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Osteoinductive Properties of PLGA/HA/β-TCP Composite Polymer Scaffolds

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Abstract: The composite polymer poly(lactide-co-glycolide)(PLGA)/β-tricalcium phosphate(β-TCP)/hydroxyapatite (HA) was designed to ensure efficient bone tissue bioengineering. It was synthesized using microwave radiation and hydrothermal conditions. Polymer scaffolds were produced using an electrospinning method. PLGA had ratio of monomers - 80/20. The aim of this investigation is to study the osteoinductive properties of newly sintezed scaffolds. Osteoinductive properties were revealed by assessing the appearance of differentiation markers in mesenchymal stem cells (MSCs) during *in vitro* growth on the scaffolds. The non-transformed human MSCs line – FetMSC was used. Real-time PCR revealed the increasing of Runx2 and YAP1 expression on 14 days after cultivation human FetMSCs on PLGA/HA/β-TCP scaffolds. By means of fluorescence microscopy it was shown that transcription factors Runx2, Osterix and YAP1 expression was higher with the growth of cells on the PLGA/HA/β-TCP composite scaffolds than in negative control and increased in the process of culture of cells during 14 days. Calcification was revealed histochemically using staining by Alizarin red in cells which were grown on the surfaces of both types of PLGA scaffolds in basal medium at the 28th day of cultivation. On the PLGA/HA/β-TCP scaffolds staining was detected earlier (at the 21st day). Osteoinductive activity of PLGA/HA/β-TCP composite scaffolds is considered to be established.

***Keywords*:** Bone tissue engineering, Human bone marrow MSCs, PLGA-copolymer of lactide (l- or dl-form) and glycolide, β-tricalcium phosphate, Hydroxyapatite.

1. Introduction

Bone transplantation is gradually shifting from using of natural transplants to synthetic bone tissue substitutes and biological factors [1, 2]. Nowadays there is a necessity to develop materials which will have optimal mechanical properties and will influence on the cell proliferation and differentiation in osteogenic direction. Osteoinduction process, i.e. the recruitment of immature cells and the stimulation of these cells to develop into preosteoblasts, plays an important role in the initial part of the healing response, that starts immediately after the injury and is very active during the first week [3]. Thus it is important to study whether new material possesses osteoinductive characteristics [4].

Polyhydroxy ester-based scaffolds, including copolymers of lactide and glycolide, have long attracted attention of researchers with aim of developing materials for bone engineering [5]. However, despite their biocompatibility and mechanical properties, the clinical use of pure PLGA for bone regeneration is difficult because of its poor adhesiveness for cells and not always reproducible osteoconductiveness. In order to improve their suitability for use in bone engineering, calcium phosphates are added to the composition, which give the material of hardness and have good biocompatibility, due to its chemical and crystalline similarity to the mineral component of bone [6]. A method of synthesizing a new material with desired properties was developed. As a result, a composite scaffold consisting of a copolymer of lactide (l- or dl-form) and glycolide (80/20), hydroxyapatite (HA), tricalcium phosphate in the β-modification (β-TCP) was created. This material had a fibrous structure and was intended for stromal cells adhesion. It was assumed that it will exhibit osteoinductive properties. Previously, a study of similar material, differing only in fiber

absence and the ratio of PL and GA (50/50), was tested on the rat MSCs. There was shown its biocompatibility and biodegradability *in vivo* and osteoinduction (calcification under the influence of osteogenic inducers) *in vitro* [7].

This study based on revealing of key genes responsible for stem cells osteogenic differentiation. It is known that during the early stages of osteogenic induction expression of genes-transcription factors Runx2 (cbfa-1), Osterix (Osx, which is also known as Sp7), YAP1 (yes-associated protein 1) (which is also known as YAP or YAP65) and anothers in stem progenitor cells is stimulated. [8, 9, 10]. The process of differentiation ends by forming of extracellular matrix and their mineralization (deposition of calcium salts in matrix [11].

