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Growth of Mesenchymal Stem Cells on Poly(3-Hydroxybutyrate) Scaffolds Loaded with Simvastatin

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We studied the effect of porous composite scaffolds based on poly(3-hydroxybutyrate) (PHB) loaded with simvastatin on the growth and differentiation of mesenchymal stem cells. The scaffolds have a suitable microstructure (porosity and pore size) and physicochemical properties to support the growth of mesenchymal stem cells. Scaffold loading with simvastatin suppressed cell growth and increased alkaline phosphatase activity, which can attest to their osteoinductive properties.

Key Words: *poly(3-hydroxybutyrate); simvastatin; hydroxyapatite; scaffolds; osteoinductive*

Poly-3-hydroxybutyrate (PHB) is a biopolymer of bacterial origin, it is biocompatible, non-cytotoxic, and biodegradable [4], which makes it possible to use PHB for fabrication of scaffolds for cell growth. It has been shown that films made of poly-3-hydroxybutyrate promote the growth of mesenchymal stem cells (MSC) [6]. Much attention is attracted to 3D scaffolds from poly-3-hydroxybutyrate, which are capable of maintaining spatial cell growth due to their microstructure [3,14]. The need in scaffolds capable of maintaining spatial growth and differentiation of bone tissue cells is dictated by the fact that cells cannot fill the entire defect volume without porous 3D substrate.

However, the effects of PHB scaffolds of different structure and composition on the growth and differentiation of cells remain little studied. Simvastatin is mainly used as a cholesterol-lowering drug, but it is often applied for stimulation of bone growth and induction of MSC differentiation into osteoblasts. Gradual local release of simvastatin can ensure prolonged effect and help to select the necessary concentration to achieve the desired result [8,15]. Together with hydroxyapatite that strengthens the polymer backbone of the scaffold and acts as an osteogenesis inductor [1,12], simvastatin while loaded into the polymer material of the scaffold supports the differentiation of mesenchymal cells.

Our aim was to study the effect of POB-based scaffolds loaded with simvastatin and hydroxyappatite on the growth and differentiation of rat MSC.

MATERIALS AND METHODS

Materials. The polymer films and scaffolds were fabricated using microbiologically obtained PHB [4], simvastatin, hydroxyapatite, ammonium carbonate, and PBS (Merck). Biocompatibility *in vitro* was assessed in culture of MSC from Wistar rats; the cells were isolated from the adipose tissue of 3-day-old male rats [6]. The number of viable cells was evaluated using XTT Cell Proliferation Kit (Biological Industries).

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Fabrication of polymer films. Polymer films from PHB were fabricated by precipitation from a chloroform solution on glass Petri dishes. To obtain films loaded with simvastatin, simvastatin (1 and 5% w/w) was completely dissolved in a PHB solution and placed in a glass Petri dish until it completely dryed out.

Fabrication of polymer 3D scaffolds. Polymer scaffolds were fabricated by the leaching method using ammonium carbonate as a pore-forming agent. The PHB solution was mixed with hydroxyapatite (10%) and/or simvastatin (5%), and then this solution was placed in a Petri dish, mixed with ammonium carbonate, and after evaporation of the solvent washed with distilled water until ammonium carbonate was completely washed out.

Analysis of scaffold microstructure. Microphotographs of the scaffolds were obtained using a JSM-6510LV scanning electron microscope (Jeol) and a Nikon SMZ1500 light microscope (Nikon) [4].

The porosity (P, %) of the polymer scaffolds was calculated by the formula:

$$P=(1-D/d)\times 100\%,$$

where d is the theoretical density of the scaffold in the absence of pores (g/cm^3) and D is actual density calculated by the formula:

$$D=m/h\times l\times w,$$

where m is the weight of the scaffold (g), and h, l, and m are its height (or thickness), length, and width (cm).

The density of PHB is 1.243 g/cm^3. The weight was measured on an AL-64 laboratory scales (Acculab).

An ink soak test was used to determine the interconnection of pores.

