Research Article **Open Access**

Zivile Gudleviciene*, Gabrielis Kundrotas, Regina Liudkeviciene, Jelena Rascon, Marcin Jurga

Quick and effective method of bone marrow mesenchymal stem cell extraction

Abstract: Background. Mesenchymal stem cells (MSCs) are currently exploited in numerous clinical trials to investigate their potential in immune regulation, hematopoesis or tissue regeneration. The most common source of MSCs for clinical use is human bone marrow. To generate sufficient numbers of cells relevant to clinical use in most cases the high volumes (20-50 ml) of bone marrow aspirates are taken. Methods. In this pilot study, 8 healthy bone marrow donors were included. Two different MSC extraction methods were evaluated: MSCs extraction from 60 ml of bone marrow using density gradient and MSCs extraction from 6 ml using red blood cell (RBC) lysis. Results. Our results showed that after RBC lysis the efficient amount of human MSCs can be isolated from 10 times less bone marrow volume (6 ml). Moreover, using small volume of bone marrow the adequate therapeutical dose of MSCs could be achieved during similar period of time (3-4 weeks). In conclusion, we have shown that MSCs isolation using RBC lysis is an effective and more advantageous method in comparison to standard MSCs isolation using density-gradient. Using RBC lysis from small volume of bone marrow the same amount of MSCs were obtained as usually using large volume and density-gradient.

Keywords: Bone marrow, Cell therapy, Ficoll gradient, Mesenchymal stem cells, Red blood cell lysis

***Corresponding author: Zivile Gudleviciene**, National Cancer Institute, Biobank, Santariskiu 1, 08660 Vilnius, Lithuania, tel. +37052190909, fax. +37052720164, E-mail: zivile.gudleviciene@nvi.lt

Gabrielis Kundrotas, National Cancer Institute, Biobank Santariskiu 1, 08660 Vilnius, Lithuania

Vilnius University, Faculty of Natural Sciences M.K. Ciurlionio 21/27, 03101 Vilnius

Regina Liudkeviciene, National Cancer Institute, Biobank Santariskiu 1, 08660 Vilnius, Lithuania

Jelena Rascon, Children's Hospital, Affiliate of Vilnius Unversity Hospital Santariskiu Klinikos, Children Heamatology and Transplantation Center Santariskiu 7, 08406, Vilnius, Lithuania

Marcin Jurga, Cryo-Save Labs NV, Attn. The Cell Factory (R&D department) Galileilaan 19, B-2845 Niel, Belgium

DOI 10.1515/med-2015-0008
received: March 21, 2014; accepted: June 29, 2014

1 Introduction

Mesenchymal stem cells (MSCs) are multipotent stromal cells that can differentiate into variety types of cell [1]. MSCs are currently exploited in numerous clinical trials to investigate their potential in immune regulation, hematopoiesis and tissue regeneration [2]. Unfortunately, small number of MSCs are circulatig in the human body and usually they must be isolated and subsequently expanded in order to generate clinically relevant numbers of cells [3]. The most common source of MSCs for clinical use is the human bone marrow [4]. According the literature, in various clinical trials for MSCs cultivation, high volumes of bone marrow aspirates (20-50 ml) are taken [5-7]. Commonly MSCs are isolated by density gradient centrifugation [8]. However, aspiration of high volume bone marrow is invasive and painful to donors [9], during the procedure premedication or general narcosis must be used. On the other hand the density gradient centrifugation is difficult to standardize [10]. Due to these reasons application of easier standardized and less frustrating methods for patients in clinical trials is needed. The aim of our article is to present the quick and donor saving MSCs extraction from bone marrow method.

2 Materials and methods

2.1 Study design

8 healthy donors were included in our pilot study (period from the June of 2013 to November of 2013). All donors have signed an Informed patient consent form approved by the

© 2015 Z. Gudleviciene et al, licensee De Gruyter Open
This work is licensed under the Creative Commons Attribution-NonCommercial-NoDerivs 3.0 License.

Vilnius Regional Committee of Biomedical Research (Lithuania, 2011-09-06 permission No. 158200-09-381-104).

