DOI 10.1007/s10517-015-3015-5
Cell Technologies in Biology and Medicine, No. 2, August, 2015

Culturing of Mouse Mesenchymal Stem Cells on Poly-3-Hydroxybutyrate Scaffolds

N. V. Andreeva[1,2], A. P. Bonartsev[2,3], I. I. Zharkova[2], T. K. Makhina[3], V. L. Myshkina[3], E. P. Kharitonova[4], V. V. Voinova[2,3], G. A. Bonartseva[4], K. V. Shaitan[2], and A. V. Belyavskii[1]

Translated from *Kletochnye Tekhnologii v Biologii i Meditsine*, No. 2, pp. 114-119, April, 2015
Original article submitted February 27, 2014

We studied the possibility of long-term culturing of mouse mesenchymal stem cells on a porous scaffold made of biocompatible polymer poly-3-hydroxybutyrate. The cells remained viable for at least 2 months and passed more than 65 population doublings in culture. Culturing on the scaffold did not change surface phenotype of cells. 3D poly-3-hydroxybutyrate scaffolds are appropriate substrate for long-term culturing of mesenchymal stem cells.

Key Words: *mesenchymal stem cells*; *poly-3*-hydroxybutyrate; polymer *scaffold*

Transplantation of hemopoietic stem cells is used in clinical practice for the treatment of leukemia and radiation injuries for a long time [9]. Transplantation of stem cells in various pathologies, such as stroke, trauma, malignant tumors is now intensively studied in numerous clinical trials [8,17]. Due to low content of stem cells in organs, their expansion in culture in large quantities for medical purposes is a pressing problem.

Functional activity, self-replication, and differentiation of stem cells in the body requires certain microenvironment, or niche that is formed by supporting stem cells or factors produced by them (extracellular matrix molecules, growth factors, adhesion proteins, *etc*.). Various substrates and materials used for stem cell culturing, their composition and structure, can affect differentiation and growth of cultured cells [10]. Among a variety of biopolymers used for culturing of stem cells and other cell types, poly-3-hydroxyalkanoates occupy a special place, because they are environmentally safe and biocompatible, though their hydrophobicity causes certain concerns about successful biomedical application [4]. However, sufficient adaptation of mesenchymal stem cells (MSC) to scaffolds made of poly-3-hydroxybutyrate (PHB) and poly-3-hydroxybutyrate-co-3-hydroxyvalerate (PHB-HV) co-polymer characterized by developed porous structure [18] and successful differentiation of human MSC into osteoblasts on this copolymer were demonstrated [15]. PHB monomer, 3-hydroxybutyric acid, is a natural metabolite in tissues and organs (liver, kidney, spleen, brain, and muscle); moreover, even PHB oligomers are present in the plasma. This determines high biocompatibility this polymer [1]. Although PHB is highly biocompatible with body tissues, it remains unclear whether it is suitable for long-term culturing and expansion of MSC.

Here we studied the possibility of long-term culturing of MSC from the mouse adipose tissue on a porous PHB scaffold.

MATERIALS AND METHODS

Biosynthesis and isolation of the polymer for fabrication of membranes. Polymer membranes were fabricated from PHB with a molecular weight of 8×10^5 synthesized by *Azotobacter chroococcum* 7B, one of the most efficient producer strains capable of accu-

[1]V. A. Engelhard Institute of Molecular Biology, Russian Academy of Sciences; [2]Department of Bioengineering, Biological Faculty, M. V. Lomonosov Moscow State University; [3]Federal Research Centre Fundamentals of Biotechnology, Russian Academy of Sciences; [4]Physical Faculty, M. V. Lomonosov Moscow State University, Moscow, Russia. ***Address for correspondence:*** ant_bonar@mail.ru. A. P. Bonartsev

mulating up to 80% PHB of total dry cell weight. For PHB biosynthesis, Azotobacter culture was grown at 30°C under conditions of carbon excess in Burk's medium containing (in g/liter): 0.4 $MgSO_4 \times 7H_2O$, 0.01 $FeSO_4 \times 7H_2O$, 0.006 $Na_2MoO_4 \times 2H_2O$, 0.5 sodium citrate, 0.1 $CaCl_2$, 1.05 $K_2HPO_4 \times 3H_2O$, 0.2 KH_2PO_4, 40 sucrose, and 20 sodium valerate. For reducing the molecular weight, sodium acetate was added to the culture medium in a concentration of 12 g/liter.

