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THE INFLUENCE OF VARIOUS COATINGS OF HYDROXYAPATITE BONE CARRIER ON THE SUCCESS OF BONE REGENERATION IN RABBIT CALVARIAL DEFECTS: HISTOMORPHOMETRIC AND HISTOLOGICAL ANALYSIS

Uticaj različitih materijala koji oblažu hidroksiapatit koštanog nosača na uspeh koštane regeneracije defekta kalvarije zeca: histomorfometrijska i histološka analiza

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Abstract

The materials used nowadays for bone replacement do not fully meet the requirements for complete regeneration, which is why new ones are being tested. **Background/Aim.** Despite numerous attempts to improve bone tissue regeneration, no fulfilling material has been found yet. This study investigated the influence of poly-lactide-co-glycolide (PLGA) and poly-ethylene-imine (PEI) as coatings for hydroxyapatite (HAP) bone carrier onto bone tissue regenerative potential in rabbit's calvarial defect. **Methods.** Calvarial defects measuring 6 mm in diameter were made in 19 skeletally mature rabbits. Defects were filled with one of the following materials: PLGA coated HAP (HAP+PLGA), PEI coated HAP (HAP+PEI) and bovine HAP – Bio-Oss (positive control). Unfilled defects represented negative control. Histological analysis was performed in order to determine the inflammatory response of the host tissue. Formation of the new bone was evaluated by means of histomorphometric analysis. All analysis have been conducted in samples obtained 3, 6 and 9 weeks after implantation. **Results.** Three weeks post-implantation, a trend toward increased healing in HAP+PLGA group compared to other investigated materials was noticed, with no statistically significant difference between study groups ($p>0.05$). However, after six and 9 weeks, significant healing was observed in favour of the HAP coated with PLGA compared to other groups ($p<0.05$). Within this group, greater bone healing was observed comparing to HAP+PEI and Bio-Oss. **Conclusion.** PLGA has demonstrated greater coating potential comparing to PEI with respect to osteogenesis improvement in bone reconstructive surgery does.

**Key words:** bone reconstruction; calvarial defect; hydroxyapatite; poly-lactide-co-glycolide; poly-ethylene-imine.

Apstrakt

Materijali koji se u današnje vreme koriste za nadoknadu koštanog tkiva ne dovode do kompletne regeneracije čega se ispituju novi. **Uvod/Cilj.** Uprkos brojnim pokušajima da se poboljša regeneracija koštanog tkiva, još uvek nije pronađen materijal koji ispunjava sve
kriterijume. U ovom radu ispitivan je uticaj poli-laktid-koglikolida (PLGA) i poli-etilenimina (PEI) kao premaza za oblaganje hidroksiapatita (HAP) na regenerativni potencijal koštanog tkiva u defektu kalvarije zeca. 

**Metode.** Kod 19 koštano zrelih zečeva, načinjeni su defekti kalvarije dijametra 6 mm, koji su potom ispunjeni sledećim materijalima: HAP obložen PLGA (HAP+PLGA), HAP obložen PEI (HAP+PEI), I govedi HAP – Bio-os (pozitivna kontrola). Prazni defekti su predstavljali negativnu kontrolu. Histološki je ispitivana inflamacijska reakcija tkiva domaćina, dok je histomorfometrijskom analizom evaluirano formiranje nove kosti. Analizirani su uzorci dobijeni 3, 6 i 9 nedelja nakon implantacije. 

**Rezultati.** Tri nedelje nakon implantacije, uočena je tendencija boljeg zarastanja u HAP+PLGA grupi, bez statistički značajne razlike između ispitivanih grupa ($p>0.05$). Međutim, šest i 9 nedelja nakon implantacije, značajno formiranje koštanog tkiva je primećeno u korist HAP+PLGA grupe ($p<0.05$). Oblaganje HAP sa PLGA dovelo je do boljeg koštanog zarastanja u poređenju sa HAP+PEI i Bio-os. 

**Zaključak.** PLGA je pokazao veći potencijal prekrivanja defekta od PEI u pogledu stimulisanja osteogeneze u koštanoj rekonstruktivnoj hirurgiji.

**Ključne reči:** koštana rekonstrukcija; defekt kalvarije; hidroksiapatit; poli-laktid-koglikolid; poli-etilen-imin.

**Introduction**

Bone regeneration is an important issue in oral and maxillofacial surgery. Autogenous bone still presents a gold standard for bone defects repair. On the other hand, several drawbacks regarding the use of autogenous bone grafts have been described, such as donor site morbidity, limited amount of harvested bone and relatively high resorption rate of the construct. Having in mind these drawbacks, synthetic bone substitutes and xenografts have been introduced $^{1,2,3}$. An ideal bone substitute should be non-irritable and non-toxic, providing adequate microenvironment for adhesion, proliferation and differentiation of the cells $^{4}$. In addition, requirements for graft material include not easy achievable mechanic stability and high porosity $^{5}$. Likewise, ideal bone substitute is expected to resorb completely, in proper period, synchronized with new bone synthesis $^{6}$. Geistlich’s Bio-Oss is the most investigated bone substitute, characterized by desirable clinical results in comparison to other commercially available products. Despite positive
clinical outcomes obtained with Bio-Oss, this material does not provide complete bone regeneration. Furthermore, it has been shown that some particles remain within connective tissue for years.