2.2 Bone marrow collection

The bone marrow was collected in the operating room under the general narcosis. For the first 4 donors 60 ml of bone marrow was aspirated aseptically from the iliac crest: 10 vacutainers (volume 6 ml) with lithium heparin (BD Biosciences, France) were taken from each donor. For remaining 4 donors – only 6 ml of bone marrow were taken (1 vacutainer of 6 ml with lithium heparin). Procedure was performed using Standard operating procedure in the Vilnius University Santariskiu Clinic, Children Heamatology and Transplantation Center. Directly after bone marrow aspiration all vacutainers were gently vortexed and immediately (<30 min.) transported at room temperature to the Biobank of Scientific Research Center of National Cancer Institute. In the Biobank laboratory bone marrow were directly processed for the MSCs extraction. Two different methods were used for MSC extraction.

2.3 MSCs extraction using ficoll gradient

Using centrifugation through a ficoll gradient, 60 ml of bone marrow (10 vacutainers of 6 ml) was processed. The bone marrow was diluted (1:1) with standard RPMI 1640 medium (Invitrogen, UK) and transferred in the 50 ml conical centrifuge tubes (BD Biosciences, France) on the ficoll gradient (Ficoll-PaqueTM Plus, GE Healthcare Bio-Sciences AB, Uppsala). Proportion of ficoll and bone marrow was 1:3; centrifugation was performed for 20 min at 300 g. After centrifugation mononuclear cells were carefully collected at an whitish ring and transferred in the new 50 ml conical centrifugation tubes (BD Biosciences, France) and washed twice with 20 ml DMEM medium (Invitrogen, UK) containing 10% of fetal bovine serum (FBS, Invitrogen, UK). After washing, the mononuclear cells were counted with the Bürker counting chamber (LO-Laboroptik GmbH, UK) and plated into the 175 cm2 ventilated flasks (BD Biosciences, France) in the density of round cells 100x106/flask. Cells were cultivated for 24 hours in the DMEM medium (Invitrogen, UK) containing 10% of FBS in the incubator (CO2Cell, Germany) under standard condition 5% of CO2 and 37°C.

2.4 MSC extraction using red blood cell lysis

For MSC extraction using red blood cell lysis method, 6 ml of bone marrow (1 vacutainer of 6 ml) was used. The total amount of the vacutainer (6 ml) was transferred to the 50 ml conical centrifugation tube and Erythrocyte lysis buffer (remaining reagent from QIAamp RNA Blood Mini Kit, Qiagen GmbH, Germany) was added in the proportion 1:5. The tube was mixed manually for 1 minute and centrifuged immediately for 5 min at 480 g. Promptly, after centrifugation the top layer was discarded and the pellet was resuspended with 5 ml of RPMI 1640 medium and washed twice using centrifugation in same conditions (5 min at 480 g). Finally, total volume of resuspended pellet was transferred to the 175 cm2 ventilated flask and cultivated for 24 hours in the DMEM medium containing 10% of FBS in the incubator under standard condition 5% of CO2 and 37°C.

2.5 MSC passaging

Using both methods, after 24 hours the media was removed and cells were washed with phosphate buffered saline (PBS) in order to remove non-adherent cells. MSC Basal Medium (Human) (StemCell technologies Inc., Canada) containing 10% of special FBS for human mesenchymal stem cells (StemCell technologies Inc., Canada) was used for subsequent cultivation of MSC. The whole media was changed every 3-4 days. When adherent cells became confluent (monolayer was formed), MSCs were treated with trypsin-EDTA (Invitrogen, UK), washed twice with PBS, counted and distributed to the new 175 cm2 flasks under the density 2 x 106/flask, incubated in the incubator under the same condition (5% of CO2 and 37°C) (passage 0, P0). P0 means period from bone marrow processing till 1st monolayer formation. Cells from 1st monolayer trypsinization and passaging means passage 1 (P1), next monolayer formation and passaging means passage 2 (P2). During our study tryspsinization process was repeated twice, time to formation of second (P1) and third (P2) monolayer was fixed. MSCs from all 8 donors were expanded until passage 2. All procedures were performed in the class II vertical laminar air flow safety cabinet (ESCO, Singapore). After passage 2 the total number of cells was calculated, cells were collected for the flow cytometry analysis.

2.6 MSC number calculation

Cell number was determined using a Bürker counting chamber at each passage and expressed in terms of absolute cell number.

2.7 Immunophenotypic analysis

Immunophenotype of MSCs was analyzed using the 6-color flow cytometry-based identification analysis. Data acquisition was performed on a LSR II flow cytometer (BD Biosciences, Germany) equipped with three lasers (VioFlame 405 nm, Sapphire blue 488 nm and HeNe 633 nm) and capable of 8-color analysis. MSCs were identified using 7 key markers: CD105+, CD73+, CD90+, CD14-, CD34-, CD45- and HLA-DR-.