Study of mechanical properties. Mechanical properties of dry samples were studied on an Anton Paar MCR 302 rheometer equipped with a plate-plate measuring system. Typical thickness of the studied samples was 3-4 mm, diameter 2.5 cm. Young's modulus was measured by sample compression. The dependence of normal pressure on the relative deformation was plotted. Then, Young's modulus was calculated from the slope of the linear portion of the graph.

Drug release. To analyze the prolonged release of simvastatin, porous PHB scaffolds containing 5% simvastatin (w/w; 3 scaffolds per point) were placed in 10 ml PBS and incubated in a thermostat at 37°C for 14 days. A non-porous film without the drug was used as a control. Each time after measuring the amount of released drug, the scaffolds were placed in a fresh PBS solution. The amount of released simvastatin was determined spectrophotometrically at 238 nm (Nanodrop, Thermo Fisher Scientific). The calculation of the final stage of drug release was checked using the first-order Fick mathematical model.

The first-order model was used to describe drug release. The kinetics is described by the equation:

$$\partial C/\partial t=-KC,$$

where C is the concentration of the drug substance and K is the first-order kinetic constant of drug release.

Considering Fick's first law, you can get a relationship for the constant K:

$$D/Vh=K,$$

where D is diffusion coefficient in partition medium, V is the volume of the release medium, and h is the thickness of the diffusion layer.

Consequently, the first-order model is expressed as follows:

$$M_t/M_o=1-k_3\exp(-kt),$$

where $k_3=e^k$ is the factor in front of the exponential, k is the value inverse to the decay time [7,9].

Evaluation of cell growth. Evaluation of cytotoxicity and study of the growth of MSC on the obtained films and scaffolds were carried out. MSC obtained by the method described earlier [6] were cultured in standard DMEM medium supplemented with 10% fetal bovine serum and 1% antibiotic (penicillin+streptomycin) until passage 3. To evaluate the cytotoxicity of the material, MSC were seeded into a 96-well plate (2000 cells per well). After 24-h incubation, cell adhesion was checked, the medium was changed, and the polymer samples previously sterilized with 96% ethanol for 1 h was placed into the well. Cell growth in the presence of the sample was determined by the standard XTT method (XTT Cell Proliferation Kit, Biological Industries) on days 1, 3, and 7.

To evaluate cell growth on films and scaffolds, the samples were sterilized with 96% ethanol, cut to well size, and placed in a 96-well plate. MSC were seeded on films and scaffolds at a rate of 2000 cells per well. Cell growth was assessed by the standard XTT method on days 1, 3, and 7.

Evaluation of alkaline phosphatase activity. Before the experiments, the cells were phenotyped by flow cytometry using antibodies to surface markers CD45 and CD90 as described previously [5]. The culture medium for osteogenic differentiation of MSC was prepared on the basis of DMEM containing 10% fetal bovine serum (Biological Industries), 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM dexamethasone (KRKA), 10 mM β-glycerophosphate (Merck), and 50 mM ascorbic acid (Merck) [13]. For measuring alkaline phosphatase activity, MSC were seeded to polymer scaffolds samples with a diameter of 1.5

cm at a rate of 7000 cells per sample. On days 7, 14 and 28, samples with growing cells were washed 2 times in PBS, placed in lysis buffer (250 mM NaCl, 0.1% Triton X-100, 50 mM HEPES; pH 7.5), and subjected to 3 freezing/thawing cycles. Then, the samples were centrifuged for 10 min at 10,000 rpm and alkaline phosphatase activity was measured. To this end, 100 µl lysate and 50 µl reagent (15 mM n-nitrophenyl phosphate (Merck) and 2 mM MgCl; pH 10) were added to the wells of a 96-well plate, incubated in a thermostat for 2 h, and optical density was measured at 405 nm.

Statistical analysis. The results were processed statistically using Prism 6 software. Normality of distribution of the obtained data was verified using the Shapiro—Wilk test. The data were analyzed using Tukey's test for multiple comparisons. The rusults are presented as $M \pm SEM$. The differences were significant at $p<0.05$.