As an effort to obtain material with degradation level synchronized by new bone formation, novel bone tissue substitute (scaffold) based on calcium hydroxyapatite (HAP) was synthesized. In order to activate the surface, HAP can be layered with various surface-active substances, such as poly-lactide-co-glycolide (PLGA). It had been suggested that PLGA coating did not induce any inflammatory effects 12 weeks after implantation. In addition, accelerated cell adhesion when HAP was coated with PLGA (HAP+PGLA) has been documented. Through activation of the Runx2=CBFA-1 transcriptional activator, HAP+PLGA promotes osteogenic differentiation of preosteoblastic cell lineage. This combination can be used as a tissue engineering scaffold material and delivery carrier of pro-osteogenic bone morphogenetic protein 2 (BMP2) and pro-angiogenic gene of vascular endothelial growth factor as well. The issue whether new bone formation could be obtained in shorter extent of time remained unclear. Despite the fact that coating provides certain advantages, a choice of adequate coating material is still at the centre of researchers’ interests.

Another option for bone substitute coating is poly-ethylene-imine (PEI). This material belongs to the next generation of gene-activated scaffolds, which might include multiple genes to promote synergistic cell-mediated protein production and facilitate neo-vascularisation of the damaged bone. Linear PEI enriched scaffolds have promoted cell growth by mimicking biological function of the native extracellular matrix. Modified PEI also exhibits a number of key advantages, like low immunogenic, low cytotoxic, non-carcinogenic properties and is considered safe for clinical use. In addition, PEI contains a large number of amino nitrogen atoms in the molecular chain, leading to a strong affinity to cells.

The aim of this study was to assess the influence of PLGA and PEI when used as HAP coatings on osteogenesis improving. The ultimate goal was to determine the ratio of the newly formed bone in rabbits’ calvarial defects after implantation of HAP+PLGA and HAP coated with PEI (HAP+PEI).

Methods
**Materials synthesis**

HAP synthesis and PLGA coating were performed as reported previously\(^{11}\). In short, powders of calcium and \((\text{NH}_4)_2\text{HPO}_4\) (p.a. Merck), were used for the hydrothermal synthesis of HAP. The precursor solutions were prepared as a combination of corresponding mixtures of \(\text{Ca(OH)}_2\) and aqueous solution of \((\text{NH}_4)_2\text{HPO}_4\). Afterward, surface-active substance PEVA/PEVV was added for further hydrothermal processing in the autoclave at a temperature of 120 °C for 2 h. The obtained particles were filtrated through a filter with a pore size of 200 nm. HAP granules were obtained using polyurethane foam template and HAP suspension. After immersion of template in the HAP suspension and its drying, the composition was thermally treated to pyrolyse polyurethane template, followed by sintering of porous HAP after thermal treatment at 1200 °C. Finally, HAP+PLGA coating was obtained by pouring the PLGA solution in chloroform over the HAP granules.

Coating with PEI included presumably slight PEI modification. Briefly, the solution of modified PEI was prepared by dissolving branched PEI (3 g) in 15 mL water by heating and stirring. Carbon dioxide \((\text{CO}_2)\) was bubbled into this solution at ambient temperature and stirring was continued for 5 h until the reaction was complete. The contents were transferred to an Eppendorf tube, freeze dried to form solid PEI-CO2, and later dissolved in ethanol. HAP+PEI coatings were obtained by immersion of HAP granules in prepared solution. Amino content and subsequently cytotoxicity of PEI were reduced by modifying with \(\text{CO}_2\).

**Study design and surgical procedures**

Nineteen adult, skeletally mature, male rabbits weighing 2-3 kg were included in the study. Experiments were performed in accordance with EU Directive 2010/63/EU for animal experiments, which was approved by Ethic committee of the Faculty of Veterinary Medicine, Belgrade University (number of the study: 323-07-08477/2015/3, issued on 08/03/2016.). The total anaesthesia was maintained after premedication with intramuscular
injection of 2% Xylazin, 5 mg/kg, (Cp pharma, Bergdorf, Germany), with the combination of 35 mg/kg Ketamine (Laboratorio Sanderso S.A., Santiago, Chile) and 0.75 mg/kg Acepromazine (Boehringer Ingelheim Vetmedica, Inc., St. Joseph, Mo 64506 U.S.A). The average duration of anesthesia was 100 minutes. Intramuscular injection of 500 mg/kg Penicillin-Streptomycin (Penstrep) was administered. The four circular calvarial defects with 6 mm in diameter were created in parietal bones of each animal. The first defect was filled with HAP+PLGA (also known as ALBO-OS), the second defect with HAP+PEI, the third defect with Geistlich Bio-Oss as a positive control, and forth defect was left empty as a negative control. The first six rabbits were sacrificed after 3 weeks, other 6 rabbits after 6 weeks and seven rabbits were sacrificed 9 weeks following the implantation. The biopsy specimens were obtained from each animal with an oscillating saw, including entire cranial vault for histology and histomorphometric analysis. In addition, the dura mater, galea and periosteum remained intact in each of the animal.

