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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 regeneracije koštanih defekta kalvarije zeca: histomorfometrijska i histološka analiza

[1]Sanja Milutinović-Smiljanić*, [1]Djordje Antonijević[†‡], Milutin Mićić[‡], Vladimir Biočanin[§], Nikola Sjerobabin[ǁ], Božana Petrović[†], Vesna Danilović*, Vukoman Jokanović[†]

University of Belgrade, *Faculty of Dental Medicine, Department of General and Oral Histology and Embryology, [†]Institute for Nuclear Science "Vinča", Laboratory for Atomic Physics, [‡]Faculty of Medicine, Laboratory for Anthropology, Belgrade, Serbia; [§]University of Business Academy, Faculty of Dentistry, Pančevo, Serbia; [ǁ]University Clinical Hospital Center "Dr. Dragiša Mišović", Belgrade, Serbia

[1]Equally contributed first author

Abstract

Background/Aim. The materials used nowadays for bone replacement do not fully meet the requirements for complete regeneration, which is why new ones are being tested. 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 polyethyleneimine (PEI) as coatings for hydroxyapatite (HAP) bone carriers on bone tissue regenerative potential in rabbits' 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. The formation of the new bone was evaluated using histomorphometric analysis. All analyses have been conducted in samples obtained 3, 6, and 9 weeks after implantation. **Results.** Three weeks post-implantation, a trend toward increased healing in the HAP + PLGA group compared to other investigated materials was noticed, with no statistically significant difference between the study groups ($p > 0.05$). However, after 6 and 9 weeks, significant healing was observed in favor of the HAP coated with PLGA compared to other groups ($p < 0.05$). Within this group, greater bone healing was observed compared to the HAP + PEI and Bio-Oss® groups. **Conclusion.** PLGA demonstrated greater coating potential compared to PEI with respect to osteogenesis improvement in bone reconstructive surgery.

Key words:
bone regeneration; histological techniques; materials testing; polymers; rabbits.

Apstrakt

Uvod/Cilj. Materijali koji se u današnje vreme koriste za nadoknadu koštanog tkiva ne dovode do kompletne regeneracije, zbog čega se ispituju novi. Uprkos mnogobrojnim pokušajima da se poboljša regeneracija koštanog tkiva, još uvek nije pronađen materijal koji ispunjava sve kriterijume. Cilj rada bio je da se utvrdi uticaj poli(laktid-ko-glikolida) (PLGA) i polietilenimina (PEI), kao premaza za oblaganje hidroksiapatita (HAP), na regenerativni potencijal koštanog tkiva u defektu kalvarije zeca. **Metode.** Kod 19 zečeva „zrelog" skeleta načinjeni su defekti kalvarije dijametra 6 mm. Defekti su potom ispunjeni jednim od sledećih materijala: HAP obložen PLGA (HAP + PLGA), HAP obložen PEI (HAP + PEI) i goveđi HAP – Bio-Oss® (pozitivna kontrola). Prazni defekti su predstavljali negativnu kontrolu. Inflamacijska reakcija tkiva domaćina je ispitana histološkom analizom. Formiranje nove kosti je procenjivano

Correspondence to: Vukoman Jokanović, Institute for Nuclear Sciences "Vinča", Laboratory for Atomic Physics, Mike Petrovica Alasa 1 Str. 2-14, 11 000 Belgrade, Serbia. E– mail: vukoman@vin.bg.ac.rs

histomorfometrijskom analizom. 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, 6 i 9 nedelja nakon implantacije, primećeno je značajno formiranje koštanog tkiva 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-Oss®. **Zaključak.** U pogledu stimulisanja osteogeneze u rekonstruktivnoj hirurgiji kostiju, PLGA je pokazao veći potencijal prekrivanja defekta od PEI.

Ključne reči:
kost, regeneracija; histološke tehnike; materijali, testiranje; polimeri; zečevi.

