ORIGINAL ARTICLE

# Isolation and characterization of mesenchymal stem cells obtained from reusable and disposable bone marrow collection filters

## Isolamento e caracterização de células-tronco mesenquimais de filtros reutilizáveis e descartáveis de medula óssea

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## ABSTRACT

**Objective:** To compare human mesenchymal stem cells obtained from reusable and disposable filters and to characterize them according to the criteria of the International Society of Cellular Therapy. **Methods:** Human mesenchymal stem cells were isolated from bone marrow collection reusable sets and compared with those obtained from disposable sets by washing the filters with cell culture media. The isolated cells were characterized according to the criteria of the International Society of Cellular Therapy using flow cytometry, differentiation *in vitro,* and cytochemistry techniques. **Results:** Samples were obtained from disposable (n=3) and from reusable collection sets (n=3). All samples obtained from bone marrow disposable sets successfully produced mesenchymal stem cells. All bone marrow derived mesenchymal stem cells were characterized and fulfilled the criteria established by International Society of Cellular Therapy. **Conclusion:** This study showed that mesenchymal stem cells can also be obtained from reusable collection sets (which are still used in several centers around the world) to be employed in research as an alternative and ethical source.

**Keywords:** Mesenchymal stem cells; Bone marrow; Filtration; Disposable equipment; Recycling

## RESUMO

**Objetivo:** Comparar as células-tronco mesenquimais humanas obtidas de filtros de coleta reutilizáveis àquelas coletadas em filtros descartáveis e caracterizá-las utilizando os critérios da *International Society for Cellular Therapy*. **Métodos:** Foram isoladas células-tronco mesenquimais humanas de *kits* de coleta de medula óssea reutilizáveis e descartáveis, pela lavagem dos filtros com meio de cultura. As células isoladas foram caracterizadas de acordo com os critérios estabelecidos pela *International Society for Cellular Therapy*, por meio das técnicas de citometria de fluxo, diferenciação *in vitro* e citoquímica. **Resultados:** As amostras foram obtidas de filtro descartável (n=3) e reutilizável (n=3). Todas as amostras obtidas de filtros descartáveis produziram células-tronco mesenquimais, e todas as células-tronco mesenquimais humanas derivadas de medula óssea preencheram os critérios estabelecidos pela *International Society for Cellular Therapy*. **Conclusão:** Este estudo mostrou que as células-tronco mesenquimais também podem ser obtidas de *kits* de coleta reutilizáveis (que permanecem em uso em vários centros, no mundo inteiro), para serem empregadas em pesquisa como uma fonte alternativa e ética.

**Descritores:** Células-tronco mesenquimais; Medula óssea; Filtração; Equipamentos descartáveis; Reciclagem

## INTRODUCTION

The best characterized source for adult stem cells is the bone marrow, which contains a heterogeneous population of cells, including hematopoietic progenitor and stem cells.

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In addition to these cell types, the bone marrow also contains a subset of non-hematopoietic stem cells with multilineage potential. These stem cells are called "marrow stromal stem cells" or "human mesenchymal stem cells" (hMSC)[1].

The hMSCs are primitive cells originated from the mesodermal germ layer, and they have been described as giving rise to connective tissue, skeletal muscle, and vascular system cells[2,3].

In 2006, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) proposed minimal criteria to characterize hMSCs[4].

The hMSCs must be plastic-adherent when maintained in standard culture conditions. In addition, they must express CD29, CD73, CD90, and CD105, and lack expression of CD34, CD45, CD14, and human leukocyte antigen HLA-DR on the surface. Furthermore, they must also have the potential to differentiate into osteoblasts, adipocytes, and chondroblasts *in vitro*. Presently, most studies characterize bone-marrow-derived hMSCs according to these criteria. However, many unanswered questions remain such as the true nature and identity of hMSCs, including their location, origin, and multipotential capacity. Isolation of hMSCs has been reported from several tissues, such as bone marrow, adipose tissue, liver, muscle, amniotic fluid, placenta, umbilical cord blood, and dental pulp[5-7].

Since bone marrow derived hMSCs are the most commonly used cells in clinical trials, these cells probably will be the usual source in further studies. In addition, they are an important control for defining other possible hMSC sources.