**Histological analysis**

All specimens were optimally decalcified using formic acid. Each specimen was embedded in paraplast, sectioned in 4 μm thick slices by rotary microtome (Leica SM2000R, Leica Microsystems, Wetzlar, Germany). Thereafter, the preparations were de-waxed, processed to Hematoxylin-Eosin (HE) and Goldner's trichrome staining technique and qualitatively analysed under light microscope to determine the level of host tissue inflammatory response.

**Histomorphometric analysis**

The histological parameters were evaluated at 40 x magnification under a microscope (Leitz Labor Lux S Fluorescence Microscope, Ernst Leitz Wetzlar GMBH, Germany), with exception of inflammatory cell infiltrates which were counted on total magnification of 400x. 2D images were captured at 40x magnification using digital colour camera (Leica DFC295, Germany) and merged to create a single image for each histological section. Thereafter, images were analysed using software (Leica University Suite, version 4.3, Leica Microsystems, Germany) running on the personal computer. Four sections from central defect region and 4 from peripheral defect region were analysed with
a spacing of 50 µm between sections. The following parameters were measured: total bone volume in percentages (TB%), mineralized bone in percentages (MB%), nonmineralized bone in percentages (NMB%), connective tissue in percentages (CT%) and blood vessels in percentages (BV%). Within the connective tissue, macrophages, giant cells, plasma cells, lymphocytes and neutrophil granulocytes were counted.

Statistics

Statistical analysis was performed using SPSS for windows - version 18.0 software (SPSS, Inc., Chicago, IL, USA). All data were presented as the mean ± SD. Two-way ANOVA was performed at 95% level of significance, followed by Tukey post hoc comparisons.

Results

Histological and histomorphometric analysis after 3 weeks post bone replacement material implantation

In all specimens 3 weeks after implantation the demarcation line and area of the defect filled with connective tissue containing the graft particles could clearly be noticed (Figs. 1 and 2). In addition, islands of new bone tissue, mainly localized at the particle surfaces or near them were detected. In HAP+PLGA group particles of the graft were almost completely surrounded by new bone tissue, with trabeculae and osteoblasts.

Histomorphometric analysis of specimens after 3 weeks is shown in Table 1. Although HAP+PLGA group showed higher percentage of total bone area comparing to Bio-Oss, the difference was not statistically significant (p>0.05). Likewise, there was no statistically significant difference in any other parameter 3 weeks after implantation (Figs. 1 and 2, Tab. 1).

Inflammatory infiltrate (macrophages, giant cells, lymphocytes, plasmocytes, neutrophils) was mainly localized in close proximity to the particles of the material. The
number of the cells was ranging from 0 to 10 in the field of view under the light microscope magnification of 400x. (Tab.2).

*Histological and histomorphometric analysis after 6 weeks post bone replacement with implanted material*

The results of histological analysis 6 weeks after implantation showed that defects were filled by connective tissue with still unabsorbed particles of the graft and new bone tissue. The amount of newly formed bone was the largest in the HAP+PLGA group, followed by HAP+PEI and Bio-Oss, whilst the lowest amount was observed in empty defects. In all tested groups, newly formed bone had lamellar structure with osteocytes, which indicated on bone vitality. In majority of the samples, the absence of inflammatory cells in connective tissue was detected (Figs. 3 and 4).

Histomorphometric analysis of the specimens obtained after 6 weeks demonstrated that the amount of newly formed bone in HAP+PLGA group was 18.9±1.3 %, which was statistically higher comparing to the other groups ($p<0.05$) (Tab. 3).

Decreasing tendency of inflammatory reaction has been noted. In 4 out of 12 cases no inflammatory cells were noticed. In the rest of the specimens the number of cells was minimal (Tab.4).

*Histological and histomorphometric analysis 9 weeks post bone replacement with implanted material*

The results of histological and histomorphometric analysis 9 weeks after implantation showed the highest amount of newly formed bone in the HAP+PLGA group and the lowest in the Bio-Oss group and empty defect. The difference between HAP+PLGA group and Bio-Oss group was found to be statistically significant ($p<0.001$) (Figs. 5 and 6; Tab.5).

Nine weeks after the implantation of the materials, in 10 out of 12 specimens no histological signs of inflammatory reaction were discovered (Tab.6).

**Discussion**
The present study evaluated regenerative potential of various coatings for HAP bone substitutes. In HAP+PLGA group, the greatest amount of new formed bone was noticed, accompanied with the lowest number of inflammatory cells in all the investigated time cut offs.

Obtained results are the extension of the previous investigation that evaluated HAP+PLGA influence on the calvarial defect healing in period of 12 weeks after implantation \(^{12}\). The objectives of the current study were to determine whether bone healing could be achieved in three to nine weeks post-implantation, as well as to assess the influence of PEI coating on regeneration capacity of previously designed HAP scaffold. It has been demonstrated that the type of coating exhibits considerable impact on regeneration potential of the bone substitute. For instance, it should be observed that the newly formed bone ratio 6 weeks following the implantation was 3 times higher in the PLGA than in PEI coated group (4.3±0.7 % vs. 1.4±0.3 %, respectively). Similarly, the rate of mineralized bone at 9 weeks cut-off was almost 2 times higher in HAP+PLGA comparing to HAP+PEI group (28±4 % vs. 16.9±0.8 %, respectively). The results of the current study are promising: when compared to the “gold standard”, Bio-Oss, ~50% more mineralized bone is observed in the HAP+PLGA group after six weeks and ~70% more mineralized bone at week 9.

