

Effect of implants of hydroxyapatite with tricalcium phosphates alloyed with Si on histomorphological and biochemical parameters in cases of bone defects of rabbits

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Article info

Received 08.05.2021

Received in revised form
04.06.2021

Accepted 05.06.2021

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Chemerovskiy, V. O., Rublenko, M. V., Rublenko, S. V., Ulanchych, N. V., Firstov, S. O., & Kolomiets, V. V. (2021). Effect of implants of hydroxyapatite with tricalcium phosphates alloyed with Si on histomorphological and biochemical parameters in cases of bone defects of rabbits. *Regulatory Mechanisms in Biosystems*, 12(2), 281–288. doi:10.15421/022137

Complex comminuted fractures are accompanied by development of bone defects and loss of reparative potential of the bone tissue in the region of the trauma. This brings the necessity of using implants with optimum osteoconductive and osteointegration properties. The objective of the study was determining the condition of biochemical bone markers and peculiarities of histomorphological changes under the influence of ceramic hydroxyapatite (HA) implants with various physical-chemical properties in the conditions of diaphyseal bone defects in rabbits. We composed control and experimental groups of rabbits with 10 individuals in each with diaphyseal bone defects (3 mm) of the radial bones formed under general anesthesia. In one experimental group, they were filled with granules of hydroxyapatite with α -tricalcium phosphate, and in the second group – with β -tricalcium phosphate, alloyed with Si. In the control rabbits, the defects healed under a blood clot. Blood was analyzed on the 3rd, 7th, 14th, 21st and 42nd days, and as reference we used biochemical parameters of blood of clinically healthy rabbits ($n = 10$). Bone biopsied materials were taken on days 21–42 under general anesthesia. When using hydroxyapatite with β -tricalcium phosphate, alloyed with Si, we determined early intensification of the levels of nitrogen oxide, angiogenesis and development of bone regenerate in conditions of shortening of inflammatory resorption phase, which was verified according to the level of tartrate-resistant acid phosphatase. According to the level of bone isoenzyme of alkaline phosphatase in the blood serum of animals of the control group, the reparative osteogenesis developed slowly and peaked on day 42, whereas in animals implanted with α -tricalcium phosphate, its development peaked on days 14–42, and when using Si-alloy – on days 7–14. Histomorphologically, on the 21st day, in the case of replacement of bone defect with hydroxyapatite with α -tricalcium phosphate, coarse-fibered type of bone regenerate developed with no dense contact with the elements of the regenerate, while spongy bone trabeculae occurred when hydroxyapatite was applied with β -tricalcium phosphate alloyed with Si, and the control rabbits were observed to be in the stage of cartilaginous callus. On the 42nd day, under the influence of implants of hydroxyapatite with α -tricalcium phosphate, the spongy bone tissue transformed into compact tissue with further mineralization. With implants alloyed with Si, there occurred compact bone tissue, and bone regenerates of the control animals were regions of coarse-fibered and spongy bone tissue without dense contact with the parent bone. This study revealed that hydroxyapatite with β -tricalcium phosphate alloyed with Si had notable osteoinductive and osteointegrating properties, as indicated by early angiogenesis and osteoblast reaction, positive dynamics of the marker biochemical parameters with faster and better development of bone regenerate as spongy bone trabeculae.

Keywords: bone markers; nitrogen oxide; tartrate-resistant acid phosphatase; bone isoenzyme of alkaline phosphatase.

Introduction

Generalized data of the recent studies conducted in the scope of university programs in Ukraine (Rublenko & Yeroshenko, 2012; Teliatnikov, 2013; Semeniak et al., 2014) indicate a high percentage of pathologies of the locomotor system in small pets, particularly 17.7% in the bone-joint system. The commonest bone fractures of various localization and complexity account for 71.4% of the total. The share of fractures that localize on the thoracic limbs makes up 29.9% and the share of those on pelvic limbs is 52.5%. At the same time, around 90.1% of the cases are diaphyseal fractures. Those data correlate with the foreign monitoring of orthopedic pathology in small pets (Libardoni et al., 2016), according to which 79.6% of 955 cases of fractures in dogs were diaphyseal fractures of long bones. In the monitoring of 342 cases of fractures (Mohammed et al., 2019), their localization on the thoracic limbs, particularly the humerus accounted for 5.5%, and 30.2% on the forearm bones. Nonetheless, most fractures were recorded on pelvic limbs, amounting to 27.1% of fractures of the tibia and fibula and 37.1% of femur. The most complicated fractures are considered the comminuted ones (Haaland et al., 2009), the

specific weight of which ranges within – 25.1–60.2%. In the cases of open fractures, post-traumatic osteomyelitis frequently occurs as a result of direct contamination of bone during trauma (Gielsing et al., 2019). Typically, non-viable bone fragments are removed from the area of the fracture, leading to large-scale defect of bone tissue, significant damage to endosteal and periosteal nutrition and loss of reparative potential of the bone tissue. Healing of such fractures may result in pseudoarthroses (Schlundt et al., 2018). Different ways of osteogenesis in the conditions of comminuted fractures provide fixation of the trauma site, but have no effect on induction of osteogenesis (Roberts & Rosenbaum, 2012).

As is known (Sun et al., 2016; Bahney et al., 2018), the basis of the reparative process of osteogenesis is cellular type of regeneration which is the formation of a fibrin clot in the fracture site, which contains a significant amount of undifferentiated mesenchymal cells and growth factors. Then, they differentiate to fibroblasts that synthesize collagen fibers. In the region of lysis of marginal fragments, a vascular network grows that forms from the margin of the bone to the center of formed fibrous callus, where the osteoblasts migrate, and then trabeculae of the bone begin to develop in the fibrous callus. The entire zone of reconstruction at this moment is

divided into three main zones: 1) central zone, where the vascular network grows between the developed cartilaginous callus; 2) remodeling zone where the spongy bone restructures into compact bone tissue; 3) mineralization zone, where the bone regenerate is finally saturated with mineral component (Kanczler & Oreffo, 2008; Lafuente et al., 2009).