Introduction

Bone regeneration is an important issue in oral and maxillofacial surgery. Autogenous bone still presents a gold standard for bone defect repair. On the other hand, several drawbacks regarding the use of autogenous bone grafts have been described, such as donor site morbidity, a limited amount of harvested bone, and a relatively high resorption rate of the construct. Having in mind these drawbacks, synthetic bone substitutes and xenografts have been introduced [1–3]. An ideal bone substitute should be non-irritable and non-toxic, providing an adequate microenvironment for adhesion, proliferation, and differentiation of the cells [4]. In addition, requirements for graft material include not easy achievable mechanical stability and high porosity [5]. Likewise, the ideal bone substitute is expected to resorb completely, in a proper period, synchronized with new bone synthesis [6]. Geistlich 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 [7, 8]. Furthermore, it has been shown that some particles remain within connective tissue for years [9, 10].

As an effort to obtain a material with degradation levels synchronized by new bone formation, a novel bone tissue substitute (scaffold) based on calcium hydroxyapatite (HAP) was synthesized [11, 12]. 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 [12]. 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 (BMP-2) and the pro-angiogenic gene of vascular endothelial growth factor [13]. The issue of whether new bone formation could be obtained in a shorter extent of time remains unclear. Even though coating provides certain advantages, a choice of adequate coating material is still at the center of researchers' interests.

Another option for bone substitute coating is polyethyleneimine (PEI) [14, 15]. 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 the neo-vascularisation of the damaged bone [16]. Linear PEI-enriched scaffolds have promoted cell growth by mimicking the biological function of the native extracellular matrix [17]. Modified PEI also exhibits a number of key advantages, like low immunogenic, low cytotoxic, and non-carcinogenic properties, and is considered safe for clinical use [18]. In addition, PEI contains a large number of amino nitrogen atoms in the molecular chain, leading to a strong affinity to cells [19, 20].

The aim of this study was to assess the influence of PLGA and PEI when used as HAP coatings on osteogenesis improvement. 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 $(NH_4)_2HPO_4$ (p.a. Merck) were used for the hydrothermal synthesis of HAP. The precursor solutions were prepared as a combination of corresponding mixtures of $Ca(OH)_2$ and the aqueous solution of $(NH_4)_2HPO_4$. Afterward, the surface-active substance poly(ethylene-vinyl acetate)/poly(ethylene-) (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 a polyurethane foam template and HAP suspension. After immersion of the template in the HAP suspension and its drying, the composition was thermally treated to pyrolyze polyurethane template, followed by sintering of porous HAP after thermal treatment at 1,200 °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 (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-CO_2, and later dissolved in ethanol. HAP + PEI coatings were obtained by immersion of HAP granules in a prepared solution. Amino content and subsequently cytotoxicity of PEI were reduced by modifying with CO_2.

Study design and surgical procedures

A total of 19 adult skeletally mature male rabbits weighing 2–3 kg were included in the study. Experiments

were performed in accordance with the European Union Directive 2010/63/EU for animal experiments, which was approved by the Ethics Committee of the Faculty of Veterinary Medicine, the University of Belgrade (number of the study: 323-07-08477/2015/3, issued on March 8, 2016). Total anesthesia was maintained after premedication with an intramuscular (IM) injection of xylazine 2%, (CP-Pharma, Burgdorf, Germany) 5 mg/kg, with the combination of 35 mg/kg ketamine (Laboratorio Sanderson S.A., Santiago, Chile) and 0.75 mg/kg acepromazine (Boehringer Ingelheim Vetmedica, Inc., St. Joseph, Mo 64506 USA). The average duration of anesthesia was 100 min. IM injection of 500 mg/kg penicillin-Streptomycin (Penstrep™) was administered. The four circular calvarial defects of 6 mm in diameter were created in the 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 was filled with Geistlich Bio Oss® as a positive control, and the fourth defect was left empty as a negative control. The first six rabbits were sacrificed after 3 weeks, the 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 the entire cranial vault for histology and histomorphometric analysis. In addition, the *dura mater*, *galea*, and *periosteum* remained intact in all animals.

Histological analysis

All specimens were optimally decalcified using formic acid. Each specimen was embedded in paraplast and 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 analyzed under a light microscope to determine the level of host tissue inflammatory response.