Isolation and purification of the polymer from *Azotobacter chroococcum* biomass included the following stages: PHB isolation from the bacterial mass by chloroform extraction with shaking over 12 h at 37°C; filtration of PHB solution from cell debris; precipitation of PHB from chloroform extract with isopropyl alcohol; repeated dissolution in chloroform and precipitation with isopropanol; drying at 60°C [5,6].

Fabrication of membranes. For fabrication of polymer membranes of desirable shape and internal geometry, we applied the method of pore formation by using blowing agent, ammonium carbonate (Khimmed). Ammonium carbonate (0.1 g) was grinded in a mortar and placed in a 50-mm Petri dish, then 4 ml 3% solution of the polymer in chloroform was added and the solvent was allowed to evaporate completely. Then the Petri dish was placed in a glass with distilled water, heated to gassing, and leaved until complete cessation of gas generation. The obtained porous membrane was washed 5 times with distilled water and dried.

Morphology and porosity of membranes. The surface geometry of the obtained structures was initially studied by light microscopy (Biomed 1 microscope; Biomed) using a MYscope 300Mdigital eyepiece (Webbers).

Microphotographs were obtained by scanning electron microscopy on a Supra 50 VP LEO microscope with INCA Energy+Oxford microanalysis system (Carl Zeiss) at accelerating voltage of 20 kV and maximum resolution of 10 nm after routine carbon coating.

Membrane porosity (MP) was calculated by the formula:

$$P = 1 - \frac{m_{memb}}{V_{memb} \times \rho_{PHB}} \times 100\%,$$

where m_{memb} is membrane weight, V_{memb} is membrane volume calculated from its linear dimensions measured with calipers (KRINO), and ρ_{PHB} is the mean density of the polymer equal to 1.26 g/cm^3. Pore size was evaluated by scanning electron microscopy and light microscopy using Cool Ruler software.

Analysis of the thermal properties of membranes by differential scanning calorimetry. The thermal characteristics of the membranes (melting and crystallization temperatures, heat of melting and crystallization, crystallinity) were analyzed on a DSC 204 F1 Phoenix differential scanning calorimeter (Netzsch) in nitrogen atmosphere at scanning rate of 10°C/min; sample weight was ~5 mg. The instruments were calibrated using the calibration set and Netzsch In (Indium) standard (T_m=156.6°C). Crystallinity was calculated using a theoretical value of thermodynamic melting enthalpy for a 100%-crystalline PHB sample (146.6 J/g) [12,13].

Isolation and culturing of MSC on the scaffold. MSC were isolated from the adipose tissue of 2-month-old female C57BL/6 mice. The adipose tissue was cut with scissors into small fragments, placed in a test tube with 15 ml DMEM medium and collagenase IA (Sigma) (final concentration 2 mg/ml) under aseptic conditions, and incubated for 40-60 min at 37°C and constant shaking. Then, the suspension was passed through a Nylon filter with pore diameter of 100 μ. The cells were washed by centrifugation in serum-free DMEM (Sigma) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml fungizone (Life Technologies). The cells were centrifuged at 367*g* (3×7 min) and transferred to 10-cm Petri dishes. Culturing of zero passage cells was carried out in low-glucose DMEM containing 10% fetal calf