In cases of a simple fracture, to induce the reparative osteogenesis and ensure its success, the presence of a natural matrix the role of which is played by fibrin is needed at its initial stage (Onopryenko & Voloshyn, 2017). However, in cases of complex comminuted fractures, defects need to be repaired with osteoplastic materials (Saglsyn et al., 2016). Currently, (Talashova et al., 2012; Rahmati et al., 2018) the following types of materials are proposed: 1) bioorganic – demineralized bone matrix, collagen, fibrin components; 2) ceramic materials – oxides of aluminum and zirconium, calcium-phosphate ceramics; 3) synthetic polymers – polymethyl methacrylate, polyglycolide, polydioxanone; 4) multi-component materials of various types (Reznik et al., 2019; Kadiroglu et al., 2020).

In general, they should correspond to the following demands: be biocompatible; able to carry out osteointegration, resorption and gradual replacement by bone tissue, have osteoconductive and osteoinductive properties, be relatively simple to introduce to the defect site and be able to be used for modeling during surgery, and have adequate biomechanical properties. These requirements are mostly met by the materials based on calcium-phosphate ceramics, which for their biological properties are called bioactive ceramics (Huryn et al., 2012; Oliveira et al., 2017; Deveci et al., 2020). Earlier, we (Rublenko et al., 2018; Chemerovskiy, 2020; Rublenko et al., 2020) conducted X-ray, macromorphological and hematological evaluations of osteointegrational properties of samples of calcium-phosphate ceramics of hydroxyapatite and β -tricalcium phosphate and alloyed with Si with various physical-chemical properties, which were used on rabbits with modeled fractures and dogs with complex diaphyseal bone fractures of long bones, which were brought to the University Clinic of Small Pets.

The objective of the study was the condition of biochemical bone markers and peculiarities of histomorphological changes under the effect

of implants of hydroxyapatite ceramics with various physical-chemical properties in the conditions of diaphyseal bone defects in rabbits.

Materials and methods

The study was performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Official Journal of the European Union L276/33, 2010). The protocol of the study was approved by the Ethics Committee of Bila Tserkva National Agrarian University (Conclusion No. 3 of 31.05.2018). The study was carried out at Bila Tserkva National Agrarian University at the Department of Surgery and Diseases of Small Pets in 2019 on 3 month old clinically healthy Californian rabbits weighing 2.5 kg. The animals were kept in the University vivarium cells in rooms with obligatory ventilation and combined light and daily feeding. Feeding was done using specialized compound feed for rabbits in the dose of 200 g per individual a day. The animals had unlimited access to water. We formed two experimental and control groups of rabbits with 10 individuals in each. In animals of all groups, in diaphyseal radial bones, we made defects of the compact bone tissue. Surgery was done on the dorsal-lateral surface according to the rules of asepsis and antiseptics.

Before the surgical modeling of bone defects, anesthesia comprised intramuscular injection of 2% solution of xylazine (1–3 mg/kg), subcutaneous injection of 1% solution of butorphanol (0.2–0.4 mg/kg) and intravenous injection of thiopental (5–8 mg/kg, sodium thiopental, TLC Brovafarma, Ukraine) and local infiltrational anesthesia with 0.5% solution of lidocaine (3–4 mg/kg). After the dissection of the periosteum, we formed a bone defect using a drill (3 mm). In rabbits of the control group ($n = 10$), bone defects were left to heal under the blood clot, and in rabbits of the first experimental group ($n = 10$) these defects were filled with hydroxyapatite with α -tricalcium phosphate (HA/ α -TCP-500) and the second experimental group ($n = 10$) – hydroxyapatite with β -tricalcium phosphate alloyed with Si (HA/ β -TCP/I-Si-700, Fig. 1). Wounds were stitched using noose suture technique.



Fig. 1. Macromorphological picture of the formed defects in rabbits: *a* – control group, the defect has been healing under the blood clot; *b* – the first experimental group – HA/ α -TCP-500; *c* – the second experimental group – HA/ β -TCP/I-Si-700

After surgery, the stitches of the rabbits were twice a day treated with ioddicerin (antiseptic dug, one gram of which contains 5 mg of iodine and additional substances: dimethyl sulfoxide, glycerol and potassium iodide) for five days. Throughout the study, daily general clinical examination was conducted, including: time of each animal's recovery from anesthesia, development of inflammatory reaction in the region of the conducted surgery, functional condition of the limbs, and conducted palpation study for presence of densification in the region of the formed defect. Visual assessment of the healing process was made before removing the sutures. The wounds in the rabbits of all groups were treated by per primam intentionem technique, the sutures were removed on day 7 of the study. For biochemical assays of blood of rabbits, we drew blood from the jugular vein before anesthesia and on 3rd, 7th, 21st and 42nd days after forming the experimental model bone defects.

Materials for implantation were synthesized at the Institute of Problems of Materials Science named after I. M. Frantsebych of the National Academy of Sciences of Ukraine (Kyiv). The material implanted to ani-

mals of the first experimental group was two phase calcium-phosphate granules of HA/ α -TCP-500, which according to the results of X-ray phase analysis (Fig. 2a) comprised 70% of phase of hydroxyapatite and 30% of α -tricalcium phosphate. The size of the granules equaled 400–600 μ m. Adsorbing activity of granules of HA/ α -TCP-500 equaled 118.7 mg/g. Adsorptive activity of granules of bioactive ceramics was determined according to GOST 4453-74. Saturation of the granules was done using aqueous solution of methylene blue.