The aim of this investigation is to study of the osteoinductive properties of new PLGA/HA/β-TCP composite scaffold by means assessing the appearance of osteogenic differentiation markers in human mesenchymal stem cells (hMSCs) during cultivation *in vitro*.

2. Materials and Methods

PLGA scaffolds were prepared by a wet forming method proposed by Morgan et al. [12] in our modification. The main stages of laboratory technique of composite matrix-scaffold fabrication are: a) dissolving and mixing; b) polymerization and molding; c) freezing and drying. The copolymer poly(D,L-lactide-co-glycolide) was dissolved using magnetic stirrer Biosan MSH-300 (Latvia), hydroxyapatite and β-tricalcium phosphate were added under constant stirring. The resulting solution was placed into a coagulation bath filled with deionized water for homogeneous layer formation, then the plate was prepared via molding and after that samples of matrix-scaffold were extruded using punch. The obtained scaffold samples were placed in container in dewar with liquid nitrogen for freezing, after 60 minutes the samples were transferred into vacuum drying cabinet LT VO-90 Labtex (Russia) for drying and specimens were dried at a temperature of 47°C and at a pressure of 1 bar until constant weight. The biphasic HA/β-TCP ceramic was synthesized using microwave radiation and hydrothermal conditions. Composite scaffolds with different material compositions were produced using an electrospinning method (PLGA has ratio of monomers 80/20).

The ability to osteogenic differentiation during growth on scaffolds *in vitro* was studied using non-transformed cell line FetMSC (Vertebrate Cell Culture Collection, Institute of Cytology, Russian Academy of Sciences), derived from stromal cells of a 5-6 week embryo`s bone marrow. Cell line was characterised according all the parameters which are the minimal requirements of International Society for Cellular Therapy, which determine its belonging to mesenchymal stem cells (MSCs) [13]. It was revealed that: 1) adhesion to plastic of standard culture dishes occurred; 2) the clusters of differentiation CD73, CD90 и CD105 expressed; 3) the absence of CD34, HLA-DR expression was observed; 4) the ability to differentiate in osteogenic and adipogenic direction *in vitro* was confirmed. Karyotypic analysis, which was carried out at the 12-14 passage of cultivation, showed normal karyotype: 46, XY [14]. FetMSC were cultured in DMEM/F12 (Rosmedbio, Russia) containing 10% fetal bovine serum (HyClone, США), 1% antibiotic penicillin-streptomycin (Gibco, USA) and 1% glutamine (Lonza, Switzerland). The ability of FetMSC to osteogenic differentiation was studied *in vitro* during growth on PLGA/HA/β-TCP composite scaffold. FetMSC cell line (4-5th passage after thawing) was seeded on surface of samples (scaffolds), putted into the wells of 24-well plate in the amount of 10^5 per sample and cultured in standard conditions. As a positive control cells which were growing on the standard culture surface in osteogenic differentiation medium were used at the same time as the cells which were growing on the composite matrices. PLGA scaffold (without fillers) or standard cultural surface were used as a negative control.

Real-time polymerase chain reaction. RNA from the samples of human mesenchymal stem cells was extracted using "Lira" reagent (Biolabmix, Russia), which contains phenol and guanidine thiocyanate. Reverse transcription was carried out using OT M-MuLV–RH reagent kit (Biolabmix, Russia). Specific PCR-products were obtained using self-designed primers to *PPIA* (reference gene), *RUNX2* and *YAP1* genes (Table 1). Real-time PCR was conducted using AriaMx amplifier (Agilent Technologies, USA).

Table 1: Primers for real-time PCR.