HMSCs have been efficiently recovered, unaffected by the gender of the donor, from bone marrow aspirates of healthy individuals, and also of patients suffering from serious diseases and injuries. However, hMSCs are an extremely rare cell type, accounting for less than 0.1% of nucleated cells in bone marrow aspirates[8-10].

For clinical applications, the bone marrow is aspirated from the posterior iliac crest and collected in disposable or reusable collection sets containing culture media supplemented with anticoagulant.

For economical reasons, bone marrow collection reusable sets are widely employed in several centers around the world. During this procedure, the bone marrow graft is filtered and transferred to a transport bag, mainly to remove clogs and bone particles from the material that will be infused into the patient. The material remaining in the filter is usually discarded.

Under standard protocols, hMSCs are isolated from filtered bone marrow samples. Interestingly, it has been proposed that in bone marrow aspirates there is also the formation of hematons, which are a stromal-web tightly packed with hematopoietic progenitor cells and differentiated post-mitotic cells. Since these clusters are more than 50mm in diameter, they are probably removed by disposable filters, whose pore sizes range from 500 to 200$\mu$m. Reusable filters have smaller pore sizes (250 or 150$\mu$m)[11]. Dvorakova et al., Capelli et al., Sundin et al., and others reported successful isolation of hMSCs from bone marrow collection disposable sets, but no one has reported this from reusable filters[12-14].

In this study, we isolated and characterized hMSCs obtained from bone marrow collection filters of both disposable and reusable sets. Since collecting bone marrow from health volunteers for research is a complex issue due to the morbidity of the procedure (and several centers still use reusable sets for collecting bone marrow grafts), this study present the reusable sets as an alternative cell source for clinical and research purposes.

## OBJECTIVE

To compare hMSCs obtained from reusable and disposable sets and to characterize them according to the criteria of the ISCT.

## METHODS

### Cell harvesting and isolation

The procedure to obtain cells from the filter was approved by the *Hospital Israelita Albert Einstein* Ethical Committee (HIAE), process number 10/1,412. After harvesting for transplant purposes and filtering bone marrow cells from health volunteers, the filter was washed with 20mL of cell culture medium DMEM-LG (Gibco®, Carlsbad, CA) to recover the cells trapped inside the filter together with blood clogs and small bone fragments (Figures 1 and 2). The disposable

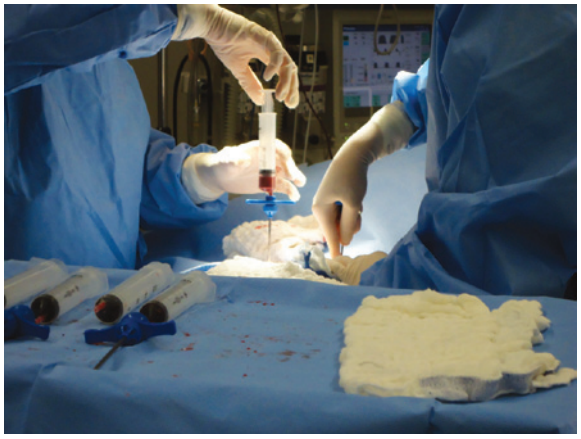

**Figure 1.** Bone marrow aspiration

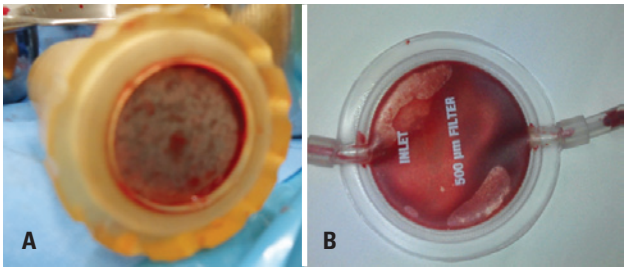


**Figure 2.** (A) Reusable filter. (B) Disposable filter

filter used presented two types of pores sized 200 and 500$\mu$m (Fenwal, Lake Zurich, IL), and the reusable one presented two pore sizes of 150 and 250$\mu$m. The 150-$\mu$m filter was employed in this study (University of Washington, Seattle, WA).