RESULTS

Polymer PHB films without and with 1 and 5% simvastatin were prepared (Fig. 1, *a*). Scanning electron microscopy showed that the film surface was smooth and without roughness (Fig. 1, *b*).

Cell growth on films. The growth of MSC on films containing 1 and 5% simvastatin was studied. Although the increase in simvastatin content in the polymer composition slightly decreased cell growth, the difference in the number of cells was insignificant (Fig. 1, *c*), and in further experiments, we used films containing 5% simvastatin. Hydroxyapatite, an inevitable component of the bone tissue commonly used for fabrication of scaffolds for bone tissue engineering [12], was also added to the scaffold together with simvastatin that supports MSC differentiation.

3D porous scaffolds consisting of PHB alone and PHB with hydroxyapatite simvastatin (10 and 5% w/w; PHB/HA/Sim) had rigid structure, were not fragile, and retain their shape (Fig. 2, *a*). All types of scaffolds have interconnecded pore system; ink-wetting method revealed no closed pores looking like white areas (Fig. 2, *b*). All porous scaffolds had irregular 3D porous structure (Fig. 2, *c*, *d*).

Porosity. The pore size of natural bone is on average 50-100 µm [2], which is close to the experimental vaues obtained by us (Table 1). The porosity of natural

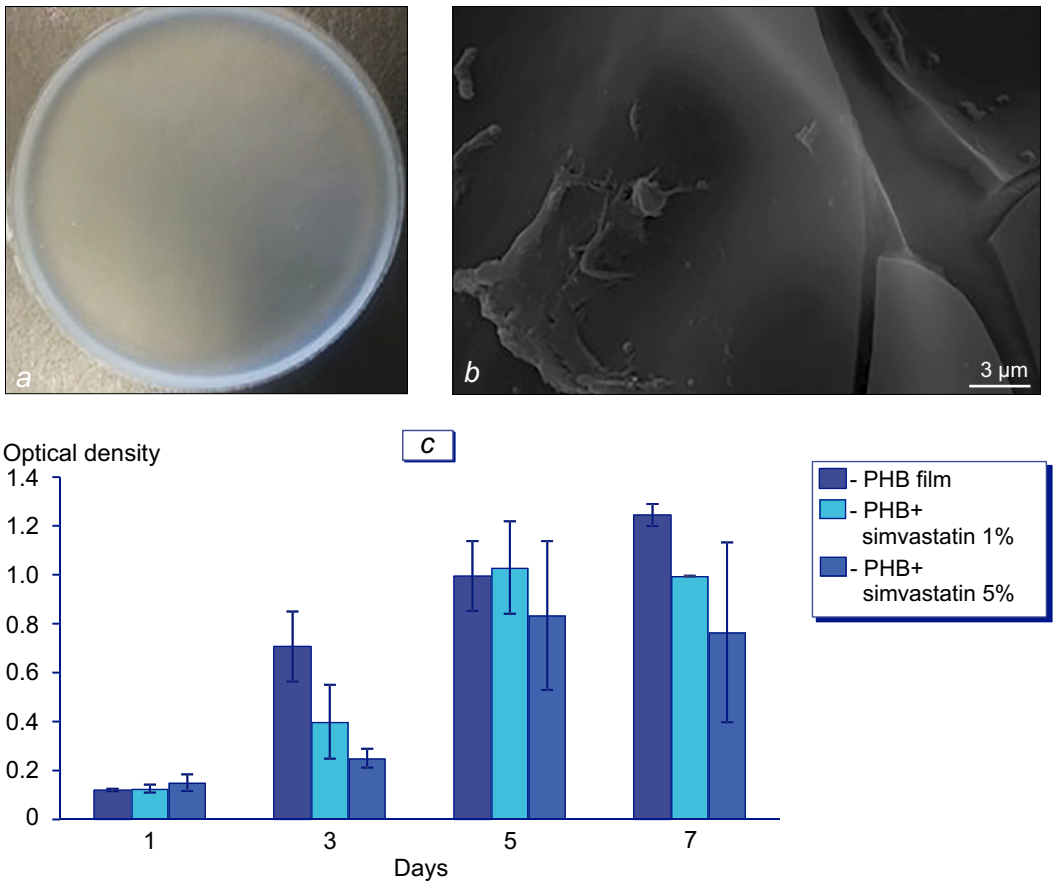

Fig. 1. Photograph (*a*) and microphotograph (*b*) of the film; cell growth on films with the inclusion of simvastatin (1 and 5% w/w) (*c*). Scanning electron microscopy, ×2500 (*b*).

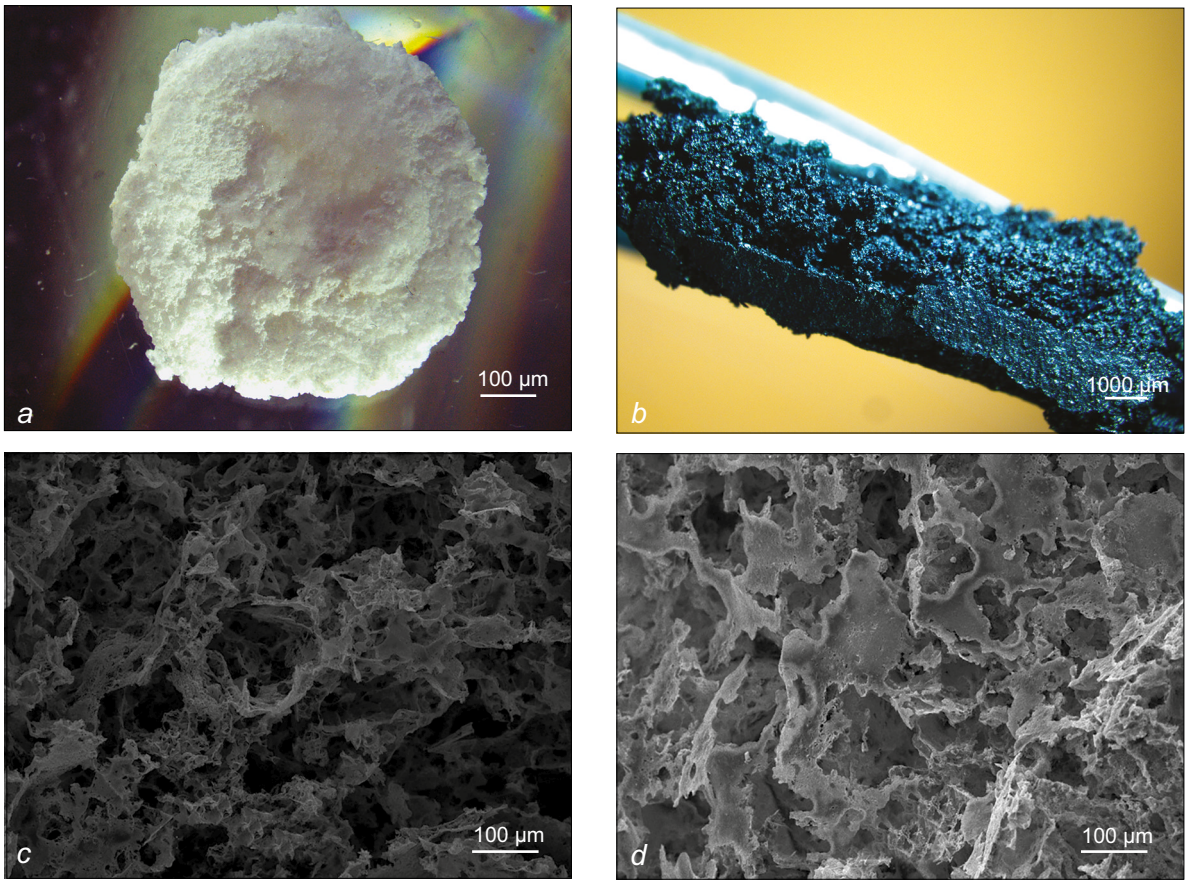

Fig. 2. Microstructure of PHB scaffolds with inclusion of hydroxyapatite and simvastatin (PHB/HA/Sim). Wide-field light microscopy, ×20 (*a*, *b*), scanning electron microscopy, ×150 (*c*, *d*). *a*) PHB/HA/Sim scaffold; *b*) PHB/HA/Sim scaffold, ink-wetting test; *c*) microstructure of PHB scaffold; microstructure PHB/HA/Sim scaffold.