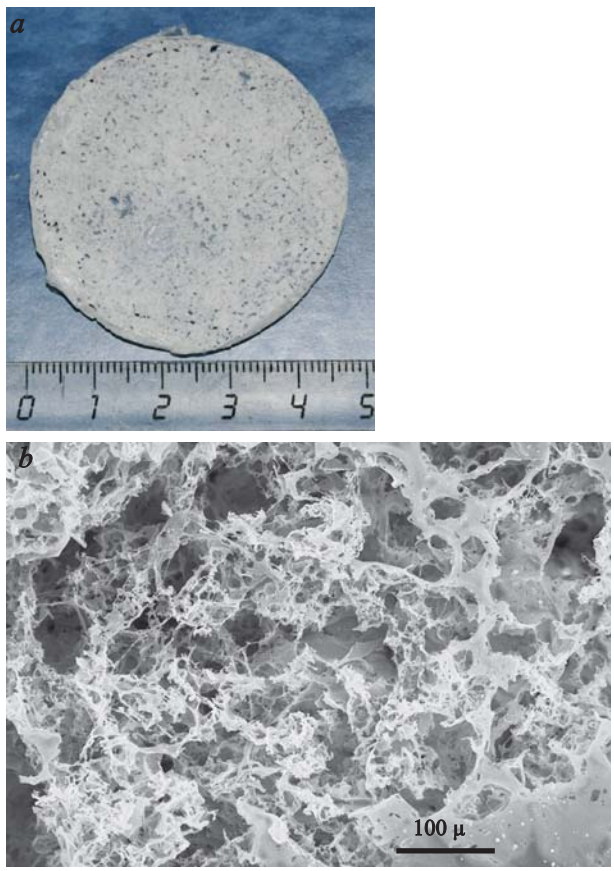

Fig. 1. Appearance (*a*) and microstructure (*b*) of the porous polymer membrane.

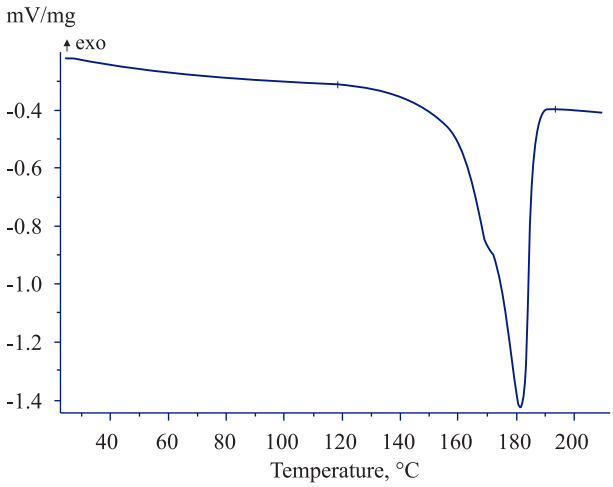

Fig. 2. Thermogram of the PHB polymer material. Differential scanning calorimetry.

serum (FCS), 2 mM glutamine, 100 U/ml penicillin, streptomycin (100 μg/ml) and fungizone (2.5 mg/ml) at 37°C in a CO_2 incubator under hypoxic conditions (5% CO_2, 5% O_2). The cells were cultured for 4-6 days to 70-80% confluence, then removed by incubation with 1x TrypLE Express (Life Technologies) for 10 min at 37°C, and washed by centrifugation in DMEM for 7 min at 367*g*. Cells were counted in the Goryaev's chamber, seeded at a density of 500 cells/cm^2 in 3.5-cm Petri dishes with the scaffold, cultured under hypoxic conditions for 4-6 days, and then subcultured to the next passage. The medium was replaced every 3 days. The experiment was performed until passage 12.

The scaffold prior to cell seeding was washed with water and sterilized by 1-h incubation in 70% ethanol followed by UV irradiation for 2 h (1 h from each side of the membrane). After culturing, the cells were removed from the scaffold with 1x TrypLE Express; to this end, the medium was removed, the scaffolds were washed three times with PBS and incubated with 1x TrypLE Express for 10 min at 37°C. The harvested cells were washed by centrifugation in DMEM (7 min; 367*g*), suspended in PBS, stained with trypan blue, and counted in a Goryaev's chamber.

For scaffold regeneration after cell removal, it was additionally treated with 1x TrypLE Express for 20 min at 37°C, then washed twice with water and 70% ethanol, and dried at room temperature; then, the scaffolds were stored in a Petri dish until next use.