The material that was implanted to the animals of the second experimental group was HA/ β -TCP/I-Si-700 – rinsed two-phase granules, Si-alloyed, which according to the results of the X-ray analysis (Fig. 3b) consisted of 72% of hydroxyapatite and 28% of β -tricalcium phosphate, burned at the temperature of 850 °C; size of granules was 600–800 μ m. Adsorbing activity of granules of HA/ β -TCP/I-Si-700 – 229.1 mg/g. Content of silica 1.3% in granules after sintering was determined using X-ray fluorescent express analyzer Expert3L, manufactured by INAM, Ukraine, 2019, equaling 1.2%. It has to be noted that despite increase in

the size of the granules, adsorption activity of HA/ β -TCP/l-Si-700 granules significantly exceeded the adsorption activity of HA/ α -TCP-500 due to increase in porosity, which is clearly shown by the photos of microstructure of the materials.

In the blood serum, we determined the content of nitrogen oxide (NO) according to the level of metabolites. As restorative agent, we used

granules of metabolic cadmium, which were added to the samples of blood serum after sedimentation of protein in it and held for 15 h. Concentration of nitrates was determined using Green's technique (Grand et al., 2001) modified by Holykov (2004). The coloured complex formed as a result of interaction of nitrites of serum with Grease reagent underwent colorimetry in Stat Fax 4500 spectrophotometer at the wave length of 540 nm.

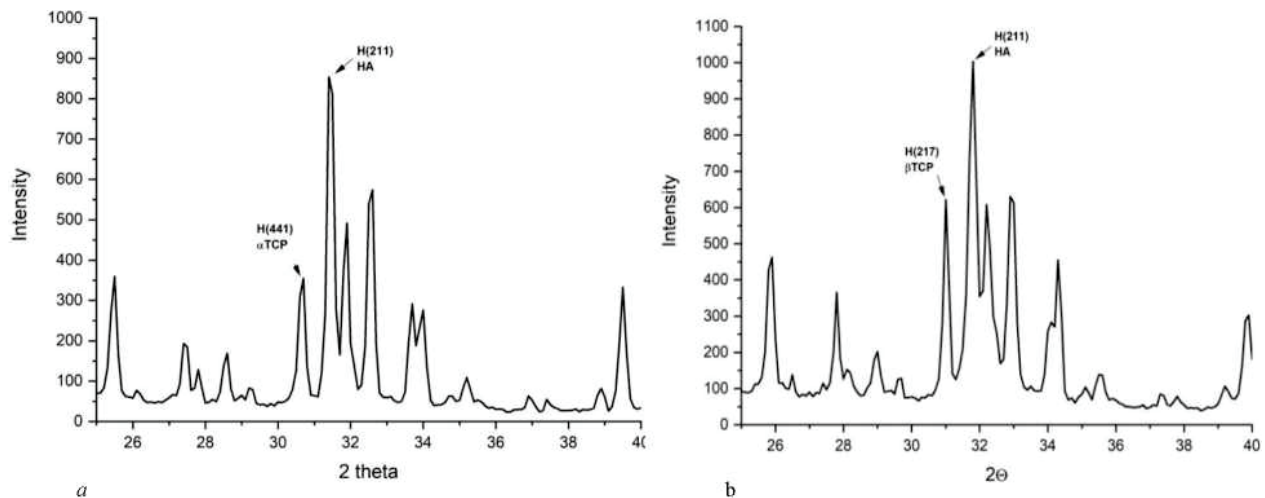


Fig. 2. Diffractograms of granules used on rabbits of the experimental groups: *a* – the first experimental – hydroxyapatite with α -tricalcium phosphate (HA/ α -TCP-500); *b* – the second experimental – hydroxyapatite with β -tricalcium phosphate, alloyed with Si (HA/ β -TCP/l-Si-700); abscissa axis indicates diffractory peaks; ordinate axis – intensity of diffraction

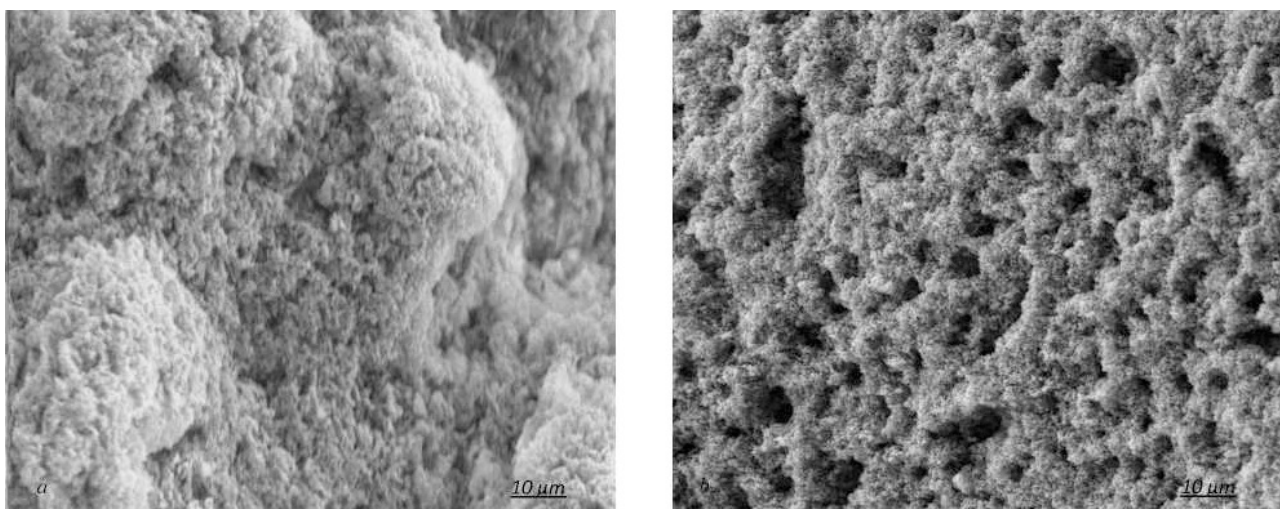


Fig. 3. Microstructures of the external surface of granules of composite materials used on rabbits of the experimental groups were obtained using scanning electron microscope (SEM): *a* – the first experimental – hydroxyapatite with α -tricalcium phosphate (HA/ α -TCP-500); *b* – second experimental – hydroxyapatite with β -tricalcium phosphate alloyed with Si (HA/ β -TCP/l-Si-700)