bone is 80% [11], which is also close to the obtained values of scaffolds porosity. It was shown that higher porosity promotes cells migration into the sample [5,10]. The obtained samples were tested for rigidity by the method of sample compression. Samples containing hydroxyapatite showed higher values in Young's modulus, which indicates their greater rigidity.

Simvastatin release from the scaffolds. The intensity of drug release was high in the initial phase (starting from day 3) and attained a "plateau" during further incubation (Fig. 3, *a*). As can be seen from the kinetic curve of drug release, so-called "burst effect" of drug release on the first day was not obsereved, its release begins from the third day and corresponds to the kinetics described by the mathematical model of the first order release.

There are many therapeutic systems that display this type of release of soluble drugs included in porous scaffolds, when the amount of drug released is proportional to the amount of drug remaining in the scaffold. Thus, the amount and proportion of actively released drugs tended to decrease with time. The first-order model is more suitable for describing the final stage of drug release, because it describes the late stage of Fick diffusion [9] (Fig. 3, *b*).

Cell growth on films and scaffolds. For evaluatin of the cytotoxicity of polymeric porous scaffolds, MSC were cultured in the presence of scaffolds for a week. Hydroxyapatite and simvastatin included into the

TABLE 1. Porosity of Obtained Scaffolds (*M*±*m*)

Scaffold composition	Porosity, %	Pore sizes, μl	Young's modulus, kPa
PHB	93±5	116±26	8.7±0.1
PHB/HA/Sim	89±7	102±23	36.6±0.6*

Note. *$p<0.05$. Films are non-porous and are not included in the Table.

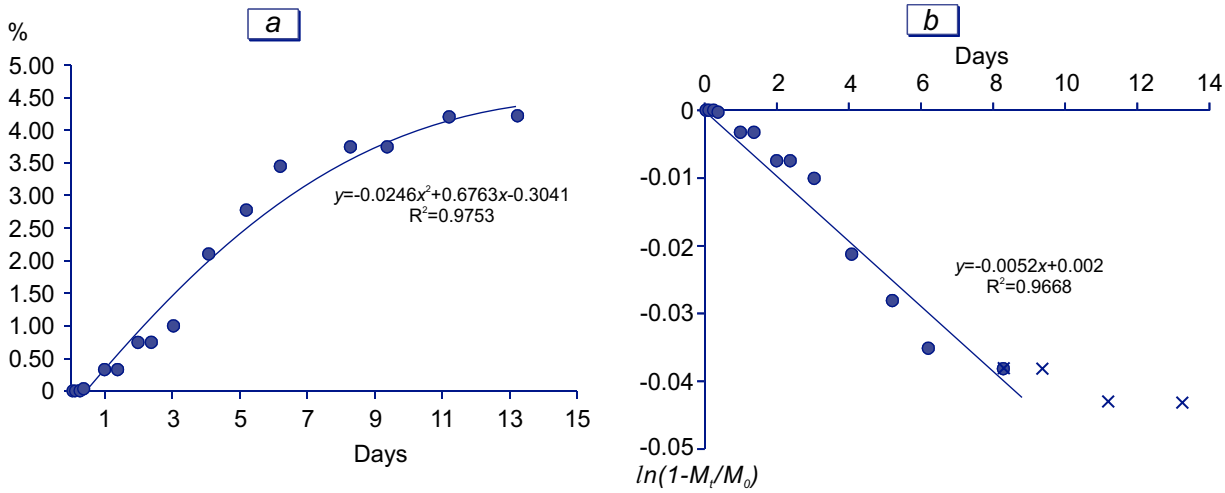

Fig. 3. Simvastatin release from porous PHB scaffolds including 5% simvastatin (*a*) and approximation of the kinetics of simvastatin release from scaffolds according to the first order model (*b*).