Histomorphometric analysis

The histological parameters were evaluated at ×40 magnification under a microscope (Leitz Labor Lux S Fluorescence Microscope, Ernst Leitz Wetzlar GMBH, Germany), with the exception of inflammatory cell infiltrates, which were counted on a total magnification of ×400. Using a digital color camera (Leica DFC295, Germany), 2D images were captured at ×40 magnification and merged to create a single image for each histological section. Thereafter, images were analyzed using software (Leica University Suite, version 4.3, Leica Microsystems, Germany) running on a personal computer. Four sections from the central defect region and four from the peripheral defect region were analyzed 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.

Statistical analysis

Statistical analysis was performed using SPSS for Windows - version 18.0 software (SPSS, Inc., Chicago, IL, USA). All data were presented as the mean ± standard deviation (SD). Two-way ANOVA was performed at a 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 be clearly noticed (Figures 1 and 2). In addition, islands of new bone tissue mainly

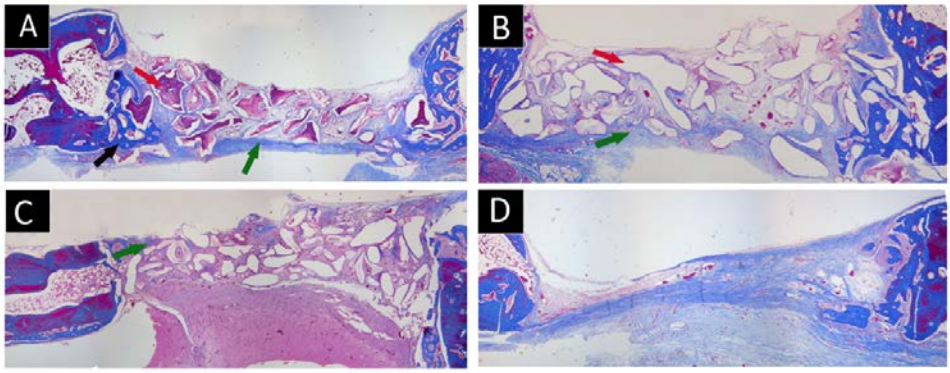

Fig. 1 – Histological specimens obtained after 3 weeks of regeneration: HAP + PLGA (A), HAP + PEI (B), Bio-Oss® grafted defects as a positive control (C) and non-grafted calvarial defect as negative control (D).
Initial signs of ossification, with newly formed bone tissue (black arrows), occurred at 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 a minimal number of inflammatory cells or without them. The empty defect of control specimen (D) was filled with connective tissue. (Goldner's Trichrome staining, ×40 magnification).
HAP– hydroxyapatite; PLGA – poly lactide-co-glycolide; PEI – polyethyleneimine.

localized at the particle surfaces or near them were detected. In the 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 the HAP + PLGA group showed a higher percentage of total bone area compared to the Bio-Oss® group, the difference was not statistically significant ($p > 0.05$). Likewise, there was no statistically significant difference in any other parameter 3 weeks after implantation (Figures 1 and 2, Table 1).