Flow cytofluorometry analysis of cell surface phenotype. MSC cultured on plastic and scaffolds (10^5 cells) were suspended in 100 μl PE buffer (2 mM EDTA, 0.5% FCS, and PBS) and incubated with FITC-labeled antibodies to murine cell surface markers CD29, CD44 (BioLegend), CD105, CD45, CD11b (Miltenyi Biotec), and CD34 (eBioscience) in the dark for 40 min at 5°C. The cells were washed by centrifugation in PBS and analyzed on a Gallios flow cytometer (Beckman Coulter). The data were processed using Flowing Software 2.5.1.

RESULTS

The yield of bacterial biomass containing the polymers was 4.5±0.5 g/liter, the content of PHB was 78.6±3.4% of bacterial dry weight. The mean molecular weight of the polymer was 8.1×10^4. High degree of polymer purification (>99.5%) for its use in biomedical research was achieved.

Porous membrane were prepared from the purified PHB (Fig. 1, *a*) with pore size of 170±30 μ and porosity of 89±4%. Membrane surface had numerous pores connected to each other (Fig. 1, *b*). Porous 3D structure of the implant is important for both cell attachment and stimulation of their *in vitro* growth, and for replacement regeneration of the bone tissue *in vivo* [3,11].

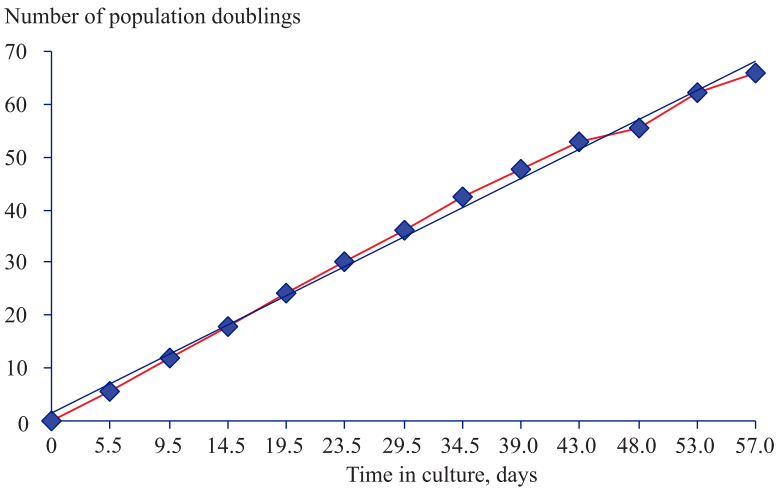

Fig. 3. Dependence of the number of population doublings of mouse MSC on the time of culturing on PHB scaffold. Symbols on the curve correspond to subculturing on the new scaffold. Straight line shows theoretical dependence of the number of MSC doublings on the time of their culturing without replicative senescence.