3 Statistical methods and experimental procedures

3.1 Statistical analysis

For statistical analysis SPSS software (version 21) was used. All results are expressed as mean values ± SD. To estimate the probability of differences we have adopted the nonparametric Mann-Whitney U-test. Significance was set at $P < 0.05$.

4 Results

In this pilot study conducted from the June of 2013 to November of 2013, 8 healthy donors were included. Any complications were not stated during procedures of bone marrow aspiration. MSCs were extracted using two methods: by centrifugation through ficoll gradient (CfFG) (4 samples of 60 ml volume) and by red blood cell (RBC) lysis (4 samples of 6 ml volume). During this experiment the cell morphology and phenotype was evaluated, time to the first monolayer (P0) and total number of cultivated cells (P2) were calculated and compared between these two methods.

Time to the first monolayer (P0). During our observation mesenchymal stem cells (MSCs) isolated from 60 ml of BM by CfFG formed monolayer after 13±2.16 days; 16.125±6.79 x 106 of cells were obtained. MSCs isolated by RBC lysis of 10 times lower BM volume (6 ml) formed a monolayer after 11±3.3 days; 13.75±6.29 x 106 of cells were obtained. The difference between these two groups was not statistically significant (p-value=0.7728). According to this result we can say that although the bone marrow volume used in RBC lysis method was much lower, the first monolayer with the same amount of MSCs was obtained during the same time.

Total number of cultivated MSCs. Using CfFG method, at P1 there were 74±19.6 x 106 of MSCs (after 24±5.5 days in culture) and 144.23±52.41 x 106 at P2 (after 29±5.97 days in culture) were obtained. Using RBC lysis method, there were 39.375±8.26 x 106 MSCs at P1 (after 19±4.9 days in culture) and 87.375±7.36 x 106 MSCs at P2 (after 27±4.83 days in culture). However, there was no statistically significant difference between respective passages of CfFG and RBC methods according to cell number and days in culture (Fig. 1): p-value=0.1489 between P1 and p-value=0.5637 between P2. There also was no statistically significant difference between CfFG P1 and RBC P2 (p-value=1). This suggests that the final number of cells and cultivation time, having different volume of bone marrow, depends on the MSCs isolation method: even using small volume of bone marrow and RBC lysis the same number of MSCs can be expanded during the same time of cultivation.

According the literature, expanded MSCs usually were identified according the morphology, plastic adherence and expression of surface markers [11, 12].

Morphological analysis of cultivated MSCs In our study, all MSCs were adhered to the plastic surface, had typical spindle shaped morphology (Fig. 2, A). There was no significant difference observed in MSC morphology between different isolation methods and different passages of each donor. However, it was noticed that MSCs

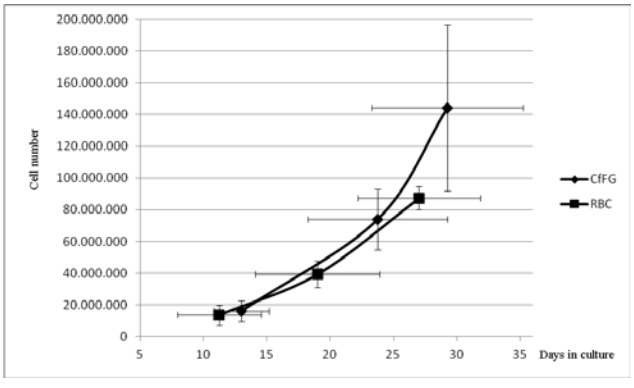


Figure 1: MSCs amount obtained at different passages (P0, P1, P2) using centrifugation through ficoll gradient (CfFG) and red blood cell (RBC) lysis methods. Results are expressed as mean values ± SD.

isolated by RBC lysis method from colonies earlier, some were visible even the next day after isolation (Fig. 2, B).