Gene	Forward primer	Reverse primer	Product size, bp
PPIA	AGACAAGGTCCCAAAGAC	ACCACCCTGACACATAAA	118
RUNX2	ACCGAGACCAACAGAGTCATTTA	GTGTCACTGTGCTGAAGAGG	119
YAP1	AACAGCAAGAACTGCTTCGG	GCAGGGCTAACTCCTGACAT	84

Fluorescence microscopy. Investigation of Osterix, Runx2 and YAP1 transcription factors accumulation in human MSCs was carried out via indirect immunofluorescent labeling with an antibody and visualisation of staining. Composite matrices loaded with cells were cultured in 48-well plate until the analysis (at the 7th and 14th days). At the end of

cultivation period the samples were fixed with 10% formalin and incubated with primary antibodies to RUNX2, Sp7 / Osterix and YAP1 (Abcam, UK), and after that with secondary fluorescent antibodies. Study of fluorescence had been conducted using inverted confocal microscope ZOE™ Fluorescent Cell Imager (Bio-Rad, USA). Quantitative investigation of the fluorescence intensity was carried out via analysis of images with ImageJ 1.51j8 software, and nonparametric statistical methods (the Mann-Whitney U test).

Histochemical detection of the calcification during the osteogenic differentiation was performed at the 21st and 28th days after the seeding the cells on the composite matrices. At the end of the cultivation the samples were transferred into new well-plate, fixed with 10% formalin, stained with 2% alizarin red solution.

3. Results and Discussion

Runx2 (cbfa-1) and Osterix transcription factors are the key regulators of osteogenesis at a molecular-genetic level. Runx-2 is activated under the influence of TGF-β1 and BMP-2, binds with specific cis-acting elements of osteoblasts and controls genes expression, which code the main proteins of osseous matrix, for example, osteocalcin. Osterix (Osx, which is also known as Sp7) is the second transcription factor, which is absolutely required for osteoblasts differentiation. Osterix takes part in Runx2-independent osteogenesis regulation pathways and acts at later stages of differentiation, than Runx2. YAP1 (yes-associated protein 1), which is also known as YAP or YAP65, is a transcription regulator protein, which activates transcription of genes involved in cells proliferation [10]. YAP1 was identified as functional partner of Runx and αA1 as an osteocalcin transcription coactivator during osteogenesis.

Using real-time PCR it was revealed that *RUNX2* gene expression in the FetMSC cultured on the PLGA/HA/β-TCP composite scaffolds was in 5.5 times higher at 14 days than it was on standard cultural surface (negative control). Expression of gene *YAP1* was similar and it was 2-4 times higher than in negative control.

Analysis of fluorescent revealing of early osteogenesis transcription factors Runx2, Osterix and YAP1 was revealed that during FetMSC line cultivation on PLGA matrices, transcription factors were expressed in cells at the 7th and 14th days (Table 2). Wherein during the growth of FetMSC on composite matrices PLGA/β-TCP(HA) significant increasing of Runx2 and YAP1 production at the 14th day was revealed in comparison with PLGA-matrices.

Table 2: Relative fluorescence of transcription factors during the in vitro cultivation of FetMSC on the composite matrices surfaces.

	Relative fluorescence, %, Mean ± Standard Error of Mean, M±m					
	Runx2		***Osterix***		***YAP1***	
	7 days	*14 days*	*7 days*	*14 days*	*7 days*	*14 days*
Negative Control	0.12±0.05	0.21±0.03	0.09±0.04	0.18±0.05	0.12±0.04	0.15±0.02
PLGA	7.31±1.01*	5.93±1.13*,**	8.56±1.91*,**	10.10±1.91*,**	3.01±0.32*	4.44±0.73*,**
PLGA/HA/β-TCP	8.88±1.35*	10.30±1.80*	2.88±0.31*	4.80±0.62*	3.26±0.60*	7.83±1.34*

Notes: PLGA – polylactide glycolide, PLGA/HA/β-TCP – samples from PLGA, which contain β-TCP and HA. Negative control – the surface of the composite matrix without cells.