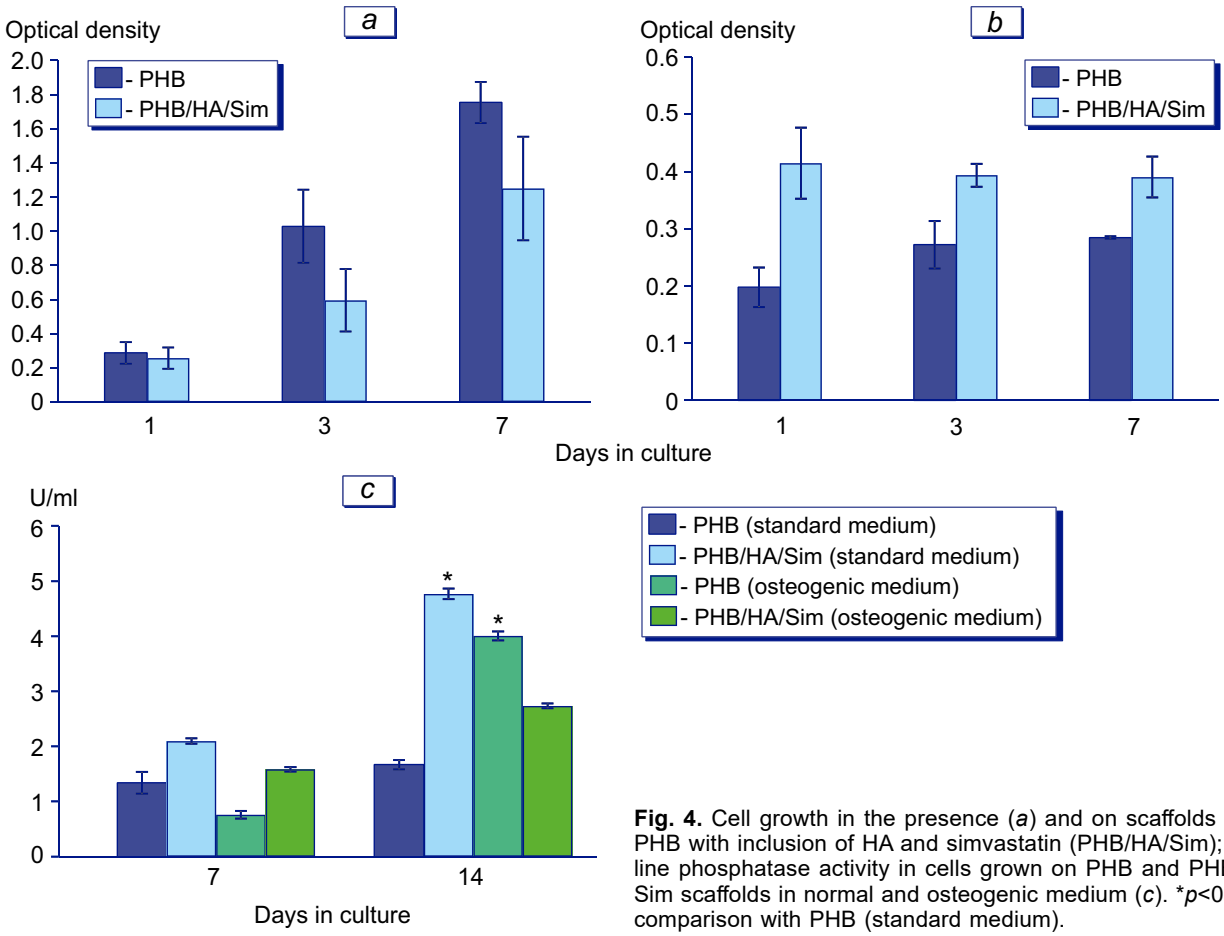

Fig. 4. Cell growth in the presence (*a*) and on scaffolds (*b*) of PHB with inclusion of HA and simvastatin (PHB/HA/Sim); alkaline phosphatase activity in cells grown on PHB and PHB/HA/Sim scaffolds in normal and osteogenic medium (*c*). **p*<0.05 in comparison with PHB (standard medium).