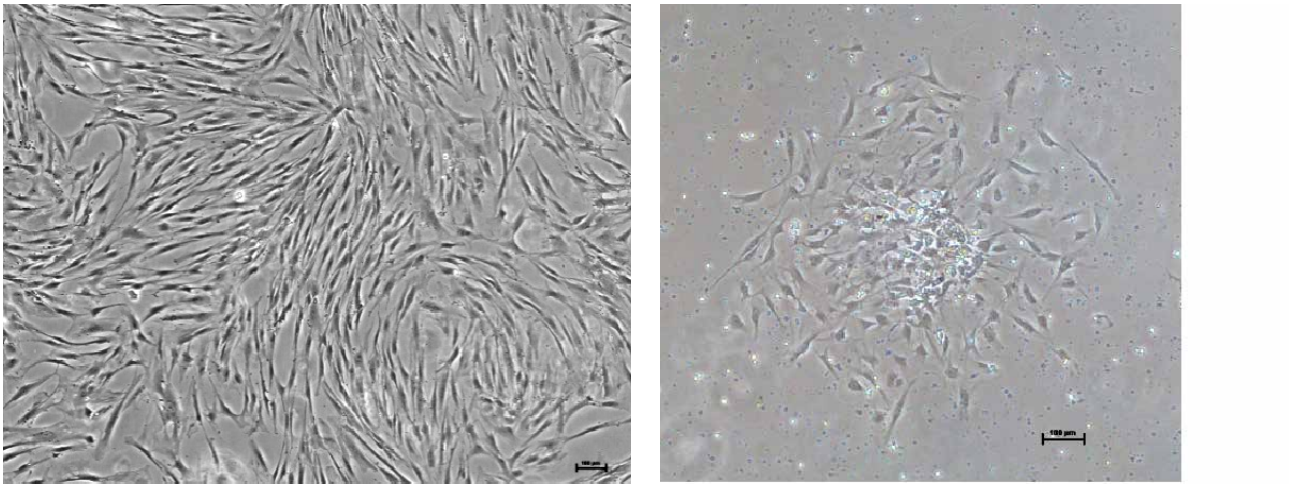
Flow cytometry analysis. All cells were tested for MSC key surface markers. Expression of CD105+, CD73+, CD90+ (>95%) and loss of CD14-, CD34-, CD45- and HLA-DR- (<2%) was stated in all analyzed cases.

5 Discussion

The commonly applied preparation method for the generation of MSCs from bone marrow is density gradient centrifugation [8]. Usually, to isolate the required amount of human MSCs, the high volume (of 20-60 ml) of bone marrow aspirates are taken [5, 7, 13]. The procedure is time consuming, the premedication and anesthesia for donors and working in operating room is obligatory. Some authors suggest avoiding low aspiration volume (< 8mL) to reduce the risk of obtaining aspirates with low cell number [14]. However, other authors showed that RBC lysis of 10-20 ml bone marrow volumes could be superior alternative [10]. In this study we have demonstrated that using RBC lysis the efficient amount of human MSCs can be isolated from small volumes (just 6 ml) of bone marrow in comparison with typically used larger volumes (60 ml) and ficoll gradient centrifugation.

As described in the literature for therapeutical applications currently applied doses are in the range of 1–5 × 106 MSCs/kg body weight [2, 5, 15]. In our pilot study the possible therapeutic doses was reached in the same period of time using both methods: 144 x 106 of cells were obtained after 29 days by CfFG method and 87 x 106 of MSCs were obtained after 27 days using RBC lysis. There was no statistically significant difference between these P2. The Fig. 2 shows, that number of cultivated CfGF and RBC cells increases in parallel, so the therapeutic dose using RBC lysis should be achieved within a few days of next passage. It can be stated that by using RBC lysis, the efficient amount of human MSCs can be isolated from small volume of bone marrow and expanded during similar period of time, in comparison with usually used large volume and density-gradient centrifugation.

There could be several possible explanations of RBC lysis efficiency. Firstly, during RCB lysis all mononuclear cells are used whereas during density-gradient centrifugation mononuclear cells are collected at an interphase ring in the plasma fraction and some MSCs can be lost due to procedure itself. Secondly, during cell lysis various growth factors are released. Unfortunately, the complete growth factor needs for MSCs culture are still unknown [16]. There are some suggestions that platelet lysate is a very effective supplement for MSCs growth [10]. This might be attributed to growth factors such as PDGF or others. Bone marrow lysate could have similar impact as platelets lysate used in other studies [17]. It may have the impact on faster formation of the first monolayer that was also observed in our study. Other authors also discuss about platelet lysate as possible source of various cytokines and grow factors to the expansion and differentiation of MSCs [18]. One more advantage is that platelet lysate or RBC lysis not only contains the high levels of various growth factor, but is a safer method in comparison to largely used MSCs cultivation with fetal serum [19]. Disadvantage and risk of fetal serum are largely discussed in the literature: here is the risk of xenogenic immune reactions or transmission of bovine