* – the differences are statistically significant comparing with the group "negative control" (the Mann-Whitney U-test, $p < 0.05$); ** – the differences are statistically significant comparing with PLGA/HA/β-TCP (the Mann-Whitney U-test, $p < 0.05$).

It was found that the expression (relative fluorescence) of all studied transcription factors increases the cells during cultivation (up to 14 days) on PLGA/HA/β-TCP composite scaffolds. In the case of Runx2 – in 1.16, Osterix - 1.67, and most of all - YAP1 – 2.4 times. Also the expression of the factors Runx2 and YAP1 was higher than in the negative control (PLGA) – in 1.7 times over a period of 14 days. Unusually, Osterix expression on composite scaffolds was lower than on pure scaffolds. It is not yet clear what this may be connected with. It is known that Osterix and Runx2/YAP1 are activated on two alternative pathways of osteogenic differentiation. It is possible that there are molecules in the composition of scaffolds that switch cells to use on another differentiation mechanism.

Differentiation of MSCs into osteogenic cells is accompanied by calcium ions accumulation in cytoplasm at first and further in extracellular matrix [15]. Because of it, detection of local calcium salts deposition in extracellular matrix (its mineralization) is an evidence of osteogenic differentiation. Evaluation of calcifications formation during directed FetMSC differentiation was carried out using staining with Alizarin red solution, because it forms insoluble bright coloured varnishes in conjunctions with salts of divalent metals, which let to determine local deposition of calcium salts. The method of calcifications revealing during differentiation into osteocytes is generally accepted for MSCs characterization and is used actively now [16].

The study of calcifications formation was performed at the 21st and 28th days of FetMSC cultivation on composite scaffolds *(Figure)*. Cultivation was performed in growth medium to reveal supposed osteoinductive effect of matrices chemical components. During the cultivation of cells on both types of scaffolds the number of calcifications increases. Dynamics of calcifications appearance was different. During the growth of cells on PLGA/HA/β-TCP staining appeared after 21 days of cultivation, while on PLGA it was not observed at that time. After 28 days the staining was revealed on both types of scaffolds, but on matrix with PLGA/HA/β-TCP it was higher (Figure). This may evidence that the pure polymer itself has some osteoinductive properties. Calcium phosphate addition to it accelerates the process of differentiation.