scaffold slightly inhibited, but not arrested cell growth (Fig. 4, *a*). The observed growth inhibition can be explained by MSC differentiation under the action of simvastatin and hydroxyapatite released into the medium.

For evaluation of the biocompatibility of porous scaffolds, MSC were cultured on the scaffolds containing hydroxyapatite and simvastatin (Fig. 4, *b*). PHB scaffold is capable of maintaining the growth and proliferation of cells. However, when hydroxyapatite and simvastatin were included into the scaffold, intensive cell growth does not occur and the number of living cells does not decrease. This can also be explained by

differentiation of MSC on scaffold under the influence of hydroxyapatite and simvastatin and as we can see from the kinetics of drug release was actively released from the scaffolds on the third day.

Alkaline phosphatase activity in cell growth on scaffols. Before the experiments, MSC were tested for osteomarkers CD45 and CD90 and corresponded by their profile to undifferentiated MSC [5]. On day 14, incubation in an osteogenic medium has a significant effect on MSC cultured on scaffolds (Fig. 4, *c*). During culturing in osteogenic medium, activity of alkaline phosphatase increased in cells grown on PHB scaffolds, but significantly decreased in cells grown on scaffolds containing simvastatin. The type of substrate also had a strong influence on enzyme activity in cells. In standard medium, addition of hydroxyapatite and simvastatin to PHB almost 3-fold increased activity of alkaline phosphatase in cells, while in osteogenic medium, the process of osteogenic differentiation on this scaffold was inhibited compared to cell growth on a PHB scaffold without additives, which can be explained by the initiation of cell differentiation.

Thus, simvastatin included into scaffolds inhibited cell growth and increased activity of alkaline phosphatase, which may indicate their osteoinductive properties.