Activity of bone isoenzyme of alkaline phosphatase was determined in blood serum using the method based on splitting of phenyl phosphate with formation of phenol. Alkaline phosphatase is able to split substrate-4-nitrophenyl phosphate, formed of 4-nitrophenole and phosphate. Reaction was stopped using 30 mM of solution of trilon B in 1 M NaOH. The extent of activity of the enzyme is the amount of released product of the reaction – 4-nitrophenol that provides yellow colour in alkaline environment, intensity of which is determined using spectrometer at $\lambda = 410$ nm. Activity of alkaline phosphatase was expressed in units/L, where units were the amount of μmol of 4-nitrophenol, which forms in 1 min at the temperature of $+37^\circ\text{C}$ under the effect of alkaline phosphatase contained in 1 L of blood serum. To determine the activity of thermostable alkaline phosphatase, we performed thermal inhibition of blood serum in $+56^\circ\text{C}$ for 15 min, for which we transferred the samples into an ice bath for 5 min. Activity of bone isoform of alkaline phosphatase was calculated according to the difference between the activities of the total and thermostable alkaline phosphatases.

Tartrate-resistant acidic phosphatase was determined in blood serum using Vital reagents kit (Russia), the working principle of which is the

value of p-nitrophenol formed in unit time, proportional activity of enzyme, which is determined by optical density of sample at 405 nm.

Total calcium was determined in blood serum using Filisit-Diagnostyka (Ukraine). The technique works as follows: ions of calcium in alkaline environment react with o-cresolphthalein complexone and form a coloured complex. Intensity of colouration of the violet complex was proportional to calcium concentration in the experimental sample.

Inorganic phosphorus in blood serum was determined using Filisit-Diagnostyka (Ukraine). The method works as follows: by acting with molybdic acid, inorganic phosphorus forms phosphomolybdic acid, which reduces in the presence of iron (II) into molybdenum blue. Proteins that settled using the indicator reagent dissolve after adding stabilizer triethanolamin. Optical density of reactive solution was proportional to the concentration of inorganic phosphorus in the sample.

The animals were euthanized on days 21–42 by intravenous injection of thiopental in the dose of 50 mg/kg. The samples of bone tissue extracted on days 21–42 were studied histologically. Bone biopsied materials were fixated in neutral formaline, de-calcinated using specialized solution (Rapid decalcifier, manufactured by Kaltek, Italy), and then they

were dehydrated in alcohols of increasing concentration, exposed to xylol and embedded in paraffin (PlastiWax, manufactured by Kaltek, Italy). Then, on rotational microtome, we made paraffin 5–10 μmol histological sections of the biopsied materials, which were stained with hematoxylin and alcohol solution of eosin. Histological analysis of the sections was performed under trinocular microscope Fusion FS 7530 manufactured by Micromed (China) with specialized camera Microscope digital eyepiece MDC-500 and using software Vividia Able Scope. Histological conclusions were evaluated by scoring, as indicated in the Table 1.

Table 1

System of points of the histological evaluation of the bone regenerates in the microscopic study, proposed by the authors of the article

Points	Type of tissues in the bone regenerate
1	Dense fibrous connective tissue
2	Developed fibrous callus, emergence of centers of hyaline cartilage
3	Hyaline cartilage developed, remains of the fibrocartilage callus are present
4	Coarse-fiber bone tissue has developed, the spongy bone trabeculae begin to develop
5	Spongy trabeculae formed, the remains of coarse-fiber bone tissue are present
6	Spongy trabeculae have developed, the compact bone tissue begin to form
7	Compact bone tissue has formed, the remains of spongy trabeculae are present
8	Only compact bone tissue

The results were statistically analyzed in Statistica software (StatSoft Inc., USA, 2011). The data are presented in the tables as $x \pm SD$ ($x \pm$ standard deviation). Differences between the groups were determined using ANOVA, considering significant those at $P < 0.05$ (taking into account Bonferroni correction).

Table 2

Dynamics of biochemical markers in blood serum of rabbits using reparative osteogenesis ($x \pm SD$, $n = 5$)