A B

Figure 2: A–Typical spindle shape morphology of human BM-MSCs. B–Human bone marrow MSCs forming a colony 1 day after isolation by RBC lysis. Magnitute 400x, scale 100 µm.

pathogens (various viruses or bacteria), on the other hand the huge differences between various manufacturers of even between different lots of serum of same manufacturer may impact reproducibility of results [17, 20]. So, according to the results of various authors and observation during our study, it is worth investigating the composition of the lysate and identify factors important for MSCs proliferation. These further investigations would be interesting for biotechnology companies and manufacturers which develop defined serum-free cell culture media.

In the recent years, researchers have been looking for new and safer methods for MSCs expansion from bone marrow. The new filtration method for MSC extraction from bone marrow was published [21].The authors showed that this method and novel filter device is a fast, efficient and reliable system to isolate MSCs. Using this filter device generated significantly greater initial cell recovery requiring less investigator time and resulted in approximately 2.5-fold more MSCs after the second passage. Since RBC lysis method is as effective as centrifugation, it is worthwhile to use the lysis method advantages over centrifugation. Since all mononuclear cells are isolated and subsequently the composition of heterogeneous MSC mixture is less affected, this technique simplifies the standardized and Good Manufacturers Practice (GMP) conformed culture isolation for clinical application of MSCs. Novel clinical grade and automated MSC extraction devices could be developed and manufactured based on this lysis method. Such standardized methods will reduce the risk of contamination and increase the reproducibility of MSCs. Secondly, since less bone marrow is needed, the patient inconveniences caused by biopsy procedure are reduced because of shorter procedure time and shorter duration of anesthesia. General complications following bone marrow aspiration procedure could be bleeding, bruises in the place of aspiration, infection or traumatical lesion of bone. In this pilot study any complications for both groups of bone marrow donors were not stated. Moreover, lysis is a faster, more straightforward method, and centrifugation is time consuming because of frequent manual interventions and manipulation [10]. Finally, the lysis method can have no additional cost because reagents left after standard laboratory procedures can be used, with no additional devices or laboratory supplies needed.

In this pilot study we showed that RBC lysis is effective, less time consuming and a safer method for MSCs expansion from small amount of bone marrow comparing to centrifugation through ficoll gradient method. According to our results it can be assumed, that bone marrow lysate could have the same impact on MSCs expansion and cultivation time as platelete lysate methods described in the literature. These our primary observations need additional research and future evaluation to develop clinical grade protocol to use the bone marrow lysate as medium supplement.

In conclusion, we have shown that MSCs isolation using remaining RBC lysis buffer from QIAamp RNA Blood Mini Kit (Qiagen GmbH) is an effective and more advantageous method over standard MSCs isolation using density-gradient. Using RBC lysis from a small volume of bone marrow, the same amount of human MSCs were obtained as when using large volumes and density-gradient method.

Acknowledgements: This pilot sudy was performed by support of Lithuanian Ministry of Education and Science, Grant „LASTER“ (No. VP1-3.1-ŠMM-10-V-02-027).

To dr. Eiva Bernotiene and Jaroslav Denkovskij from Innovative Medicine Center (Vilnius University, Vilnius, Lithuania) for flow cytometry analysis of MSC.

Conflict of interest statement: Authors state no conflict of interest

Abbreviations

BD – Becton Dickinson
CD – surface antigens
CfFG – centrifugation through ficoll gradient
DMEM – Dulbecco's Modified Eagle Medium
EDTA – ethylenediaminetetraacetic acid
FBS – fetal bovine serum
HLA-DR – human leukocyte antigen, major histocompatibility complex
MSC – mesenchymal stem cell
PBS – phosphate buffered saline
RBC – red blood cell
RNA – ribonucleic acid
RPMI – Roswell Park Memorial Institute

References

[1] Beyer N.N., Meirelles S.L., Mesenchymal Stem Cells: Isolation, In Vitro Expansion and Characterization, In: Wobus A.M. and Boheler K.R. (Eds), Stem cells, vol. 174, Handbook of experimental pharmacology, New York: Springer-Verland Berlin Heidelberg, 2006, 249–282