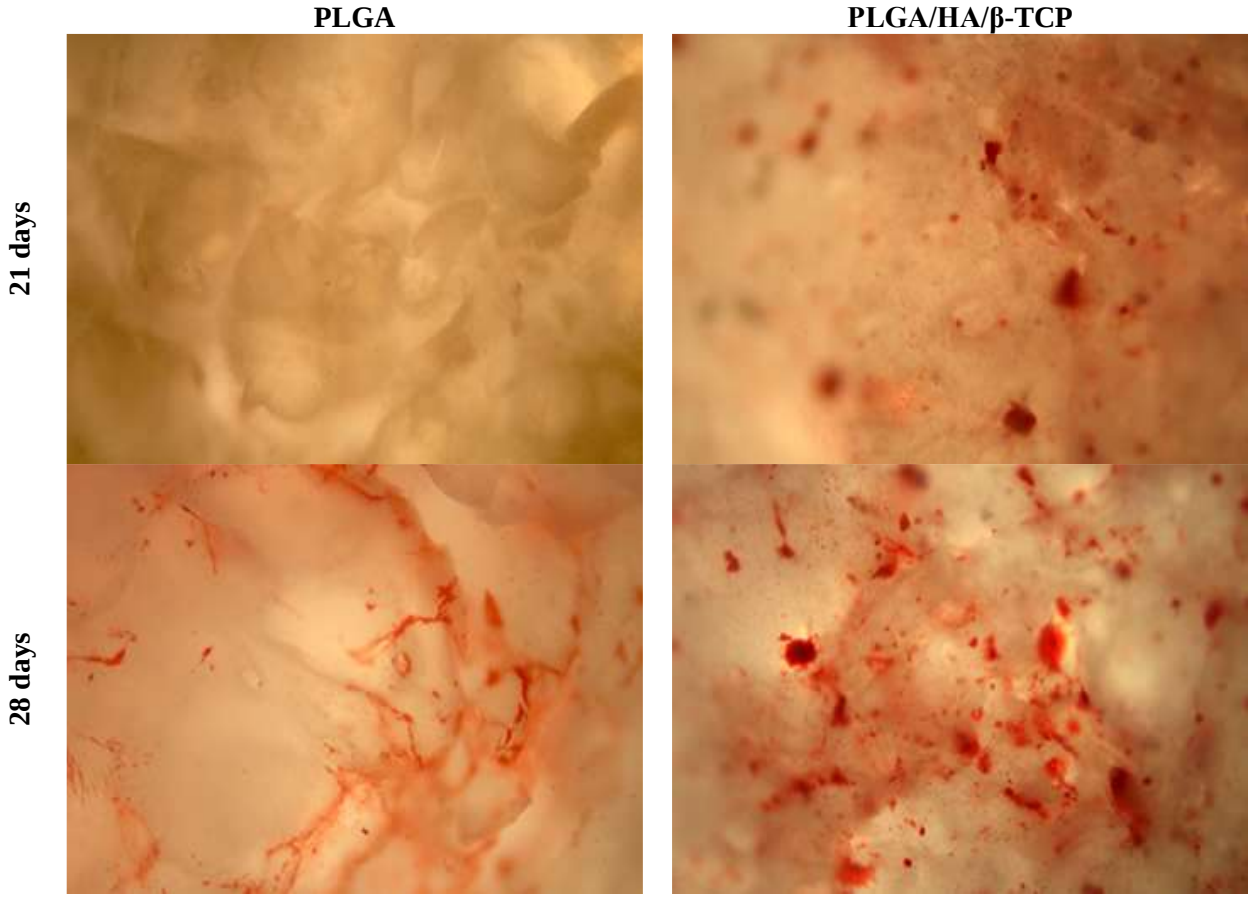

Fig. 1: FetMSC during the growth on the composite scaffold surfaces with various composition in basal growth medium. Staining with Alizarin Red. 40x magnification.

4. Conclusion

This paper presents the results of a study in which for the first time the osteinductive properties of a new material based on PLGA and including on the original calcium phosphate composite (HA and β-TCP) was studied. Activation of transcription factors Runx2, Osterix and YAP1 was detected in the early stages of differentiation using real-time PCR and fluorescence microscopy. The effectiveness of the process of osteodifferentiation was confirmed at longer periods of cell cultivation for the formation of calcification. The findings suggest that the new material demonstrates osteoinductive properties and may be promising materials for tissue engineering of bone.

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References

[1] V. Uskoković, V. M. Wu, "Calcium Phosphate as a Key Material for Socially Responsible Tissue Engineering," *Materials (Basel)*, vol. 9, no. 6, pp. 434, 2016.

[2] H. Yuan, H. Fernandes, P. Habibovic, J. de Boer, A. M. C. Barradas, A. de Ruiter, W. R. Walsh, C. A. van Blitterswijk, J. D. de Brujin, "Osteoinductive ceramics as a synthetic alternative to autologous bone grafting," *Proc. Natl. Acad. Sci.*, vol. 107, pp. 13614–13619, 2010.

[3] T. Albrektsson, C. Johansson, "Osteoinduction, osteoconduction and osseointegration," *Eur. Spine J.*, vol. 10, pp. 96-101, 2001.

[4] M. E. Thomas, P. W. Richer, J. Crooks, U. Ripamonti, "Smart calcium phosphate-based bioceramics with intrinsic osteoinductivity," *Bioceramics*, vol. 13, pp. 441-444, 2001.