## Isolation of hMSC

The bone marrow sample obtained after filtering was diluted (1:3) with phosphate-buffer saline (PBS), and then transferred to a 50mL conical tube containing 20mL of Ficoll/Hypaque (GE Healthcare) and centrifuged for 30 minutes at 500g, 22°C. After centrifugation the cells were transferred to another tube and centrifuged again for 5 minutes at 500g, 22°C; the supernatant was discarded and cells were resuspended with DMEM-LG (Gibco®, Carlsbad, CA) supplemented with 10% FBS (Gibco®, Carlsbad, CA) in order to achieve a concentration of 1x10$^5$cells/mL. Next, the cells were cultivated in 25cm$^2$ flasks maintained in humidified 5% $CO_2$ incubators at 37°C to favor the attachment of the hMSC to the flask bottom.

## Culture and cell differentiation

The medium was changed for the first time after 48 hours of culture, and every other day after that. The flask containing the cells was verified by optical microscopy every day in order to confirm the adherence of hMSC colonies. After the establishment of hMSC cultures on the fourth passage, the cells differentiated in adipocytes, osteoblasts, and chondrocytes.

Adipogenesis was induced by addition of an adipogenic medium, comprised by Alpha-MEM (Gibco®, Carlsbad, CA) supplemented with 10% FBS (Gibco®, Carlsbad, CA), 1$\mu$m dexamethasone (Sigma, St Louis, MO), 100$\mu$g/mL 3-Isobutyl-1-methylxanthine IBMX (Sigma, St Louis, MO), 10$\mu$g/mL insulin (Sigma, St Louis, MO), and 100$\mu$M indomethacin (Sigma, St Louis, MO). The adipogenic medium was changed every other day for 3 weeks.

Osteoblast differentiation was induced by addition of an osteogenic medium, comprised of Alpha-MEM (Gibco®, Carlsbad, CA) supplemented with 10% FBS (Gibco®, Carlsbad, CA), 1$\mu$m dexamethasone (Sigma, St Louis, MO), 2$\mu$g/mL ascorbic acid (Sigma, St Louis, MO), and 10$\mu$m beta-glycerophosphate (Sigma, St Louis, MO). The osteogenic medium was changed every other day for 3 weeks.

Chondrocyte differentiation was induced by addition of chondrogenic medium, that was Alpha-MEM (Gibco®, Carlsbad, CA) supplemented with 10% FBS (Gibco®, Carlsbad, CA), 1$\mu$m dexamethasone (Sigma, St Louis, MO), 2ug/mL ascorbic acid (Sigma, St Louis, MO), 6,25ug/mL insulin (Sigma, St Louis, MO), and 10ng/mL TGF-beta (Sigma, St Louis, MO). Chondrogenic medium was changed every other day for 3 weeks.

## Immunohistochemical staining methods

### Oil Red O

Adipogenic differentiation was demonstrated by staining lipid droplets after 3 weeks in culture. The cells were fixed in 4% paraformaldehyde for 30 minutes, washed, dehydrated in 60% isopropanol for 2 to 5 minutes, and stained with 0.5% Oil Red O (O-0625; Sigma) in 100% isopropanol previously diluted in water.

### Alizarin Red

Osteogenic differentiation was evaluated by Alizarin Red staining after 3 weeks in culture. For Alizarin Red, the cells were fixed in 4% paraformaldehyde for 30 minutes, washed with distilled water, stained with Alizarin Red (2g in 100mL of distillated water) pH 4.2 (A5533; Sigma) for 5 to 10 minutes and thoroughly washed.

### Toluidine blue

Chondrogenic differentiation was evaluated by toluidine blue staining after 3-week culture. For toluidine blue, the cells were fixed with ethanol 70% for 1 minute, ethanol 90% for 1 minute and absolute ethanol for 1minute, then toluidine blue was added (1g toluidine blue, 1g sodium borate/100mL of water) (198161; Sigma).

### Flow cytometry

Cells from passage four were used to analysis of cell surface markers. The cells were washed with PBS, and then detached from the plastic with TryPLE (Gibco®).