Parameters	Clinically healthy rabbits, $n = 10$	Groups	Day 3	Day 7	Day 14	Day 21	Day 42
Nitrogen oxide, $\mu\text{mol/L}$	29.5 ± 0.5	control	$35.9 \pm 1.9^{**}$	30.1 ± 1.5	29.7 ± 0.6	30.3 ± 1.1	27.9 ± 1.3
		HA/ α -TCP-500	$36.7 \pm 1.5^{***}$	31.1 ± 2.5	32.6 ± 1.4	$35.6 \pm 0.3^{****}$	30.3 ± 1.6
		HA/ β -TCP/I-Si-700	$38.7 \pm 1.6^{***}$	30.7 ± 1.3	$36.5 \pm 1.2^{****}$	31.1 ± 1.2	31.1 ± 1.2
Tartrate-resistant acid phosphatase, U/L	32.4 ± 0.4	control	36.1 ± 1.7	31.6 ± 1.1	$36.6 \pm 0.8^{***}$	$44.5 \pm 1.8^{***}$	$41.8 \pm 1.8^{***}$
		HA/ α -TCP-500	$37.2 \pm 2.1^*$	$38.7 \pm 1.2^{****}$	$40.9 \pm 1.4^{***}$	$43.5 \pm 3.5^{**}$	37.3 ± 5.6
		HA/ β -TCP/I-Si-700	33.5 ± 1.6	31.1 ± 2.1	$43.5 \pm 1.5^{***}$	$40.4 \pm 0.8^{***}$	$36.3 \pm 1.1^{**}$
Bone isoenzyme of alkaline phosphatase, U/L	41.5 ± 1.3	control	$48.9 \pm 1.5^{***}$	$46.5 \pm 1.7^*$	$45.9 \pm 1.3^*$	$47.6 \pm 2.2^*$	$63.1 \pm 1.5^{***}$
		HA/ α -TCP-500	45.9 ± 3.9	44.5 ± 3.3	$57.9 \pm 1.1^{****}$	$56.1 \pm 1.5^{***}$	61.7 ± 2.1
		HA/ β -TCP/I-Si-700	45.9 ± 2.7	$51.5 \pm 2.4^{**}$	$58.8 \pm 0.7^{****}$	45.5 ± 2.3	$45.3 \pm 1.6^{***}$
Total calcium, mmol/L	2.43 ± 0.04	control	2.35 ± 0.15	2.43 ± 0.17	2.41 ± 0.19	2.25 ± 0.12	2.34 ± 0.12
		HA/ α -TCP-500	2.24 ± 0.31	2.36 ± 0.13	2.54 ± 0.13	2.75 ± 0.25	2.26 ± 0.22
		HA/ β -TCP/I-Si-700	2.36 ± 0.15	2.64 ± 0.23	$2.77 \pm 0.13^*$	$2.76 \pm 0.14^*$	2.23 ± 0.11
Inorganic phosphorus, mmol/L	1.74 ± 0.06	control	1.54 ± 0.22	$1.47 \pm 0.08^{**}$	$1.35 \pm 0.15^*$	1.56 ± 0.14	1.53 ± 0.15
		HA/ α -TCP-500	$1.35 \pm 0.07^{***}$	1.53 ± 0.13	$1.46 \pm 0.15^{**}$	1.65 ± 0.13	1.57 ± 0.09
		HA/ β -TCP/I-Si-700	$1.43 \pm 0.08^{**}$	$1.53 \pm 0.05^*$	1.64 ± 0.09	1.47 ± 0.19	$1.34 \pm 0.06^{***}$

Note: the control group – the defect was healing under the blood clot; HA/ α -TCP-500 – the first experimental; HA/ β -TCP/I-Si-700 – the second experimental; * – $P < 0.05$; ** – $P < 0.01$; *** – $P < 0.001$, compared with the parameters of the control group in this period of the study using ANOVA and Bonferroni correction; • – $P < 0.05$; •• – $P < 0.01$; ••• – $P < 0.001$, compared with the parameters of clinically healthy animals using ANOVA and Bonferroni correction.

In the cases of healing under a blood clot, the activity of tartrate-resistant acid phosphatase started to increase ($P < 0.001$) on the 14th day, reached the peak on day 21, and remained at approximately the same level until day 42. In the first experimental group, the activity of tartrate-resistant acid phosphatase increased ($P < 0.05$) already on the 3rd day and continued to dynamically increase up to the peak occurring on day 21 – 43.5 ± 3.5 U/L. Then, on the 42nd day, it was no different from the parameters of the healthy rabbits. At the same time, in the second experimental group, the level of tartrate-resistant acid phosphatase reached the peak on day 14 – 43.5 ± 1.5 U/L, and then dynamically dropped. The activity of bone isoenzyme of alkaline phosphatase in the control group began to gradually increase on the 3rd day and reached the peak on the 42nd day, equaling 63.1 ± 1.5 U/L. When applying HA/ α -TCP-500 (the first experimental group), it rapidly increased on the 14th day and remained near the achieved level till the 42nd day. In the second experimental group, the activity of bone isoenzyme of alkaline phosphatase significantly increased starting on day 7 and reached the peak on day 14. Changes in the content

Results

For the first 6 h after surgery, the bodies of the animals were in a spatially natural position. The general condition was satisfactory throughout the period of research. On the 1st day, in the region of the formed defect, we recorded gradual inflammatory reaction of the surrounding tissues, no differences in the reactions between the groups of animals were observed. When moving, the animals were observed to have no functional damage to the limb on which the defect was formed. Sutures on the animals were removed on the 7th day, because no edema or soreness were present, the margins of the wound were held by the adhesion of the connective tissues in the condition of epithelialization. In all the animals of all groups, palpation revealed painless densification of the tissues in the area of the formed defect, which in the control groups later somewhat increased in size by the 21st day. When using hydroxyapatite with α -tricalcium phosphate, present until the 21st day, and in the case of β -tricalcium phosphate and Si alloy, palpation revealed insignificant densification on the 14th day.

In the conditions of healing of the bone defect under blood clot (the control group) (Table 2), we determined an increase in the level of nitrogen oxide on the 3rd day ($P < 0.01$), which reflects the course of post-traumatic inflammatory process. However, by the 42nd day, it had decreased ($P < 0.05$) compared with clinically healthy animals. When using hydroxyapatite with α -tricalcium phosphate (HA/ α -TCP-500 – the first experimental), the group was observed to have two peaks of increase in the concentration of nitrogen oxide on days 3–21, which was 1.2 ($P < 0.01$) higher compared with the control. In (the second experimental) group – hydroxyapatite with β -tricalcium phosphate alloyed with Si (HA/ β -TCP/I-Si-700), the peak parameters of nitrogen oxide were recorded on days 3–14 of the reparative osteogenesis.

of calcium in all groups and during all the periods of the study were within the physiological norm and had practically no statistically significant fluctuations. The same was seen for the dynamics of the concentration of inorganic phosphorus in the blood. However, there were periods in each group when significant changes in its content occurred. In general, a tendency towards its decrease was seen in the control – in the period of days 7–14, in the first experimental group – on days 3–14, and in the second experimental group – on day 42.