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

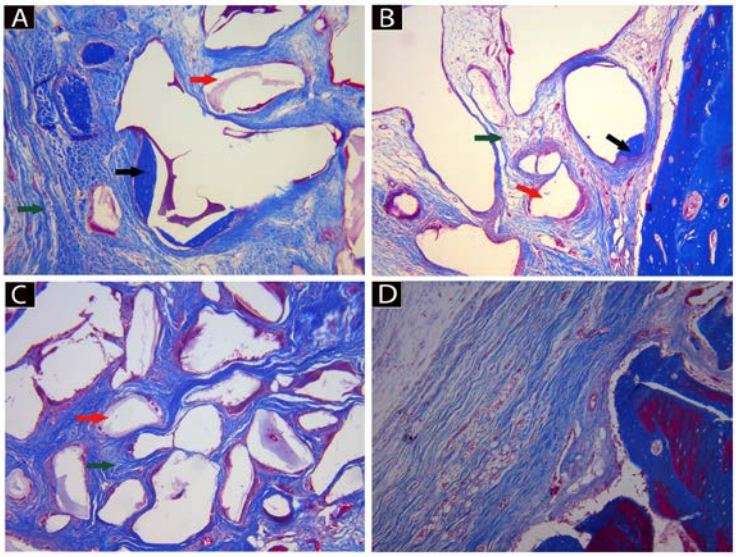

Fig. 2 – Photomicrographs of bone defects grafted with HAP + PLGA (A), HAP + PEI (B), Bio-Oss® (C) and non-grafted (empty) calvarial defect (D), obtained 3 weeks after implantation.
Although the amount of the newly formed bone differed in various test groups (with almost complete absence in empty defect), the histological structure of the 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 a minimal number of inflammatory cells, vascular stasis was observed in some specimens. (Goldner's Trichrome staining, ×100 magnification).
HAP – hydroxyapatite; PLGA – poly lactide-co-glycolide; PEI – polyethyleneimine.

Table 1

Histomorphometric analysis of the specimens 3 weeks after implantation

Variable	Bio-Oss®	HAP+PEI	HAP+PLGA	Empty defect
Graft	47 ± 2	25 ± 5 ($p < 0.001$)	47 ± 3	10 ± 1
TB	13.2 ± 0.3	8.5 ± 0.10 ($p < 0.001$)	18 ± 3	8.4 ± 0.7
MB	10 ± 0.5	6.6 ± 0.4	13 ± 3	4 ± 0.3
NB	3.2 ± 0.4	1.4 ± 0.3 ($p < 0.001$)	5 ± 1	1.30 ± 0.13
CT	39 ± 2	66 ± 6 ($p < 0.001$)	34.5 ± 1.4	85 ± 0.4
BV	0.5 ± 0.7	0.4 ± 0.40	0.68 ± 0.16	0.26 ± 0.02

All values are expressed as percentages (mean ± standard deviation).
TB – total bone; MB – mineralized bone; NB – nonmineralized bone; CT – connective tissue; BV – blood vessels; HAP – hydroxyapatite; PLGA – poly-lactide-co-glycolide; PEI – polyethyleneimine.

Table 2

Amount of inflammatory cells (ICs) under the light microscope, 3 weeks after implantation (magnification ×400)

ICs (number)	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)

All values are expressed as numbers (percentages).
HAP – hydroxyapatite; PLGA – poly-lactide-co-glycolide; PEI – polyethyleneimine.

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 the HAP + PEI and Bio-Oss® groups, while the lowest amount was observed in empty defects. In all tested groups, the newly formed bone had a lamellar structure with osteocytes which indicated bone vitality. In the majority of the samples, the absence of inflammatory cells in connective tissue was detected (Figures 3 and 4).

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

The 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 (Table 4).

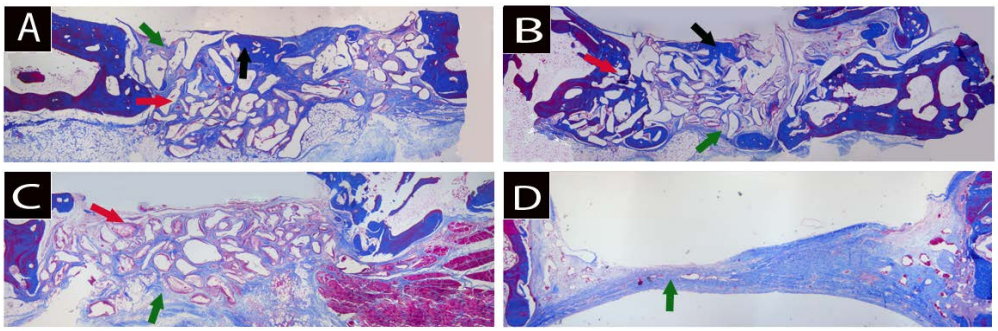

Fig.3 –Histological specimens of HAP + PLGA (A), HAP + PEI (B), Bio-Oss® (C) grafted defects, and non-grafted calvarial defect (D) obtained 6 weeks after implantation. The amount of newly formed bone tissue was larger compared to that after 3 weeks of regeneration, especially in the HAP+ PLGA group. 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 structures, which indicated the occurrence of intensive bone remodeling. Empty defect contained connective tissue without bone formation. (Goldner's Trichrome staining, ×40 magnification).
HAP – hydroxyapatite; PLGA – poly lactide-co-glycolide; PEI – polyethyleneimine