The cells obtained were stained for CD14 (FITC), CD19 (APC), CD29 (PE), CD31 (PE), CD34 (PE),

CD45 (PerCP-Cy5.5), CD73 (PE), CD90 (APC), CD105 (PE), CD106(FITC), CD117 (PE), CD133 (APC), CD166 (PE), HLA-DR (PerCP-Cy5.5) and HLA-I (FITC), and the respective isotype controls (BD Biosciences, San Jose, CA; eBiosciences, San Diego, CA, USA; Biolegend, San Diego, CA).

After staining, the tubes were incubated at room temperature for 30 minutes, followed by a wash step; the cell pellet was resuspended, measurements were performed using a FACSARIA (BD Biosciences, San Jose, CA), and analyses were performed using FlowJo software (Tree Star, OR).

**Chart 1.** Expansion of hMSCs from disposable and reusable sets

| Sample | Input of mononuclear cells x$10^6$ | Output of hMSC x$10^6$ on passage 4 |
|---|---|---|
| 1 | 100 | 30 |
| 2 | 20 | 26.5 |
| 3 | 32 | 20 |
| 4 | 2.7 | 21.0 |
| 5 | 1.0 | 16.7 |
| 6 | 0.5 | - |

hMSC: human mesenchymal stem cells.

## RESULTS

### Success in obtaining hMSCs from bone marrow collection sets

Five of the six samples yielded hMSCs. The average number of mononuclear cells obtained from reusable collection sets was 1.5x$10^6$ (±0.9x$10^6$), and the average number from disposable collection sets was 50x$10^6$ (±43x$10^6$).

The sample that did not produce hMSCs was from the reusable filter group, probably due to the low number of cells obtained. However, all bone marrow derived hMSCs originated a substantial number of cells on passage 4 (over 1x$10^7$) (Chart 1).

The characterization by flow cytometry analysis showed no significant differences in the surface marker profiles of hMSCs obtained from reusable or disposable filters (Figure 3).