Figures 1 and 2 demonstrate bone tissue samples three weeks after implantation, with observable islands of the new bone tissue in a close proximity to the graft particles, could be seen, indicating on osteoconductivity of the tested materials. Although significant difference between study samples at week 3 was not found, it is interesting to note that the greatest amount of osteoid was noticed in HAP+PEI, two times higher than in Bio-Oss group (Tab. 1). This observation points out that intensive osteogenesis has been occurred in these specimens. In HAP+PLGA group, particles of the graft were completely surrounded by newly formed bone with osteoblasts, which clearly describes active osteogenesis. The main histological parameter of biocompatibility was the number of inflammatory cells. The estimated number was ranging from 6 to 11 in all samples obtained after 3 weeks (Tab. 2). This result presents mild inflammatory reaction.
Six weeks after implantation, the highest amount of new bone was detected in the HAP+PLGA group (Figs. 3 and 4, Tab.3), which can be explained by weak immune response and consequent acceleration in creating of new bone tissue. It seems that nanotopology of the HAP+PLGA coating is suitable for cell adhesion. In tested specimens the new bone had lamellar structure with osteocytes which indicated on bone vitality. According to the number of inflammatory cells listed in the table 4, a mild inflammatory reaction was present six weeks after implantation.

Nine weeks after the implantation, the best result was obtained in the HAP+PLGA coating group, as shown in figures 5 and 6. This can be clarified by inter-group comparison of dynamics in new bone tissue forming: after 3 weeks, the rate of the newly formed bone was approximately identical in the HAP+PLGA, HAP+PEI and Bio-Oss groups (13.2 %, 16.4 % and 18 %, respectively); at week 6, more new bone tissue was detected in the HAP+PLGA and Bio-Oss (18.9 % and 15 %) than in the HAP+PEI group (8.5 %); finally, at week 9, the rate of newly formed bone in the HAP+PLGA group obviously confirms its superiority over the HAP+PEI and Bio-Oss groups (Tab.5). Nine weeks after implantation, a number of observed inflammatory cells (Tab.6) indicated on a mild inflammatory reaction again.

The HAP used in this study reaches adequate balance between material resorption and new bone formation. It is previously demonstrated that HAP coated with PLGA promotes adequate bone healing 12 weeks after implantation. This study went a step further by introducing PEI as a novel coating substance. PEI is a typical poly-cationic polymer that contain a high density of protonated secondary amines. Despite the fact that cytotoxic effects of free PEI on many cells were documented, the protonated form has been most widely used as a gene delivery agent due to its high charge density. In our investigations, CO₂-modified PEI coatings were used in order to decrease the toxic properties of PEI.

Although the results obtained for HAP coated with PEI are inferior when confronting to HAP+PLGA, HAP+PEI showed superior healing capacity in comparison to Bio-Oss 6 and 9 weeks following implantation.
With respect to the slight toxicity of modified polyethylene, this material probably triggers the response of surrounding immune cells as well as the initial period of inflammation. Furthermore, as a result of high networking and adhesion properties, it supports osteoblast propagation via interaction between PIE and BMP 2.

Comparing the unmodified and CO₂-modified PEI form, its citotoxicity is remarkably less expressed in latter. High positive charge of unmodified PEI can damage the cell membrane and disturb critical intracellular mechanisms. CO₂ alteration mitigates this cascade, without disrupting the activity of protein kinase C ²⁴, ²⁵, ²⁶, ²⁷.

When a mixture of unmodified PEI and carbonic acid is formed, a part of positively charge is neutralized by negatively charged acid anions, which ultimately reduces material toxicity. Liu et al ²⁵, demonstrated that modified PEI promotes differentiation of multipotent stem cells to several tissue-specific pathways, including bone tissue ²⁵. Furthermore, a positive impact on growth factors such as transforming growth factor (TGF) and BMP with indirect effect on osteogenesis has been shown ²⁸.

In addition, all above mentioned results stand in line with the outcomes achieved by Tang et al. ²⁹, who used bio-inspired trimodal macro/micro/nano-porous scaffold loaded with recombinant human BMP-2 (TMS/rgBMP-2). They assumed that osteogenic promotion of TMS/rgBMP-2 mainly occurred in the first 8 weeks after implantation. Later on, tissue maturity was mostly depended on self-remodeling of the newly formed bone tissue. In the current study, the greatest amount of newly formed bone was found in the HAP+PLGA group after 6 weeks of regeneration, with the same trend prolonged to week 9 in both studies. Extensive angiogenesis and osteogenesis noticed in our specimens after 3 weeks of regeneration are in agreement with primary bone formation stage in Tang's et al study ²⁹. In the present study, lamellar bone was formed after 9 weeks of regeneration, which is close to 8 weeks found in Tang's et al investigation. Exogenous rhBMP-2 was important, but probably not the crucial factor for bone regeneration process in the mentioned study. Results of our experiments for periods of 6 and 9 weeks after implantation indicate that precise biological mechanism of bone forming after implantation remains unresolved. It is well known that bone regeneration imply biological events including bone induction and conduction, as well as several cell types and signalling
pathways. Bone grafting includes osteoinduction (BMPs and other growth factors), osteogenesis (osteoprogenitor cells) and osteoconduction (scaffold). The used scaffold has a key role in supporting cell growth and tissue formation, as well as in providing appropriate microenvironment and structural integrity; additionally, it can support during cellular colonization and tissue regeneration. Suitable scaffold should also support direct cell growth and tissue formation by many growth factors, cytokines and signal molecules. Biological mechanisms, which occur after scaffold implantation, e.g. scaffold biodegradation, still have to be elucidated.