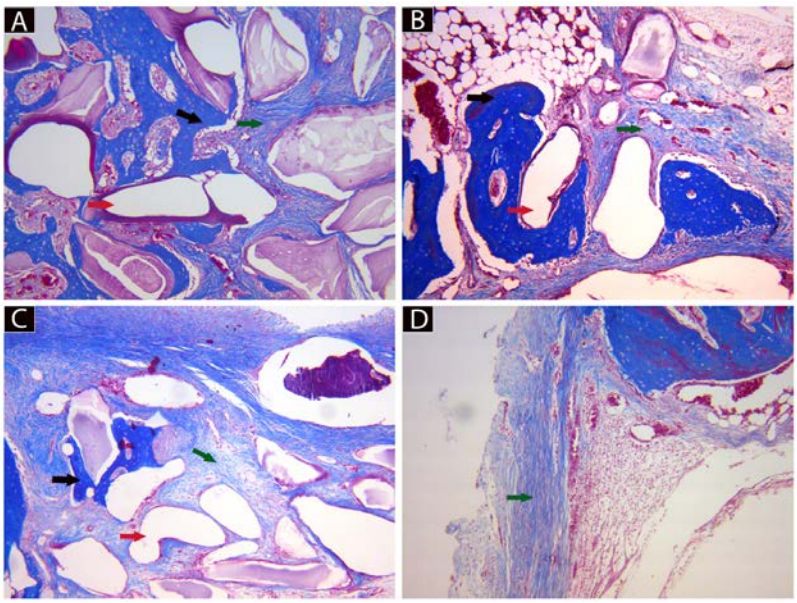

Fig. 4 – Photomicrographs of bone defects grafted with HAP + PLGA (A), HAP + PEI (B), Bio-Oss® (C), and non-grafted calvarial defect (D) obtained 6 weeks after implantation.
Defects were filled by still unabsorbed graft particles (red arrows), non-inflamed connective tissue (green arrows), and newly formed bone in close relation to particles (black arrows). The main difference in findings compared to the previous healing period (3 weeks) was an increased amount of new bone and decreased amount of graft particles due to intensive resorption via dissolution. (Goldner's Trichrome staining, ×100 magnification).
HAP – hydroxyapatite; PLGA – poly lactide-co-glycolide; PEI – polyethyleneimine.

Table 3

Histomorphometric analysis of the specimens 6 weeks after implantation

Variable	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 ($p < 0.001$)	6.6 ± 0.3
MB	10 ± 3	9 ± 0.8	14.7 ± 1.7 ($p < 0.005$)	2.64 ± 0.11
NB	4.5 ± 0.7 ($p < 0.001$)	7.5 ± 1.3	4.3 ± 0.7	3.92 ± 0.16
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

All values are expressed as mean ± standard deviation.
TB – total bone; MB – mineralized bone; NB – nonmineralized bone; CT – connective tissue;
BV – blood vessels; HAP – hydroxyapatite; PLGA – poly-lactide-co-glycolide; PEI – polyethyleneimine.

Table 4

The number of inflammatory cells (ICs) under the light microscope 6 weeks after implantation (magnification ×400)

ICs (number)	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)

All values are expressed as numbers (percentages).
HAP – hydroxyapatite; PLGA – poly-lactide-co-glycolide;
PEI – polyethyleneimine.