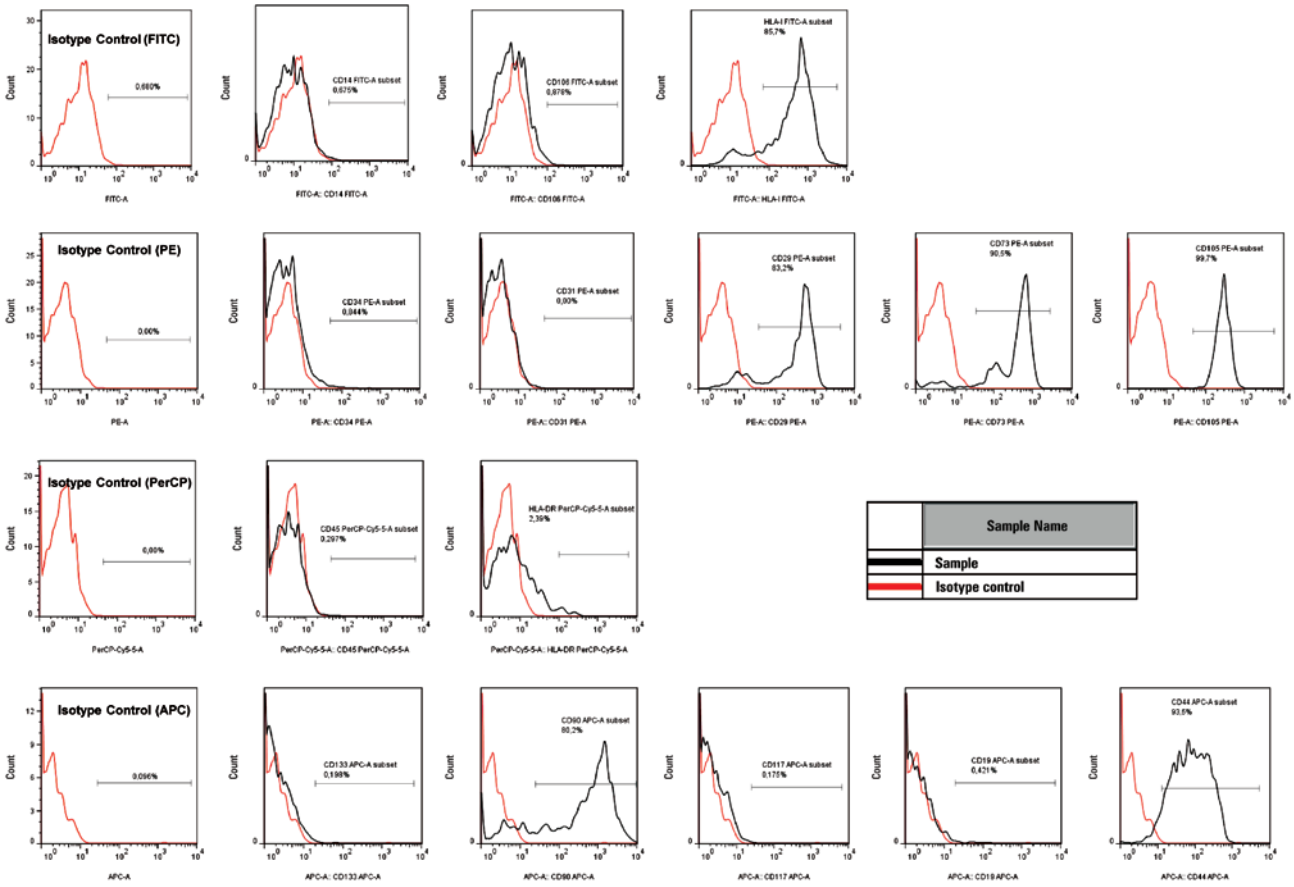


**Figure 3.** Representation of five experiments. The first row of graphics represents the isotype controls that were used for each group of antibodies. The other graphics overlay the isotype control (red) and the marker (blue). The bar represents the percentage of cells positive for each researched marker. MSCs were negative or presented very low positivity for CD14, CD19, CD31, CD34, CD45, CD117, CD133, CD106, HLA-DR; and over 89% for CD29, CD44, CD73, CD90, CD105 e HLA-I

### Differentiation of hMSC

hMSCs were also characterized by their ability to differentiate into cells of three different lineages. All five samples obtained either from reusable or disposable filters differentiated into cells of the three lineages.

### Adipogenic differentiation

Undifferentiated hMSCs (Figure 4A) were cultured in the presence of adipogenic medium for 21 days, resulting in the formation of cytoplasmic lipid droplets (Figure 4B). For better visualization, cells were stained with Oil Red O, which stains lipid droplets within the cells (Figure 4C).