According to the results of histological analysis of diaphyseal fragments, bone regenerate in the control group on day 21 (Fig. 6a) was represented on the perimeter by immature osteoid rods, chondroid structure, and also swollen and densely-fibrous connective tissue with notable inflammatory infiltration. At the same time, in the central part of the bone defect, we observed fibrous connective tissue with notable proliferation of fibroblasts and active development of collagen fibers that were filling its entire cavity. On the 21st day of using hydroxyapatite with α -tricalcium phosphate, in the (first experimental) group, the bone regenerate (Fig. 6b)

formed as a result of endosteal and periosteal osteogeneses. We recorded replacement of bone defect to a large extent by coarse-fibered bone tissue, and on the perimeter of the areas of the parent bone, we saw stages of resorption. Spongy type trabeculae on the perimeter of the defect had moderate (low) numbers of osteogenous cells. Granules of ceramics in most cases had no close contact with the bone regenerate. In the intramedullary canal, we observed a large amount of fatty tissue, among which we visualized regions of red bone marrow. It was also present and in some spaces between the rods. The area of the bone defect was clearly seen due to connection of trabecular bone rods to compact parent bone.

In the experiment with β -tricalcium phosphate alloyed with Si, on the 21st day, the second experimental group was observed to have (Fig. 6c) the newly formed bone tissue developed by spongy and coarse-fibered types, which filled the defect from the sides of the bone wound. At the same time, in the defect, we observed bone trabeculae of lamella structure which mostly had dense contact with granules of the composite material. On the periphery of coarse-fibered bone trabeculae, we found rows of osteoblasts. This indicated thickening of bone rods till the formation of compact bone tissue. Fragments of the parent bone located near the zone of traumatic damage had signs of post-traumatic reconstruction, suggesting enlargement of the vascular canals and development of "islands" of newly-formed tissue on the surface of bone trabeculae. In the inter-trabeculae spaces of the spongy bone, we observed presence of red bone marrow.

On the 42nd day, in the control group (Fig. 6d), we saw notable endosteal and periosteal osteogenesis in the form of massive growths of coarse-fibered and spongy bone tissues filling the intramedullary canal and growth from the side of the periosteum. They overlaid the parent bone tissue, clearly separately from one another. Bone regenerate had no dense contact with the parent bone. These growths from the side of periosteum

spread across the parent bone (intact) up to the perimeter of the defect, but did not fill the defect itself. The intramedullary canal was completely filled with the regenerate as a result of the endosteal osteogenesis, but with weak contact with the parent bone. This indicated absence of the developed compact lamella in the defect site. Instead, in the cavities between the rods, formed red bone marrow was seen, and the bone canal itself was mostly represented by the connective tissue that contacted with the intact bone. The external part of the regenerate was also represented by fibrous structures of the connective tissue with chaotically formed capillaries and vascular canals.

On day 42, in the first experimental group HA/ α -TCP-500, we observed (Fig. 6e) partial replacement of the bone defect with coarse-fibered but mostly trabecular spongy bone tissue. It was represented by bone rods at the stage of thickening, and rows of osteoblasts were seen on their periphery. Granules of ceramics were in the spaces between the bone rods, mostly without dense contact with bone regenerate. Also, we saw fatty tissue with regions of red bone marrow. The region of bone regenerate was clearly different by formed rods of spongy type. On day 42, in the second experimental group HA/ β -TCP/l-Si-700, in the area of the defect, we observed (Fig. 6f) partially spongy, but mostly compact bone tissue.

The periphery of the granules of the ceramics had dense contact with the grown bone tissue. At the same time, trabeculae of compact type, developed into osteoid constructions, were represented by equal concentric lines of connection between the structures of organic matrix with numerous osteocytes located between them. In the intramedullary canal, there was mostly fatty tissue, where we visualized regions of red bone marrow. Granules of the implant were in small amounts found in the central bone canal, densely contacting with bone trabeculae of the endosteum. Some of the granules contacted with the red bone marrow and fatty cells.