[5] B. Dhandayuthapani, Y. Yoshida, T. Maekawa, D. S. Kumar, "Polymeric Scaffolds in Tissue Engineering Application: A Review," *International Journal of Polymer Science*, Article ID 290602, p. 19, 2011.

[6] V. Uskokovic, D. P. Uskokovic, "Nanosized Hydroxyapatite and Other Calcium Phosphates: Chemistry of Formation and Application as Drug and Gene Delivery Agents," *J. Biomed. Mat. Res. B*, no. 96, 152–191, 2011.

[7] S. V. Nadezhdin, Yu. Ye. Burda., E. V. Zubareva, V. V. Botvin, L. A. Pokrovskaya, A. D. Latypov, N. N. Volkovnyak, E. A. Grebtsova, V. S. Beliaeva, "Response of the living systems to the osteoconductive material on the basis of nanohydroxyapatite, lactide and glycolide copolymer," *Technologies of Living Systems*, vol. 2, pp. 22-30, 2018.

[8] T. Komori, "Signaling networks in RUNX2-dependent bone development," *J Cell Biochem*, vol. 112, pp. 750-755, 2011.

[9] W. Tang, Y. Li, L. Osimiri, C. Zhang, "Osteoblast-specific transcription factor Osterix (Osx) is an upstream regulator of Satb2 during bone formation," *J Biol Che*, vol. 286, pp. 32995-33002, 2011.

[10] R. Yagi, L. F. Chen, K. Shigesada, Y. Murakami, Y. A. Ito, "WW domain-containing yes-associated protein (YAP) is a novel transcriptional co-activator," *The EMBO Journal*, no. 18, no. 9, pp. 2551–2562, 1999.

[11] R. Marom, I. Shur, R. Solomon, D. Benayahu, "Characterization of adhesion and differentiation markers of osteogenic marrow stromal cells," *J. Cell. Physiol*, vol. 202, no. 1, pp. 41-48, 2005.

[12] S. M. Morgan, S. Tilley, S. Perera, M. J. Ellis, J. Kanczler, J. B. Chaudhuri, R. O. Oreffo, "Expansion of human bonemarrow stromal cells on poly-(D,L-lactide-co-glycolide) (PDL LGA) hollow fibres designed for use in skeletal tissue engineering," *Biomaterials*, vol. 28, pp. 5332–5343, 2007..

[13] M. Dominici, C. Le Blank, I. Mueller et al., "Minimal criteria for defining multipotent mesenchymal stromal cells," *The International Society for Cellular Therapy position statement // Cytotherapy*, vol. 8, no. 4, pp. 315-317, 2006..

[14] T. A. Krylova, A. M. Kol'tsova, V. V. Zenin, A. S. Musorina, T. K. Iakovleva, G. G. Polianskaia, "Comparative characteristics of new mesenchymal stem cell lines derived from human embryonic stem cells, bone marrow and foreskin," *Tsitologiia*., no. 54, no. 1, pp. 5-162, 2012.

[15] P. Garg, M. M. Mazur, A. C. Buck, M. E. Wandtke, J. Liu, N. A. Ebraheim, "Prospective Review of Mesenchymal Stem Cells Differentiation into Osteoblasts," *Orthop Surg*., vol. 9, no. 1, pp. 13-19, 2017.

[16] L. S. Litvinova, V. V. Shupletsova, K. A. Yurova, O. G. Khaziakhmatova, N. M. Todosenko, M. Yu. Khlusova, G. B. Slepchenko, E. G. Cherempey, Yu. P. Sharkeev, E. G. Komarova, M. B. Sedelnikova, V. V. Malashchenko, E. S. Melashchenko, I. A. Khlusov, "Cell-IQ visualization of motility, cell mass, and osteogenic differentiation of multipotent mesenchymal stromal cells cultured with relief calcium phosphate coating," *Doklady Biochemistry and Biophysics*, vol. 476, pp. 310–315, 2017.