**Conclusion**

The efforts of the current research were focused on modifying surface topography of novel HAP by means of PLGA and PEI coating in order to accelerate new bone tissue forming. Results of the study suggest that PLGA presents a superior coating option capable to considerably improve the bone regenerative potential of the synthetic HAP.

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**References**

1. Sakkas A, Wilde F, Heufelder M, Winter K, Schramm A. Autogenous bone grafts in oral implantology—is it still a "gold standard"? A consecutive review of 279 patients with 456 clinical procedures. Int J Implant Dent 2017;3:23. doi: 10.1186/s40729-017-0084-4.

2. Howlader D, Vignesh U, Bhutia DP, Pandey R, Kumar S, Chandra T, Mehrotra D. Hydroxyapatite collagen scaffold with autologous bone marrow aspirate for
1. Mandibular condylar reconstruction. J Craniomaxillofac Surg 2017;45(9):1566-1572. doi: 10.1016/j.jcms.2017.06.022.

3. Birkenfeld F., Sengebuch A., Voelschow C., Möller B., Naujokat H., Wiltfang J. Scaffold implantation in the omentum majus of rabbits for new bone formation. J Craniomaxillofac Surg, 2019;47(8):1274-1279. doi: 10.1016/j.jcms.2019.04.002.

4. Nezhurina E.K., Karalkin P.A., Komlev V.S., Sviridova I.K., Kirsanova V.A., Akhmedova S.A., et al. Physicochemical and osteoplastic characteristics of 3D printed bone grafts based on synthetic calcium phosphates and natural polymers. IOP Conf Ser Mater Sci Eng 2018;347. doi:10.1088/1757-899X/347/1/012047.

5. Zimmerer RM, Jehn P, Kokemüller H, Abedian R, Lalk M, Tavassol F, Gellrich NC, Spalthoff S. In vivo tissue engineered bone versus autologous bone: stability and structure. Int J Oral Maxillofac Surg 2017; 46:385-393. doi: 10.1016/j.ijom.2016.10.012.

6. Eweida A, Schulte M, Frisch O, Kneser U, Harhaus L. The impact of various scaffold components on vascularized bone constructs. J Craniomaxillofac Surg 2017; 45(6):881-890. doi: 10.1016/j.jcms.2017.02.016.

7. Kasuya S, Shihoko I, Nihoko K, Omori M, Yamamoto K. Evaluation of Guided Bone Regeneration Using the Bone Substitute Bio-Oss® and a Collagen Membrane in a Rat Cranial Bone Defect Model. Journal of Hard Tissue Biology 2018;27:79-84. doi: 10.1097/ID.0000000000000738.

8. Le BQ, Rai B, Hui Lim ZX, Tan TC, Lin T, Lin Lee JJ, Murali S, Teoh SH, Nurcombe V, Cool SM. A polycaprolactone-β-tricalcium phosphate-heparan sulphate device for cranioplasty. J Craniomaxillofac Surg 2019;47(2):341-348. doi:10.1016/j.jcms.2018.11.013.

9. Aludden HC, Mordenfeld A, Hallman M, Dahlin C, Jensen T. Lateral ridge augmentation with Bio-Oss alone or Bio-Oss mixed with particulate autogenous bone graft: a systematic review. Int J Oral Maxillofac Surg 2017;46:1030-1038. doi:10.1016/j.ijom.2017.03.008.

10. Shirmohammadi A, Roshangar L, Chitsazi MT, Pourabbas R, Faramarzie M, Rahmanpour N. Comparative Study on the Efficacy of Anorganic Bovine Bone
10. (Bio-Oss) and Nanocrystalline Hydroxyapatite (Ostim) in Maxillary Sinus Floor Augmentation. Int Sch Res Notices 2014:967091. doi: 10.1155/2014/967091.

11. Jokanović V, Čolović B, Marković D, Petrović M, Jokanović M, Milosavljević P, Sopta J. In Vivo Investigation of ALBO-OS Scaffold Based on Hydroxyapatite and PLGA. J Nanomater Article 2016. https://doi.org/10.1155/2016/3948768.

12. Jokanović V, Čolović B, Marković D, Petrović M, Soldatović I, Antonijević Dj, et al. Extraordinary biological properties of a new calcium hydroxyapatite/poly(lactide-co-glycolide)-based scaffold confirmed by in vivo investigation. Biomed Tech 2017;24; 62:295-306. doi:10.1515/sdj-2017-0004.