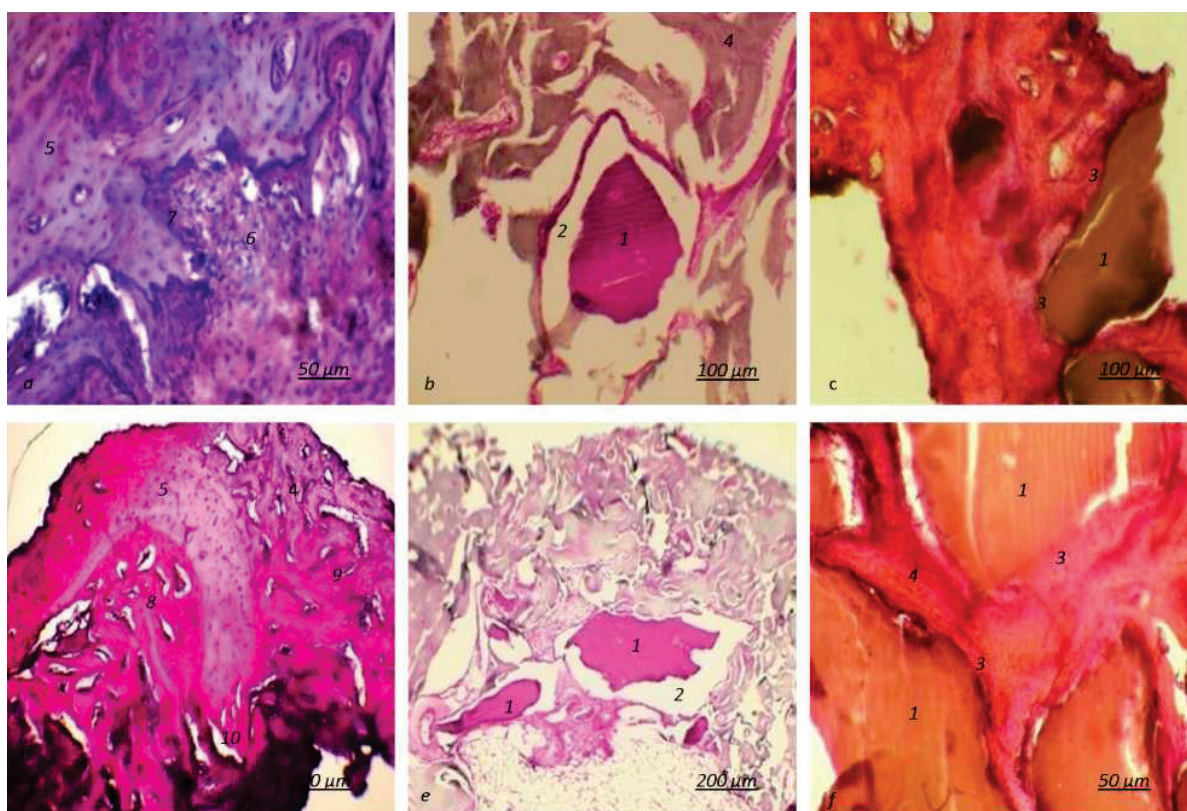


Fig. 4. Histological analysis of bone regenerates of diaphyses of radial bones of rabbits: *a* – bone regenerate on the 21st day in the control group, defects healed under the blood clot, *b* – bone regenerate on the 21st day when using hydroxyapatite with α -tricalcium phosphate, first experimental group, *c* – bone regenerate on day 21 of using β -tricalcium phosphate alloyed with Si, *d* – bone regenerate on the 42nd day in the control group, the defects were healing under blood clot, *e* – bone regenerate on day 42 of using hydroxyapatite with α -tricalcium phosphate, the first experimental group, *f* – bone regenerate on day 42 of using β -tricalcium phosphate alloyed with Si; 1 – granule of implanted material; 2 – absence of contact between granule and bone regenerate; 3 – dense contact between the granules and bone regenerate; 4 – bone trabeculae of spongy type; 5 – intact bone tissue; 6 – densely fibrous connective tissue with notable inflammatory infiltration; 7 – demarking line of separation of intact bone from regenerate; 8 – filling of intramedullary canal with coarse-fibered and spongy bone tissues; 9 – massive growths from the side of periosteum of the bone tissue of spongy type; 10 – absence of dense contact between the parent bone and bone regenerate in the area of the formed defect; hematoxylin and eosin staining

Analysis of basal and histological evaluation (Table 3) according to the tissue criteria of growth of bone regenerate indicated its more dynamic development in the groups implanted with ceramics to the bone defect. At the same time, comparing the samples of ceramics demonstrated greater efficiency of hydroxyapatite with β -tricalcium phosphate alloyed with Si.

Table 3

Dynamics of histological basal criteria of bone regenerates in rabbits on days 21–42 ($\bar{x} \pm SD$, $n = 5$)

Periods	Control, bone defect was healing under blood clot	HA/ α -TCP-500, bone defect was implanted with granules of hydroxyapatite with α -tricalcium phosphate	HA/ β -TCP/I-Si-700, granules of hydroxyapatite β -tricalcium phosphate with Si alloy was implanted into the bone defect
21 day	1.95 ± 0.33	$4.32 \pm 0.23^{***}$	$4.84 \pm 0.16^{***}$
42 day	4.63 ± 0.35	$6.43 \pm 0.14^{**}$	$7.23 \pm 0.18^{***\bullet}$

Note: * – $P < 0.05$, ** – $P < 0.01$, *** – $P < 0.001$ compared with the parameters of the control group using ANOVA and Bonferroni corrections; • – $P < 0.05$, •• – $P < 0.01$, ••• – $P < 0.001$ compared between the parameters of the first and the second experimental groups in the same period using ANOVA and Bonferroni correction.

Discussion

Surgical treatment of fractures in animals seems to be quite widely studied thanks to the introduction of various methods of osteogenesis, and recently due to the use of implants of various materials (Yawon et al., 2017; Yardımcı et al., 2018; Guiot et al., 2019). Nonetheless, high-energy fractures, particularly comminuted fractures or cases of osteomyelitis and pseudoarthrosis, are accompanied by bone defects of various sizes. In this case, regenerative potential of the bone tissue is significantly reduced. Therefore, various osteoconductive implants are used for replacement of these defects. The choice of implants is currently quite broad, but mostly, these materials are based on hydroxyapatite with β -tricalcium phosphate, α -tricalcium phosphate and collagen (Berchenko et al., 2006; Deev et al., 2015). The volume of such studies is gradually increasing due to the necessity of objective assessment of the effects of these materials on the reparative osteogenesis. The ceramics mostly used in Ukraine are based on hydroxyapatite (Shymon et al., 2020). At the same time, there is an ongoing discussion on the issues of the optimum composition, size of pores, rates of resorption of these materials, possibilities of providing them with osteoinductive properties by introduction of composites of osteotrophic microelements, particularly Si, which plays an important role in bone metabolism because of the ability to stimulate angiogenesis (Neill et al., 2018).