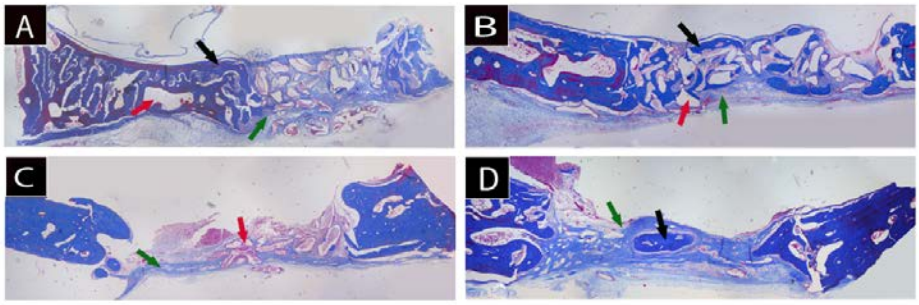

Fig. 5 – Histological specimens of HAP + PLGA (A), HAP + PEI (B), Bio-Oss® **(C) grafted defects, and non-grafted calvarial defect (D) obtained 9 weeks after implantation.**
It could be noticed that, particularly in the HAP + PLGA group, almost complete regeneration has occurred, with a large amount 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 the absence of inflammatory infiltrate. Unfilled defects predominantly resulted in connective tissue formation, with signs of spontaneous bone regeneration in the form of small islands of newly formed bone. (Goldner's Trichrome staining, ×40 magnification).
HAP – hydroxyapatite; PLGA – poly lactide-co-glycolide; PEI – polyethyleneimine.

Table 5

Histomorphometric analysis of the specimens 9 weeks after implantation

Variable	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 ($p < 0.001$)	11.94
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 ($p < 0.001$)	31 ± 3	27.3 ± 0.7	87.69
BV	0.5 ± 0.5	0.4 ± 0.7	0.5 ± 0.3	0.37

All values are expressed as mean ± standard deviation.
TB – total bone; MB – mineralized bone; NB – nonmineralized bone; CT – connective tissue; BV – blood vessels; **HAP – hydroxyapatite; PLGA – poly-lactide-co-glycolide; PEI – polyethyleneimine.**

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 the HAP + PLGA group and the Bio-Oss® group was found to be statistically significant ($p < 0.001$) (Figures 5 and 6, Table 5).

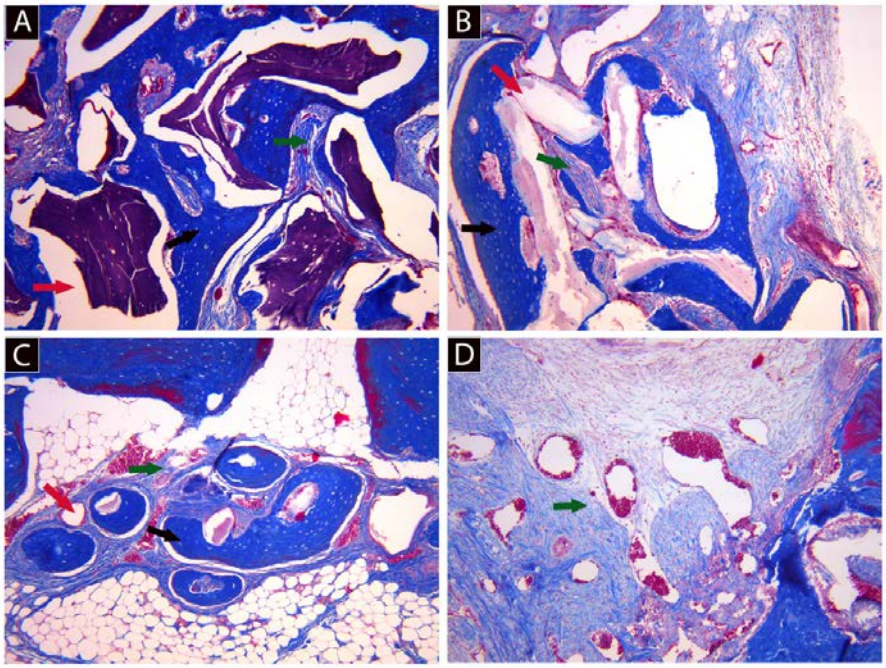

Fig. 6 – Photomicrographs of bone defects grafted with HAP + PLGA (A), HAP + PEI (B), Bio-Oss® (C), and non-grafted calvarial defect (D) obtained 9 weeks after implantation.
It could be observed that, in defects treated with HAP + PLGA and Bio-Oss, bone regeneration occurred at a significantly higher level compared to defects treated with HAP+PEI. In empty defects, bone regeneration almost completely failed, with the exception of a minor bone island in the central part of the defect. The resorption of particles with HAP+PLGA and Bio-Oss® was also remarkable. Connective tissue displayed remarkable venous stasis. (Goldner's Trichrome staining, ×100 magnification).
HAP – hydroxyapatite; PLGA – poly lactide-co-glycolide; PEI – polyethyleneimine.

