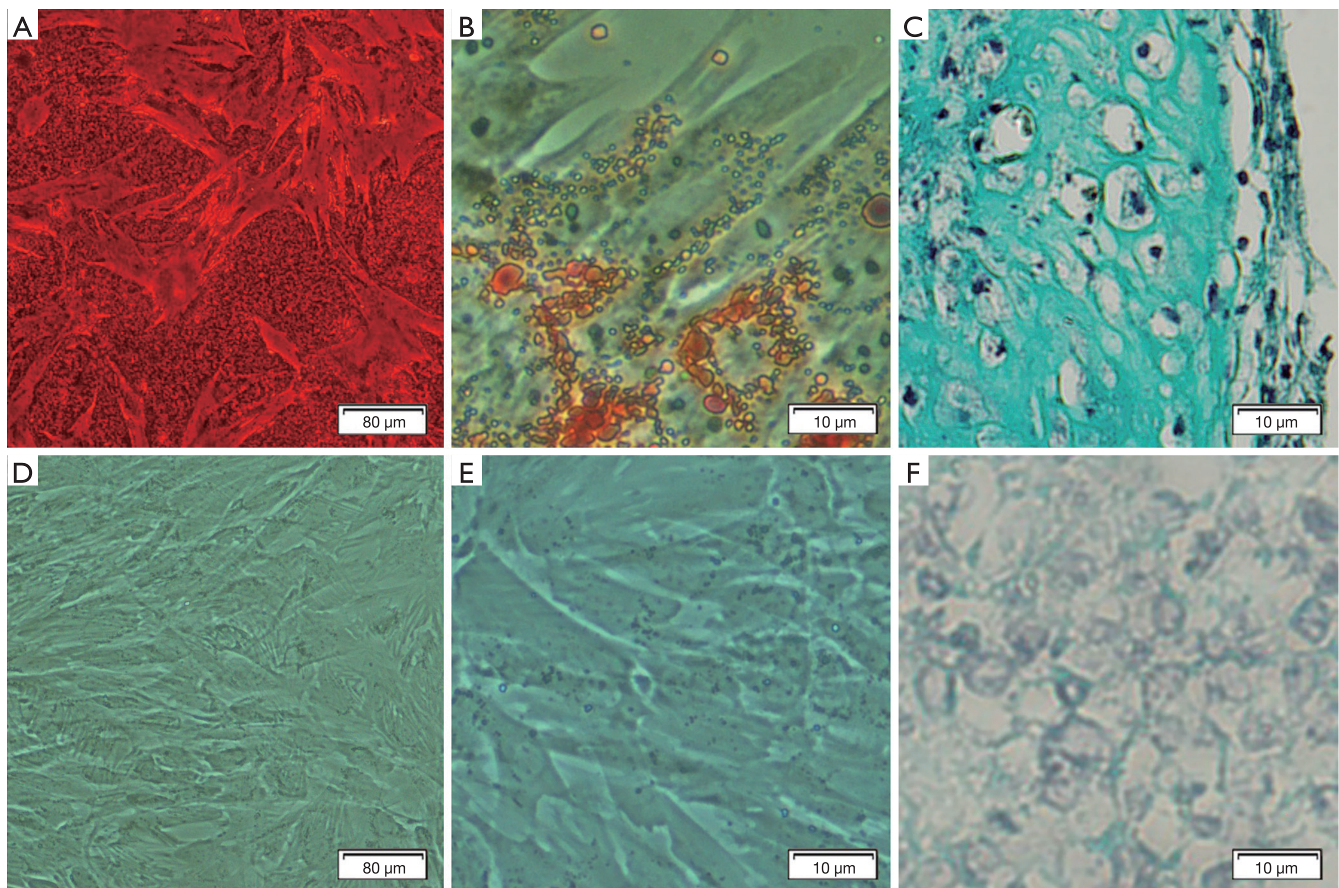

Figure 6 Tri-lineage *in vitro* differentiation assays of equine BM-MSCs at passage 3. Calcium deposition was stained by Alizarin Red S in the osteogenic treatment group (A). Intracellular neutral lipid droplets were detected by Oil Red O in the adipogenic treatment group (B). Alician blue was used to stain proteoglycans in the extracellular matrix of the chondrogenic differentiation group (C). (D,E,F) are the control group for osteogenic, adipogenic and chondrogenic, respectively.

genes (CD29, CD44, and CD90) and had trilineage differentiation potential which confirm the isolated cells are equine MSCs. Because of the lack of equine specific antibodies and/or few or no cross-reactivity with other species antibodies (33), detection of markers' expression at mRNA level by reverse transcription-PCR in equine MSCs would be a good technique that similarly replicates the findings of flow-cytometry technique (34).

Conclusions

Densities of 1×10^6 and, especially, 4×10^5 cells/cm^2 are recommended for the isolation of equine MSCs based on the amount and volume of BM sample. In addition, regarding large cell numbers, which are required for experiment or treatment, the initial density of 4×10^5 is suggestible to reach the adequate cell numbers at the end of P0. Finally, these homogenized MSCs can be used for both research and clinical purposes.

Acknowledgements

The authors gratefully acknowledge Azaraksh Sport Club, Mashhad, Iran, for their cooperation for collection of bone marrow samples from horses. We acknowledge the Ferdowsi University of Mashhad for financial support.

Footnote

Conflicts of Interest: The authors declare that there is no conflict of interest.

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Ethical Statement: The animal protocol of experiment was approved by The Ethics Committee and Animal Welfare of Ferdowsi University of Mashhad.

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doi: 10.21037/sci.2018.09.01
Cite this article as: Zahedi M, Parham A, Dehghani H, Kazemi Mehrjerdi H. Equine bone marrow-derived mesenchymal stem cells: optimization of cell density in primary culture. Stem Cell Investig 2018;5:31.

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