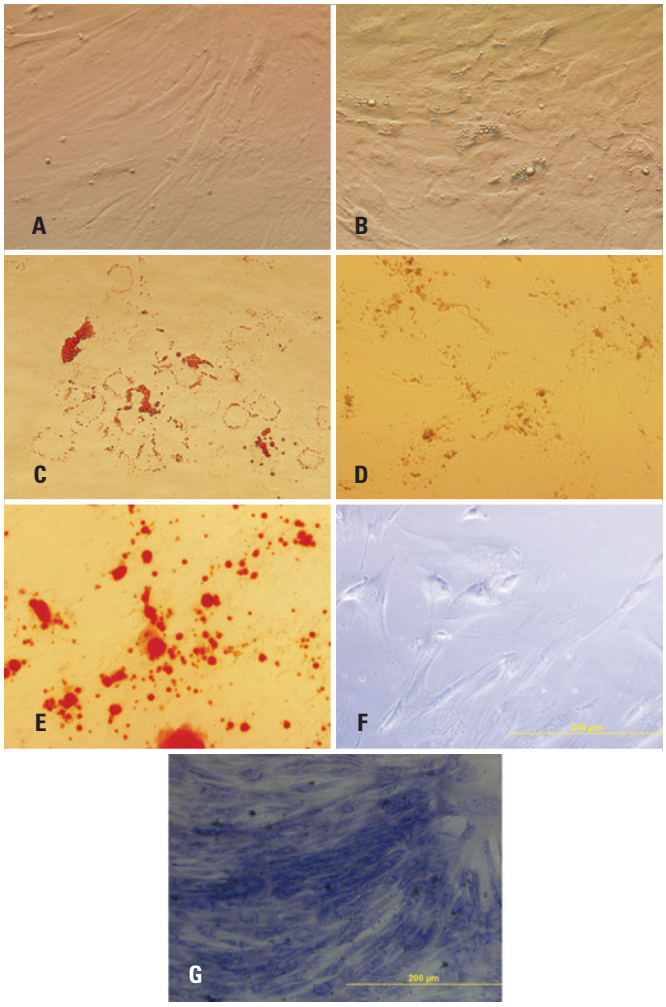


**Figure 4.** (A) Undifferentiated mesenchymal stem cells – CTRL (40x). (B) Lipid droplet formation within cells cultured in adipogenic medium (40x). (C) Lipid droplets stained with Oil Red O in cells cultured in adipogenic medium (40x). (D) Calcium deposition in cells cultured in osteogenic medium (40x). (E) Calcium deposition stained with Alizarin Red in cells cultured in osteogenic medium (40x). (F) Morphologic changes in cells cultured in chondrogenic medium (40x). (G) Proteoglycan-rich matrix stained with toluidin blue in cells cultured in chondrogenic medium (40x)

### Osteogenic differentiation

Undifferentiated hMSC were cultured in the presence of osteogenic medium for 21 days, resulting in the formation of a calcium matrix (Figure 4D). For better visualization, cells were stained with Alizarin Red, which stains calcium (Figure 4E).

### Chondrogenic differentiation

Undifferentiated hMSC were cultured in the presence of chondrogenic medium for 21 days, resulting in the formation of a proteoglycan-rich matrix (Figure 4F). For better visualization, cells were stained with toluidine blue, which stains proteoglycans (Figure 4G).

## DISCUSSION

Bone marrow derived hMSCs are the best well characterized source of stem cells, which have been used in most clinical trials, such as studies on prevention of graft *versus* host disease (GVHD) by co-infusion of hMSCs with bone marrow or peripheral blood hematopoietic stem cells. hMSCs are also being tested as a GVHD treatment, alone or in combination with corticosteroid therapy[15-17].

Besides GVHD prevention and treatment, hMSCs are involved in a variety of therapies suggestions, such as neurodegenerative disorders, cardiovascular diseases, type 1 diabetes, and others[18-21].

Therefore, bone marrow collection sets are an important source of cells, offering to the scientific community an opportunity to obtain these cells, and to clarify conflicting results – such as the implications of biological variability and differences in conditions that may affect the final therapeutic outcomes – and to provide answers to scientific and medical issues.

This study provided evidences that hMSC can be obtained from bone marrow collection reusable and disposable sets.

hMSCs were isolated by their capacity to adhere to plastic, and cultured and expanded after seeding. At the fourth passage, the cell populations were homogeneous in the expression of CD29, CD44, CD73, CD90, CD105, and HLA-I antigens. Furthermore, hMSCs were very low or negative to all hematopoietic stem and progenitor cell markers such as CD14, CD19, CD34, CD45, CD117, CD133 and HLA-DR, and they were also negative for endothelial markers, such as CD31 and CD106. This study shows that hMSCs derived from samples obtained from reusable or disposable collection sets were able to give rise, by *in vitro* differentiation, to cells of the three standard mesenchymal cell lineages – adipogenic, osteogenic, and chondrogenic. These cell

cultures obtained from filter sets have fully satisfied the minimal requirements for positive selection markers established by the ISCT[4].

Despite the fact that reusable filter sets have smaller pore size, which should retain a larger number of hematons, we were able to obtain more cells with better efficiency from the disposable filter sets. This probably occurred due to the fact that this type of filter presents larger areas for washing. Another possibility is the fact that MSCs are plastic adherent, and disposable filter sets are enclosed in plastic[22].

## CONCLUSION

This study presented evidences that hMSCs can be obtained from bone marrow reusable collection sets. hMSCs obtained from reusable sets, even with smaller initial number of cells, are able to generate a large number of hMSCs on the fourth passage, and these cells are definitely usable for research purposes as a very ethical source.