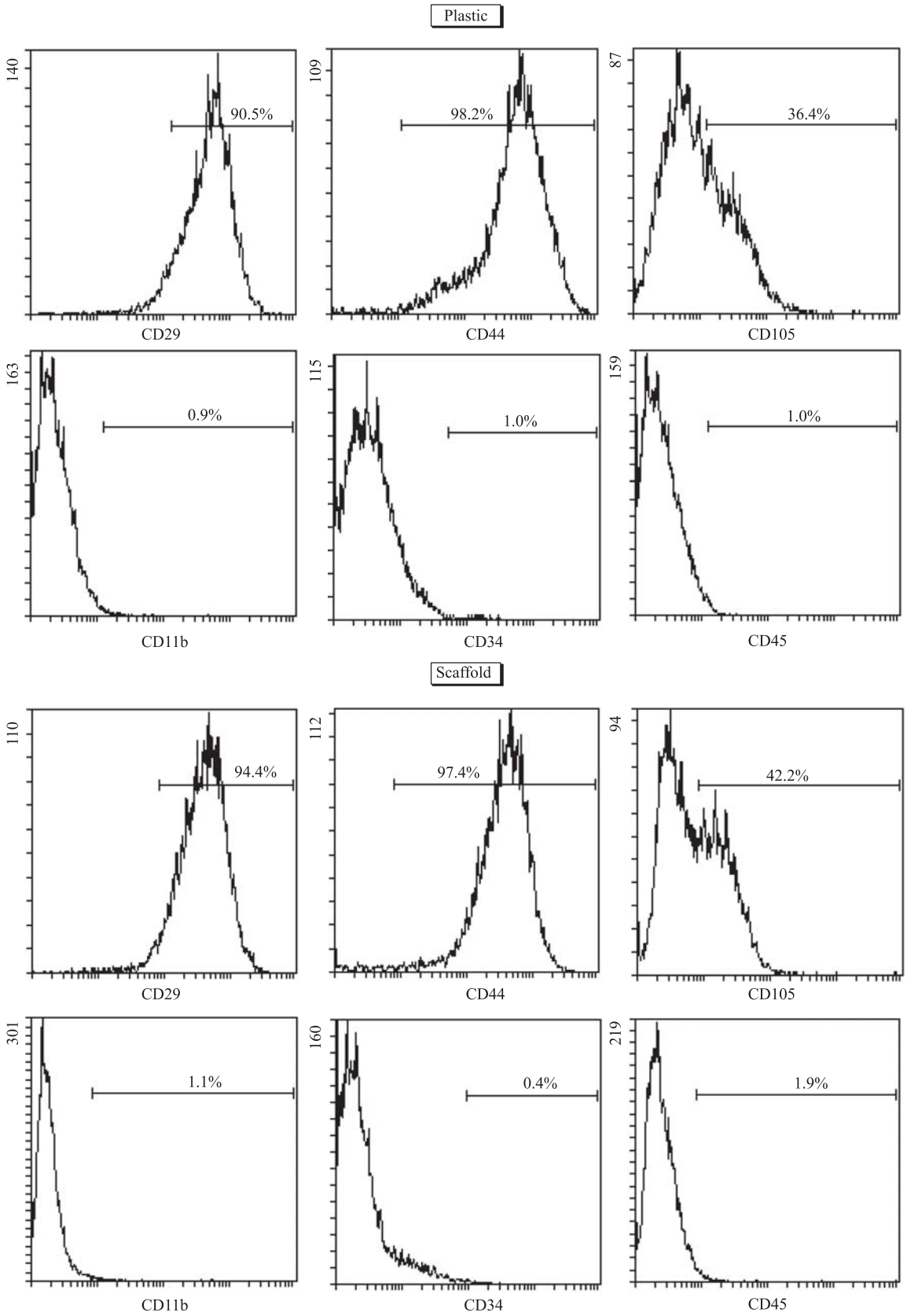

Fig. 4. Analysis of the surface phenotype of MSC cultured on plastic and PHB scaffold. Horizontal lines show the analyzed areas chosen from the cytometric profile of unstained cells. The percent of cells corresponding to the analyzed area. Abscissa: fluorescence intensity (logarithmic scale); ordinate: number of events.

The initial melting point of the membrane was 139°C and melting peak temperature 182°C (Fig. 2). The calculated crystallinity was 68%. These values correspond to the thermal characteristics obtained previously for PHB films [12], which can indicate minor influence of the method of pore formation with blowing agent on the physicochemical properties of the polymer. Hence, we can assume that biocompatibility of PHB polymer material was not significantly changed during fabrication of the polymer membrane.

For evaluation of the possibility of long-term MSC maintenance on the scaffold in culture, mouse cells were selected as the test object, because they are fastidious to culturing conditions than MSC from other species. In particular, mouse MSC, in contrast to human or rat MSC, require culturing under hypoxic conditions, because under normoxia they rapidly stop proliferating due to replicative senescence caused by oxidative stress [14,16]. The experiment was carried out under standard conditions for MSC culturing (DMEM with 10% FCS), but under hypoxic conditions (5% O_2). The cells were subcultured to new or regenerated scaffold every 4-6 days. The experiment was terminated after 2 months of constant culturing. The dependence of the number of population doublings on the time in culture is shown in Figure 3.

At the initial stages (~1 month), the rate of cell growth on the scaffolds was almost constant, but then it gradually decreased, which manifested in downward deflection of the curve from the straight line. This behavior is typical of the majority of primary cells, including MSC, and reflects the well-known phenomenon of replicative senescence of cells in the primary culture. During the experiment, the culture passed more than 65 population doublings. This attests to high efficiency of culturing on PHB scaffolds, because the number of doublings in culture for MSC from different species usually does not exceed 40-50.