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REFERENCES

1. Bonartsev AP, Muraev AA, Deyev RV, Volkov AV. Material-Associated Bone Resorption. Sovremenn. Tekhnol. Med. 2018;10(4):26-33. doi: 10.17691/stm2018.10.4.03. Russian.
2. Matveychuk IV, Rozanov VV, Litvinov YuYu. Evaluation of biophysical properties of the bone tissue for biomedical applications. Al'manakh Klin. Med. 2016;44(2):193-202. doi: 10.18786/2072-0505-2016-44-2-193-202. Russian.
3. Muraev AA, Bonartsev AP, Gazhva YuV, Riabova VM, Volkov AV, Zharkova II, Stamboliev IA, Kuznetsova ES, Zhu kov VA, Myshkina VL, Mahina TK, Bonartseva GA, Yakovlev SG, Kudryashova KS, Voinova VV, Mironov AA, Shaitan KV, Gazhva SI, Ivanov SYu. Development and Preclinical Studies of Orthotopic Bone Implants Based on a Hybrid Construction from Poly(3-Hydroxybutyrate) and Sodium Alginate. Sovremenn. Tekhnol. Med. 2016;8(4):42-50. doi: 10.17691/stm2016.8.4.06. Russian.
4. Bonartsev AP, Yakovlev SG, Myshkina VL, Mahina TK, Zernov AL, Zhuikov VA, Akoulina EA, Bonartseva GA, Zharkova II, Voinova VV, Ivanova EV, Kuznetsova ES, Shaitan KV. Biosynthesis of poly(3-hydroxybutyrate) copolymers by azotobacter chroococcum 7B: a precursor feeding strategy. Preparative Biochem. Biotechnol. 2017;47(2):173-184. doi: 10.1080/10826068.2016.1188317
5. Bonartsev AP, Zharkova II, Voinova VV, Kuznetsova ES, Zhuikov VA, Makhina TK, Myshkina VL, Potashnikova DM, Chesnokova DV, Khaydapova DD, Bonartseva GA, Shaitan KV. Poly(3-hydroxybutyrate)/poly(ethylene glycol) scaffolds with different microstructure: the effect on growth of mesenchymal stem cells. 3 Biotech. 2018;8(8). doi: 10.1007/s13205-018-1350-8
6. Bonartsev AP, Zharkova II, Yakovlev SG, Myshkina VL, Makhina TK, Zernov AL, Kudryashova KS, Feofanov AV, Akulina EA, Ivanova EV, Zhuikov VA, Volkov AV, Andreeva NV, Voinova VV, Bonartseva GA, Shaitan KV, Kirpichnikov MP. Adhesion and growth of bone marrow mesenchymal stem cells on 3D scaffolds from poly(3-hydroxybutyrate)-poly(ethylene glycol) copolymer for tissue engineering. J. Biomater. Tissue Engin. 2016;6(1):42-52. doi: 10.1166/jbt.2016.1414
7. Bruschi ML. Mathematical models of drug release. Strategies to Modify the Drug Release from Pharmaceutical Systems. Bruschi ML, ed. Woodhead Publishing, 2015. P. 63-86.
8. Chou J, Ito T, Bishop D, Otsuka M, Ben-Nissan B, Milthorpe B. Controlled release of simvastatin from biomimetic β-TCP drug delivery system. PLoS One. 2013;8(1):e54676. doi: 10.1371/journal.pone.0054676
9. Dash S, Murthy PN, Nath L, Chowdhury P. Kinetic modeling on drug release from controlled drug delivery systems. Acta Pol. Pharm. 2010;67(3):217-223.
10. Ikeda R, Fujioka H, Nagura I, Kokubu T, Toyokawa N, Inui A, Makino T, Kaneko H, Doita M, Kurosaka M. The effect of porosity and mechanical property of a synthetic polymer scaffold on repair of osteochondral defects. Int. Orthop. 2009;33(3):821-828. doi: 10.1007/s00264-008-0532-0
11. Karageorgiou V, Kaplan D. Porosity of 3D biomaterial scaffolds and osteogenesis. Biomaterials. 2005;26(27):5474-5491. doi: 10.1016/j.biomaterials.2005.02.002
12. Ramesh N, Moratti SC, Dias GJ. Hydroxyapatite-polymer biocomposites for bone regeneration: A review of current trends. J. Biomed. Mater. Res. B Appl. Biomater. 2018;106(5):2046-2057. doi: 10.1002/jbm.b.33950
13. Shakibaei M, Shayan P, Busch F, Aldinger C, Buhrmann C, Lueders C, Mobasheri A. Resveratrol mediated modulation of Sirt-1/Runx2 promotes osteogenic differentiation of mesenchymal stem cells: potential role of Runx2 deacetylation. PLoS One. 2012;7(4):e35712. doi: 10.1371/journal.pone.0035712
14. Voinova V, Bonartseva G, Bonartsev A. Effect of poly(3-hydroxyalkanoates) as natural polymers on mesenchymal stem cells. World J. Stem Cells. 2019;11(10):764-786. doi: 10.4252/wjsc.v11.i10.764
15. Volkov AV, Muraev AA, Zharkova II, Voinova VV, Akoulina EA, Zhuikov VA, Khaydapova DD, Chesnokova DV, Menshikh KA, Dudun AA, Makhina TK, Bonartseva GA, Asfarov TF, Stamboliev IA, Gazhva YV, Ryabova VM, Zlatev LH, Ivanov SY, Shaitan KV, Bonartsev AP. Poly(3-hydroxybutyrate)/hydroxyapatite/alginate scaffolds seeded with mesenchymal stem cells enhance the regeneration of critical-sized bone defect. Mater. Sci. Eng. C Mater. Biol. Appl. 2020;114:110991. doi: 10.1016/j.msec.2020.110991