## REFERENCES


1. Friedenstein AJ, Deriglasova UF, Kulagina NN, Panasuk AF, Rudakowa SF, Luriá EA, et al. Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp Hematol. 1974; 2(2):83-92.
2. Deans RJ, Moseley AB. Mesenchymal stem cells: biology and potential clinical uses. Exp Hematol. 2000;28(8):875-84.
3. Bianco P, Gehron Robey P. Marrow stromal stem cells. J Clin Invest. 2000; 105(12):1663-8.
4. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8(4):315-7.
5. Ding DC, Shyu WC, Lin SZ. Mesenchymal stem cells. Cell Transplant. 2011; 20(1):5-14.
6. Beyer Nardi N, Silva Meirelles L. Mesenchymal stem cells: isolation, in vitro expansion and characterization. Handb Exp Pharmacol. 2006;(174):249-82.
7. Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science. 1997;276(5309):71-4.
8. Kemp KC, Hows J, Donaldson C. Bone marrow-derived mesenchymal stem cells. Leuk Lymphoma. 2005;46(11):1531-44.
9. Peng L, Li H, Gu L, Peng XM, Huang YS, Gao ZL. Comparison of biological characteristics of marrow mesenchymal stem cells in hepatitis B patients and normal adults. World J Gastroenterol. 2007;13(11):1743-6.
10. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999;284(5411):143-7.
11. Blazsek I, Delmas Marsalet B, Legras S, Marion S, Machover D, et al. Large scale recovery and characterization of stromal cell-associated primitive haemopoietic progenitor cells from filter-retained human bone marrow. Bone Marrow Transplant. 1999;23(7):647-57.
12. Dvorakova J, Hruba A, Velebny V, Kubala L. Isolation and characterization of mesenchymal stem cell population entrapped in bone marrow collection sets. Cell Biol Int. 2008;32(9):1116-25.
13. Capelli C, Salvade A, Pedrini O, Barbui V, Gotti E, Borleri G, et al. The washouts of discarded bone marrow collection bags and filters are a very abundant source of hMSCs. Cytotherapy. 2009;11(4):403-13.
14. Sundin M, Remberger M, Lönnies H, Sundberg B, Ringdén O, Le Blanc K. No increased trapping of multipotent mesenchymal stromal cells in bone marrow filters compared with other bone marrow cells. Cytotherapy. 2008;10(3): 238-42.
15. Lazarus HM, Koc ON, Devine SM, Curtin P, Maziarz RT, Holland HK, et al. Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. Biol Blood Marrow Transplant. 2005;11(5):389-98.
16. Le Blanc K, Rasmusson I, Sundberg B, Götherström C, Hassan M, Uzunel M, et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet. 2004;363(9419):1439-41.
17. Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, Lanino E, Sundberg B, Bernardo ME, Remberger M, Dini G, Egeler RM, Bacigalupo A, Fibbe W, Ringdén O; Developmental Committee of the European Group for Blood and Marrow Transplantation. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet. 2008;371(9624):1579-86.
18. Kebriaei P, Isola L, Bahceci E, Holland K, Rowley S, McGuirk J, et al. Adult human mesenchymal stem cells added to corticosteroid therapy for the treatment of acute graft-versus-host disease. Biol Blood Marrow Transplant. 2009;15(7):804-11.
19. Mazzini L, Mareschi K, Ferrero I, Miglioretti M, Stecco A, Servo S, et al. Mesenchymal stromal cell transplantation in amyotrophic lateral sclerosis: a long-term safety study. Cytotherapy. 2012;14(1):56-60.
20. Williams AR, Hare JM. Mesenchymal stem cells: biology, pathophysiology, translational findings, and therapeutic implications for cardiac disease. Circ Res. 2011;109(8):923-40.
21. Mabed M, Shahin M. Mesenchymal stem cell-based therapy for the treatment of type 1 diabetes mellitus. Curr Stem Cell Res Ther. 2012;7(3):179-90.
22. Mageed AS, Pietryga DW, DeHeer DH, West RA. Isolation of large numbers of mesenchymal stem cells from the washings of bone marrow collection bags: characterization of fresh mesenchymal stem cells. Transplantation. 2007; 83(8):1019-26.