For the analysis of properties of MSC cultured on scaffolds, the cells were cultured on scaffolds and plastic for 30 days (10 passages) under hypoxic conditions, and then their surface phenotype (expression of some most common positive and negative MSC markers) was determined. The patterns of expression of surface markers of cultured MSC corresponded to accepted standards (Fig. 4). In particular, the majority of MSC were stained with antibodies to CD29 and CD44 (positive markers), at the same time, staining for negative markers CD45, CD34, and CD11b was absent. Cultured cells were heterogeneous by CD105 marker, which was also consistent with the previous reports [7]. No significant differences between the control surface phenotype of MSC cultured on plastic on PHB scaffolds were revealed, and therefore, culturing on the scaffold did not change the basic properties of MSC.

Thus, 3D scaffold made of PHB is quite suitable substrate for long-term culturing of mouse MSC and can probably be used for culturing of human MSC.

The study was supported by the Russian Foundation for Basic Research (grant No. 14-04-01855).

REFERENCES

1. L V Antonova, A. Yu. Burago, V. G. Matveeva, *et al*., *Fundamental. Issled.*, No. 7, 17-23 (2013).
2. L. V. Antonova, M. V. Nasonova, Yu. A. Kudryavtsev, and A. S. Golovkin, *Byull. Sib. Med.*, **11**, No. 1, 128-134 (2012).
3. A. P. Bonartsev, G. A. Bonartseva, K. V. Shaitan, and M. P. Kirpichnikov, *Biomed. Khimiya*, **57**, No. 4, 374-391 (2011).
4. I. I. Zharkova, A. P. Bonartsev, A. P. Boskhomdzhiev, *et al., Biomed. Khimiya*, **58**, No. 5, 579-591 (2012).
5. V. L. Myshkina, E. A. Ivanov, D. A. Nikolaeva, *et al*., *Priklad. Biokhim., Microbiol.*, **46**, No. 3, 315-323 (2010).
6. V. L. Myshkina, D. A. Nikolaeva, T. K. Makhina, *et al*., *Priklad. Biokhim. Microbiol.*, **44**, No. 5, 533-538 (2008).
7. P. Anderson, A. B. Carrillo-Galvez, A. Garcia-Perez, *et al*., *PLoS One*, **8**, No. 10, e76979, doi: 10.1371/journal.pone.0076979 (2013).
8. A. M. Auriat, S. Rosenblum, T.N. Smith, and R. Guzman, Transl. *Stroke Res*., **2**, No. 3, 250-265 (2011).
9. F. Baron and R. Storb, *Cur. Opin. Hematol*., **14**, No. 2, 145-151 (2007).
10. S. Battista, D. Guarnieri, C. Borselli, *et al*., *Biomaterials*, **26**, No. 31, 6194-6207 (2005).
11. *Biomedical Polymers*, Ed. M. Jenkins, Birmingham (2007).
12. A. Bonartsev, S. Yakovlev, A. Boskhomdzhiev, *et al*., *PLoS One*, **8**, No. 2, e57200, doi: 10.1371/journal.pone.0057200 (2013).
13. A. P. Bonartsev, S. G. Yakovlev, I. I. Zharkova, *et al*., *BMC Biochem.*, **14**, doi: 10.1186/1471-2091-14-12 (2013).
14. S. V. Boregowda, V. Krishnappa, J. W. Chambers, *et al*., *Stem Cells*, **30**, No. 5, 975-987 (2012).
15. A. C. de Paula, A. A. Zonari, T. M. Martins, *et al*., *Tissue Eng. Part A.* , **19**, Nos. 1-2, 277-289 (2013).
16. V. Krishnappa, S. V. Boregowda, and D. G. Phinney, *Cytotherapy*, **15**, No. 5, 536-541 (2013).
17. T. H. Lee*, J. Exerc. Rehabil.*, **9**, No. 2, 230-233 (2013).
18. A. Zonari, S. Novikoff, N. R. Electo, *et al*., *PLoS One*, **7**, No. 4, e35422, doi: 10.1371/journal.pone.0035422 (2012).