Table 6

The number of inflammatory cells (ICs) under the light microscope 9 weeks after implantation (magnification ×400)

ICs (number)	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)

All values are expressed as numbers (percentages).
HAP – hydroxyapatite; PLGA – poly-lactide-co-glycolide; PEI – polyethyleneimine.

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

Discussion

The present study evaluated the regenerative potential of various coatings for HAP bone substitutes. In the HAP + PLGA group, the greatest amount of newly formed bone was noticed, accompanied by 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 the 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 the regeneration capacity of previously designed HAP scaffold. It has been demonstrated that the type of coating exhibits a considerable impact on the regeneration potential of the bone substitute. For instance, it should be observed that the newly formed bone ratio 6 weeks following the implantation was three times higher in the PLGA than in the 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 two times higher in the HAP + PLGA compared to the 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 was 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 near the graft particles, indicating osteoconductivity of the tested materials. Although a 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 the HAP + PEI group, two times higher than in the Bio-Oss® group (Table 1). This observation points out that intensive osteogenesis occurred in these specimens. In the 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 ranged from 6 to 11 in all samples obtained after 3 weeks (Table 2). This result presents a mild inflammatory reaction.

Six weeks after implantation, the highest amount of new bone was detected in the HAP + PLGA group (Figures 3 and 4, Table 3), which can be explained by a weak immune response and consequent acceleration in creating new bone tissue. It seems that the nano-topology of the HAP + PLGA coating is suitable for cell adhesion. In tested specimens, the new bone had a lamellar structure with osteocytes which indicated bone vitality. According to the number of inflammatory cells listed in 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. That can be clarified by an 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® groups (18.9% and 15%, respectively) 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 (Table 5). Nine weeks after implantation, a number of observed inflammatory cells (Table 6) indicated a mild inflammatory reaction again.

The HAP used in this study reaches an adequate balance between material resorption and new bone formation. It was previously demonstrated that HAP coated with PLGA promotes adequate bone healing 12 weeks after implantation [16, 21, 22]. This study went a step further by introducing PEI as a novel coating substance. PEI is a typical poly-cationic polymer that contains a high density of protonated secondary amines. Even though 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 [23]. In our investigations, CO_2-modified PEI coatings were used in order to decrease the toxic properties of PEI [19, 24].

Although the results obtained for HAP coated with PEI are inferior when confronting HAP + PLGA, HAP + PEI showed superior healing capacity in comparison to Bio-Oss® 6 and 9 weeks following implantation.

Concerning 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 PEI and bone morphogenetic protein (BMP)-2.

Comparing the unmodified and CO_2-modified PEI form, its cytotoxicity is remarkably less expressed in the latter. A high positive charge of unmodified PEI can damage the cell membrane and disturb critical intracellular mechanisms. CO_2 alteration mitigates this cascade without disrupting the activity of protein kinase C [24–27].

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

In addition, all the abovementioned results stand in line with the outcomes achieved by Tang et al. [29], who used a bio-inspired trimodal macro/micro/nano-porous scaffold loaded with recombinant human (rh) BMP-2 (TMS/rhBMP-2). They assumed that osteogenic promotion of TMS/ rhBMP-2 mainly occurred in the first 8 weeks after implantation. Later on, tissue maturity mostly depended on the 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 the primary bone formation stage in the study by Tang et al. [29]. In the present study, lamellar bone was formed after 9 weeks of regeneration, which is close to 8 weeks found in the investigation by Tang et al. [29]. Exogenous rhBMP-2 was important but probably not the crucial factor for the bone regeneration process in the mentioned study. Results of our experiments for periods of 6 and 9 weeks after implantation indicate that the precise biological mechanism of bone-forming after implantation remains unresolved. It is well known that bone regeneration implies biological events, including bone induction and conduction, as well as several cell types and signaling pathways. Bone grafting includes osteoinduction (BMPs and other growth factors), osteogenesis (osteoprogenitor cells), and osteoconduction (scaffold) [30]. 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 cellular colonization and tissue regeneration. A 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 the surface topography of novel HAP using 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 of considerably improving the bone regenerative potential of the synthetic HAP.

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