[2] Bieback K., Schallmoser K., Klüter H., Strunk D., Clinical Protocols for the Isolation and Expansion of Mesenchymal Stromal Cells, Transfus. Med. Hemother., 2008, 35(4), 286-294

[3] Jung S., Panchalingam K.M., Rosenberg L., Behie L.A., Ex vivo expansion of human mesenchymal stem cells in defined serum-free media, Stem Cells Int., 2012, 2012:123030, DOI: 10.1155/2012/123030
[4] Thirumala S., Goebel W.S., Woods E.J., Clinical grade adult stem cell banking, Organogenesis, 2009, 5(3), 143-154
[5] Le Blanc K., Frassoni F., Ball L., Locatelli F., Roelofs H., Lewis I., at al., Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study, Lancet, 2008, 371(9624), 1579-1586
[6] Venkataramana N.K., Kumar S.K., Balaraju S., Radhakrishnan R.C., Bansal A., Dixit A., et al., Open-labeled study of unilateral autologous bone-marrow-derived mesenchymal stem cell transplantation in Parkinson's disease, Transl. Res., 2010, 155(2), 62-70
[7] Lee H., Park J.B., Lee S., Baek S., Kim H., Kim S.J., Intra-osseous injection of donor mesenchymal stem cell (MSC) into the bone marrow in living donor kidney transplantation; a pilot study, J. Transl. Med., 2013, 11-96
[8] Hass R., Kasper C., Böhm S., Jacobs R., Different populations and sources of human mesenchymal stem cells (MSC): A comparison of adult and neonatal tissue-derived MSC, Cell Commun. Signal., 2011, 9-12
[9] Cooper K., Viswanathan C., Establishment of a mesenchymal stem cell bank, Stem Cells Int., 2011, 2011, 905621
[10] Horn P., Bork S., Diehlmann A., Walenda T., Eckstein V., Ho A.D., et al., Isolation of human mesenchymal stromal cells is more efficient by red blood cell lysis, Cytotherapy, 2008, 10(7), 676-685
[11] 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), 315-317
[12] Bühring H.J., Treml S., Cerabona F., de Zwart P., Kanz L., Sobiesiak M., Phenotypic characterization of distinct human bone marrow-derived MSC subsets, Ann. N.Y. Acad. Sci., 2009, 1176, 124-134
[13] Ringden O., Le Blanc K., Mesenchymal stem cells for treatment of acute and chronic graft-versus-host disease, tissue toxicity and hemorrhages, Best Pract. Res. Clin. Haematol., 2011, 24(1), 65-72
[14] Fennema E.M., Renard A.J., Leusink A., van Blitterswijk C.A., de Boer J., The effect of bone marrow aspiration strategy on the yield and quality of human mesenchymal stem cells, Acta Orthop., 2009, 80(5), 618-621
[15] 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), 1439-1441
[16] Sensebé L., Bourin P., Tarte K., Good manufacturing practices production of mesenchymal stem/stromal cells, Hum. Gene Ther., 2011, 22(1), 19-26
[17] Horn P., Bokermann G., Cholewa D., Bork S., Walenda T., Koch C., at al., Impact of individual platelet lysates on isolation and growth of human mesenchymal stromal cells, Cytotherapy, 2010, 12(7), 888-898
[18] Fekete N., Gadelorge M., Fürst D., Maurer C., Dausend J., Fleury-Cappellesso S., et al., Platelet lysate from whole blood-derived pooled platelet concentrates and apheresis-derived platelet concentrates for the isolation and expansion of human bone marrow mesenchymal stromal cells: production process, content and identification of active components, Cytotherapy, 2012, 14(5), 540-554
[19] Fekete N., Rojewski M.T., Fürst D., Kreja L., Ignatius A., Dausend J., et al., GMP-compliant isolation and large-scale expansion of bone marrow-derived MSC, PLoS One, 2012, 7(8), e43255
[20] Schrezenmeier H., Seifried E., Buffy-coat-derived pooled platelet concentrates and apheresis platelet concentrates: which product type should be preferred?, Vox. Sang., 2010, 99, 1–15
[21] Otsuru S., Hofmann T.J., Olson T.S., Dominici M., Horwitz E.M., Improved isolation and expansion of bone marrow mesenchymal stromal cells using a novel marrow filter device, Cytotherapy, 2013, 15(2), 146-153