13. Qiao C, Zhang K, Jin H, Miao L, Shi C, Liu X, Yuan A, Liu J, Li D, Zheng C, Zhang G, Li X, Yang B, Sun H. Using poly(lactic-co-glycolic acid) microspheres to encapsulate plasmid of bone morphogenetic protein 2/polyethylenimine nanoparticles to promote bone formation in vitro and in vivo. Int J Nanomedicine 2013;8:2985-95. doi: 10.2147/IJN.S45184.

14. Chen XA, Zhang LJ, He ZJ, Wang WW, Xu B, Zhong Q, Shuai XT, Yang LQ, Deng YB. Plasmid-encapsulated polyethylene glycol grafted polyethylenimine nanoparticles for gene delivery into rat mesenchymal stem cells. Int J Nanomedicine 2011; 6:843-53. doi: 10.2147/IJN.S17155.

15. Tierney EG, Duffy GP, Hibbitts AJ, Cryan SA, O'Brien FJ. The development of non-viral gene-activated matrices for bone regeneration using polyethyleneimine (PEI) and collagen-based scaffolds. J Control Release 2012; 158:304-11. doi: 10.1016/j.jconrel.2011.11.026.

16. Cholas R, Padmanabhan SK, Gervaso F, Udayan G, Monaco G, Sannino A, Licciulli A. Scaffolds for bone regeneration made of hydroxyapatite microspheres in a collagen matrix. Materials Science and Engineering 2016; 63:499-505. doi: 10.1016/j.msec.2016.03.022.

17. Khanam N, Mikoryak C, Draper RK, Kenneth J. Balkus Jr JB. Electrosyn linear polyethyleneimine scaffolds for cell growth. Acta Biomaterialia 2007;3:1050-1059. doi:10.1016/j.actbio.2007.06.005.

18. Al-Dosari MS, Gao X. Nonviral gene delivery: principle, limitations, and recent progress. AAPS J 2009;11:671–681. doi: 10.1208/s12248-009-9143-y.
19. Kumar S, Raj S, Sarkar K, Chatterjee K. Engineering a multi-biofunctional composite using poly(ethylenimine) decorated graphene oxide for bone tissue regeneration. Nanoscale 2016; 8:6820-36. https://doi.org/10.1039/C5NR06906H.

20. Khorsand B, Nicholson N, Do AV, Femino JE, Martin JA, Petersen E, Guetschow B, Fredericks DC, Salem AK. Regeneration of bone using nanoplex delivery of FGF-2 and BMP-2 genes in diaphyseal long bone radial defects in a diabetic rabbit model. J Control Release 2017; 248:53-59. doi: 10.1016/j.jconrel.2017.01.008.

21. Jang SJ, Kim SE, Han TS, Son JS, Kang SS, Choi SH. Bone Regeneration of Hydroxyapatite with Granular Form or Porous Scaffold in Canine Alveolar Sockets. In Vivo 2017;31: 335–341. doi: 10.21873/invivo.11064.

22. Kubasiewicz-Ros P, Seeliger J, Kozak K, Jurczyszyn K, Gerber H, Dominiak M, Kunert-Keil C. New nano-hydroxyapatite in bone defect regeneration: A histological study in rats. Annals of Anatomy - Anatomischer Anzeiger 2017; 213:83-90. doi:10.21873/invivo.11064.

23. Xing H, Cheng L, Lu M, Liu H, Lang L, Yang T, Zhao X, Xu H, Yang L, Ding P. A biodegradable poly(amido amine) based on the antimicrobial polymer polyhexamethylene biguanide for efficient and safe gene delivery. Colloids Surf B Biointerfaces 2019; 182:110355. doi: 10.1016/j.colsurfb.2019.110355.

24. Guo X, Ding L, Kanamori K, Nakanishi K, Yang H. Functionalization of hierarchically porous silica monoliths with polyethyleneimine (PEI) for CO2 adsorption. Microporous and mesoporous materials 2017. doi:10.3390/ijerph17041452.

25. Liu M, Zhang L, Zhao Q, Jiang X, Wu L, Hu Y. Lower-molecular-weight chitosan-treated polyethyleneimine: a practical strategy for gene delivery to mesenchymal stem cells. Cell Physiol Biochem 2018. https://doi.org/10.1159/000494585.

26. Kunath K, von Harpe A, Fischer D, Kissel T. Galactose-PEI-DNA complexes for targeted gene delivery: degree of substitution affects complex size and transfection efficiency. J Control Release 2003. doi: 10.1016/S0168-3659(02)00458-3.

27. Fischer D, Bieber T, Li Y, Elsasser HP, Kissel T. A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethyleneimine: effect of
molecular weight on transfection efficiency and cytotoxicity. Pharm Res 1999. doi: 10.1023/a:1014861900478.

28. Tachi K, Takami M, Sato H, Mochizuki A, Zhao B, Miyamoto Y, et al. Enhancement of bone morphogenetic protein-2-induced ectopic bone formation by transforming growth factor-beta 1 2011. Tissue Eng Part A, doi: 10.1089/ten.TEA.2010.0094.

29. Tang W, Lin D, Yu Y, Niu H, Guo H, Yuan Y, Liu C. Bioinspired trimodal macro/micro/nano-porous scaffolds loading rhBMP-2 for complete regeneration of critical size bone defect. Acta Biomater 2016; 32:309-323. doi: 10.1016/j.actbio.2015.12.006.