Reparative osteogenesis, particularly production of type I bone collagen, significantly depends on the level of oxygenation. Its sufficient level provides early activation of angiogenesis. At the same time, processes of angiogenesis are induced by nitrogen oxide. Dynamic and successful reparative osteogenesis needs early angiogenesis, one of the main factors of which is nitrogen oxide (NO). It stimulates expression of interleukins and integrins by endothelial cells, thereby promoting their migration and differentiation. During synthesis of nitrogen oxide by activation of iNOS to a larger degree and e-NOS to a lower degree, endothelial cells migrate to the repairing zone, which contributes to formation of the canal network (Yang et al., 2018). In our previous work (Rublenko & Shahanenko, 2011) and the studies by other authors (Shimokawa & Godo, 2016; Yang et al., 2018), it was found that gradual increase in the level of nitrogen oxide in blood is accompanied by strengthening of the tissue activator of plasminogenesis, which has a positive effect on angiogenesis and microcirculation in the fracture zone. The results of the presented study indicate that implantation of ceramics alloyed with Si into the bone defect promoted the growth of spongy trabeculae in it already on the 21st day as a result of early activation of endothelial cells and migration of mesenchymal cells that had stimulated intense osteoblast reaction. Particularly in this group of animals, we observed the optimal dynamics of changes in the level of nitrogen oxide, and its increase on day 14 preceded the intensification of osteogenesis. That means, because the level of nitrogen oxide is a marker of endothelial function, in the case of using HA/ β -TCP/I-Si-700 we can state earlier intensification of angiogenesis and development of

bone regenerate. As is known (Torbenko & Kasavyna 1977; Lieshchova et al., 2018), levels of calcium and phosphorus in blood serum are not biochemical markers of bone metabolism, and in the conditions of reparative osteogenesis, the mineral component mobilizes and re-distributes within the bone tissue. Only increase in concentration of calcium in urea (on an empty stomach) may indicate resorption of the bone tissue. This was also reflected in the presented study.

Moreover, this study demonstrates biochemical parameters of the two main markers of bone metabolism, tartrate-resistant acid phosphatase and bone isoenzyme of alkaline phosphatase (Paskalev, 2010; Nakoskyn et al., 2018). Recently, they have been broadly used to evaluate the reparative osteogenesis. Processes of osteoresorption are provided by the cells of the bone tissue of osteoclasts (Moskalets et al., 2012), and their activity reflects the level of tartrate-resistant acid phosphatase in blood serum. This phenomenon reflects the intensity and duration of the inflammatory-resorption stage of reparative osteogenesis on the one hand, and the presence of its various complications on the other hand. It is produced by osteoclasts and generates active oxygen compounds that ruin the matrix components of the bone tissue, which reflects the degree of its resorption. Osteoblasts are responsible for the processes of osteogenesis. They produce tetrameric glycoprotein, which plays an important role in mineralization of intercellular substance of the organic matrix of the bone tissue as a result of separation of the phosphate groups from other proteins. This results in increase in the local concentration of phosphorus and decrease in concentration of pyrophosphate – mineralization inhibitor. This process provides bone isoenzyme of alkaline phosphatase (Hylev et al., 2020). According to the parameter of activity of tartrate-resistant acid phosphatase, the period of osteoresorption in the control group has been developing slowly and was stretched in time, was early in the case of using HA/ α -TCP-500, occurring during inflammatory-resorptive stage and period of early osteogenesis, and was short in the case of using ceramics with Si alloy, occurring during the transition from the inflammatory-resorptive phase into osteogenesis. Histomorphologically, on day 21, this reflected among the control animals in the stage of cartilaginous callus, whereas in the first experimental group with HA/ α -TCP-500 implantation – in formation of bone regenerate of mostly coarse-fibered type without dense contact between the ceramics and regenerate, and in the second group – in notable signs of consolidation of regenerate and the parent bone tissue of internal and external surfaces of diaphysis and ossification of young bone tissue, having the regenerate growing together with the margins of bone defect. Then, on day 42, in the second experimental group, compared with the first and control groups, we observed consolidated bone regenerate formed mostly of compact bone trabeculae with embedded osteocytes, indicating final stages of mineralization, whereas presence of coarse-fibered and spongy bone tissue in the control group indicated the prolonged time of reparative osteogenesis.

Thus, the dynamics of the activity of bone regenerate of isoenzyme of alkaline phosphatase and tartrate-resistant acid phosphatase in blood serum of rabbits completely correlates with the histomorphological picture of reparative osteogenesis when using the studied materials. At the same time, it indicates an optimum course using hydroxyapatite with β -tricalcium phosphate of ceramics alloyed with Si.

Conclusion

Ceramics based on hydroxyapatite with β -tricalcium phosphate with Si alloy, along with optimum osteo-inductive characteristics have notable osteointegrative and osteoinductive properties, as confirmed by the early reaction of endothelial cells with induction of angiogenesis, fast and successful growth of bone regenerate comprising trabeculae of spongy type as a result of early osteoblast reaction, and new compact bone tissue that integrated to the parent bone, which grew in a short period of time. At the same time, histological evaluation of the course of reparative osteogenesis in the conditions of modeled diaphyseal bone defect of radial bones in rabbits, which is expressed in scores, statistically significantly verifies the dynamic development of bone regenerate in cases of implanting hydroxyapatite with β -tricalcium phosphate and Si ceramics ions, recorded on days 21 and 42 of the consolidation of modeled fractures, compared with healing under blood clot and implantation of non-alloyed ceramics with

α -tricalcium phosphate. Currently, this is statistically significantly reflected in the dynamics of such biochemical markers as nitrogen oxide, bone isoenzyme of alkaline phosphatase and tartrate-resistant acid phosphatase, peaks of which correlate with histomorphological criteria of reparative osteogenesis in the case of damage to compact bone tissue of long tubular bones.

The obtained results allow us to extrapolate them to clinical studies with perspective of using implants in the content of hydroxyapatite with β -tricalcium phosphate alloyed with Si in cases of comminuted bone fractures in domestic and industrial animals.

This study was carried out in the scope of the State Topic "State studies of products made of developed biomaterials", financed by the National Academy of Sciences of Ukraine (State Registration No. 0119U102083).

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