30. Kharkova NV, Reshetov IV, Zelianin AS, Philippov VV, Sergeeva NS, Sviridova IK, Komlev VS, Andreeva UU, Kuznecova OA. Three-dimensional TCP scaffolds enriched with Erythropoietin for stimulation of vascularization and bone formation. Electron J Gen Med 2016; 16:em115. https://doi.org/10.29333/ejgm/108620.

Table 1. Histomorphometric analysis of the specimens 3 weeks after implantation
|                           | Bio-Oss | HAP+PEI | HAP+PLGA | Empty defect |
|---------------------------|---------|---------|----------|--------------|
| Graft (\%±SD)             | 47±2    | 25±5\*  | 47±3     | 10±1         |
|                           | \*p<0.001|         |          |              |
| TB (\%±SD)                | 13.2±0.3| 8.5±0.10\*| 18±3     | 8.4±0.7      |
|                           | \*p<0.001|         |          |              |
| MB (\%±SD)                | 10±0.5  | 6.6±0.4  | 13±3     | 4±0.3        |
| NB (\%±SD)                | 3.2±0.4 | 1.4±0.3\*| 5±1      | 1.30±0.13    |
|                           | \*p<0.001|         |          |              |
| CT (\%±SD)                | 39±2    | 66±6\*  | 34.5±1.4 | 85±0.4       |
|                           | \*p<0.001|         |          |              |
| BV (\%±SD)                | 0.5±0.7 | 0.4±0.40 | 0.68±0.16| 0.26±0.02    |

TB - total bone; MB - mineralised bone; NB - nonmineralised bone; BV - blood vessels; CT - connective tissue; SD - standard deviation. HAP+PLGA - hydroxyapatite + poly-lactide-co-glycolide; HAP+PEI - hydroxyapatite + poly-ethylene-imine.

**Table 2.** Amount of inflammatory cells under the light microscope, 3 weeks after implantation (magnification 400x)
| Number of infl. cells | Bio-Oss (n=4) | HAP+PEI (n=3) | HAP+PLGA (n=4) | Empty defect (n=1) |
|----------------------|---------------|---------------|----------------|-----------------|
| 0-5                  | 0 (0%)        | 0 (0%)        | 1 (25%)        | 1 (100%)        |
| 6-10                 | 4 (100%)      | 3 (100%)      | 3 (75%)        | 0 (0%)          |
| >11                  | 0 (0%)        | 0 (0%)        | 0 (0%)         | 0 (0%)          |

HAP+PLGA - hydroxyapatite + poly-lactide-co-glycolide. HAP+PEI - hydroxyapatite + poly-ethylene-imine.

Table 3. Histomorphometric analysis of the specimens 6 weeks after implantation
|                | Bio-Oss   | HAP+PEI | HAP+PLGA | Empty defect |
|----------------|-----------|---------|----------|--------------|
| **Graft**      | 52±3      | 51.2±1.4| 52±3     | 0            |
| **TB**         | 15±3      | 16.4±1.2| 18.9±1.3*| 6.6±0.3      |
|                |           |         |           | p<0.001      |
| **MB**         | 10±3      | 9±0.8   | 14.7±1.7*| 2.64±0.11    |
|                |           |         |           | p<0.005      |
| **NB**         | 4.5±0.7*  | 7.5±1.3 | 4.3±0.7  | 3.92±0.16    |
|                |           |         |           | p<0.001      |
| **CT**         | 33±3      | 32±3    | 29±3     | 93±3         |
| **BV**         | 0.46±0.19 | 0.6±0.10| 0.4±0.3  | 0.40±0.18    |

TB - total bone; MB - mineralised bone; NB- non mineralized bone; BV - blood vessels; CT - connective tissue. HAP+PLGA - hydroxyapatite + poly-lactide-co-glycolide. HAP+PEI - hydroxyapatite + poly-ethylene-imine.

**Table 4.** The number of inflammatory cells under the light microscope 6 weeks after implantation (magnification 400x)
| Number of infl. cells | Bio-Oss (n=4) | HAP+PEI (n=3) | HAP+PLGA (n=4) | Empty defect (n=1) |
|----------------------|---------------|---------------|----------------|-------------------|
| 0-5                  | 1 (25%)       | 0 (0%)        | 2 (50%)        | 1 (100%)          |
| 6-10                 | 3 (75%)       | 3 (100%)      | 2 (50%)        | 0 (0%)            |
| >11                  | 0 (0%)        | 0 (0%)        | 0 (0%)         | 0 (0%)            |

HAP+PLGA - hydroxyapatite + poly-lactide-co-glycolide. HAP+PEI - hydroxyapatite + poly-ethylene-imine

**Table 5.** Histomorphometric analysis of the specimens 9 weeks after implantation
|            | Bio-Oss | HAP+PEI | HAP+PLGA | Empty defect |
|------------|---------|---------|----------|--------------|
| **Graft**  | 43±0.7  | 46±3    | 39±3     | 0            |
| **TB**     | 20.4±0.9| 21.9±0.3| 34±3*    | 11.94        |
|            |         |         |         | p<0.001      |
| **MB**     | 13.7±05 | 16.9±0.8| 28±4     | 3.30         |
| **NB**     | 6.3±0.6 | 4.5±0.9 | 5.9±0.3  | 8.64         |
| **CT**     | 35.9±1.4*| 31±3    | 27.3±0.7 | 87.69        |
|            |         |         |         | p<0.001      |
| **BV**     | 0.5±0.5 | 0.4±0.7 | 0.5±0.3  | 0.37         |

TB - total bone; MB - mineralized bone; NB - non-mineralized bone; BV - blood vessels; CT - connective tissue. HAP+PLGA - hydroxyapatite + poly-lactide-co-glycolide. HAP+PEI - hydroxyapatite + poly-ethylene-imine

Table 6. The number of inflammatory cells under the light microscope 9 weeks after implantation (magnification 400x)
| Number of infl. cells | Bio-Oss (n=4) | HAP+PEI (n=3) | HAP+PLGA (n=4) | Empty defect (n=1) |
|-----------------------|----------------|---------------|----------------|-------------------|
| 0-5                   | 3 (75%)        | 2 (66%)       | 4 (100%)       | 1 (100%)          |
| 6-10                  | 1 (25%)        | 1 (33%)       | 0 (0%)         | 0 (0%)            |
| >11                   | 0 (0%)         | 0 (0%)        | 0 (0%)         | 0 (0%)            |

HAP+PLGA - hydroxyapatite + poly-lactide-co-glycolide. HAP+PEI - hydroxyapatite + poly-ethylene-imine

Legends of figures
Figure 1. Histologic specimens obtained after 3 weeks of regeneration, HAP+PLGA (A), HAP+PEI (B), Bio-Oss grafted defects as positive control (C) and non-grafted calvarial defect as negative control (D). Initial signs of ossification, with newly formed bone tissue (black arrows), occurred at the most peripheral parts of particles of the grafted material (red arrows). Particles of grafted material and newly formed bone were surrounded by non-inflamed connective tissue (green arrows), with minimal number of inflammatory cells, or without them. Empty defect of control specimen (D) was filled with connective tissue. (Goldner's Trichrome staining, x40 magnification).
Figure 2. Photomicrographs of bone defects grafted with HAP+PLGA (A), HAP+PEI (B), Bio-Oss (C), and non-grafted (empty) calvarial defect (D), obtained 3 weeks after implantation. Although the amount of the newly formed bone differed in various test groups (with almost complete absence in empty defect), histological structure of new bone was very similar in all groups. Bone resembled lamellar structure with Haversian canals (black arrows), and numerous osteocytes in lacunae. New bone was in close contact with graft particles (red arrows) and surrounded by young connective tissue (green arrows). Although connective tissue was infiltrated with minimal number of inflammatory cells, vascular stasis was observed in some specimens. (Goldner's Trichrome staining, x100 magnification)
Figure 3. Histologic specimens of HAP+PLGA (A), HAP+PEI (B), Bio-Oss (C) grafted defects, and non grafted calvarial defect (D), obtained 6 weeks after implantation. The amount of newly formed bone tissue was larger compared to that after 3 weeks of regeneration, especially in the HAP+ PLGA group. Samples of the tested groups contained islands of newly formed bone (black arrows), residual particulate bone graft (red arrows) and connective tissue (green arrows). Newly formed bone tissue had both lamellar and woven structure, which indicated the occurrence of intensive bone remodeling. Empty defect contained connective tissue without bone formation. (Goldner's Trichrome staining, x40 magnification)
Figure 4. Photomicrographs of bone defects grafted with HAP+PLGA (A), HAP+PEI (B), Bio-Oss (C) and non-grafted calvarial defect (D), obtained 6 weeks after implantation. Defects were filled by still unabsorbed graft particles (red arrows), non-inflamed connective tissue (green arrows) and newly formed bone in close relation to particles (black arrows). The main difference in findings compared to previous healing period (3 weeks) was increased amount of new bone, and decreased amount of graft particles, due to intensive resorption via dissolution. (Goldner's Trichrome staining, x100 magnification)
**Figure 5.** Histologic specimens of HAP+PLGA (A), HAP+PEI (B), Bio-oss (C) grafted defects, and non-grafted calvarial defect (D), obtained 9 weeks after implantation. It could be noticed that particularly in the HAP+PLGA group almost complete regeneration has occurred, with a large amount of newly formed bone (black arrows) with lamellar structure and osteocytes. Graft particles (red arrow) and newly formed bone were surrounded by young connective tissue (green arrows), with absence of inflammatory infiltrate. Unfilled defect predominantly resulted in connective tissue formation, with signs of spontaneous bone regeneration in form of small islands of newly formed bone. (Goldner's Trichrome staining, x40 magnification)
Figure 6. Photomicrographs of bone defects grafted with HAP+PLG (A), HAP+PEI (B), Bio-Oss (C), and non-grafted calvarial defect (D), obtained 9 weeks after implantation. It could be observed that in defects treated with HAP+PLGA and Bio-Oss, bone regeneration occurred in significantly higher level compared to defects treated with HAP+PEI. In empty defects bone regeneration almost completely failed, with exception of minor bone island in the central part of the defect. Resorption of particles with HAP+PLGA and Bio-Oss was also remarkable. Connective tissue displayed remarkable venous stasis. (Goldner's Trichrome staining, x100 magnification)
