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Histomorphological evaluation of osteoreplacement with germanium-doped calcium phosphate ceramics for model bone defects in rabbits

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Abstract: To give calcium phosphate ceramics osteoinductive properties, they are doped with silicon, germanium, zinc, and magnesium ions. The aim of this study was to evaluate the effects of doped and undoped calcium phosphate ceramics on reparative osteogenesis in rabbits. Twenty-four California white rabbits, aged 3 months and weighing 2.5 kg each, were used for the study. The rabbits were divided equally into experimental (n=12) and control (n=12) groups. In the experimental group, defects were replaced with granules of germanium-doped hydroxyapatite ceramics (HA/β-TCP/l-Ge–700), while in the control group, defects were filled with granules of unalloyed ceramics (HA/β-TCP–700). Subsequently, clinical and histological studies, along with the determination of bone metabolism markers: the activity of total alkaline phosphatase (AP), bone isozyme (BAP), and tartrate-resistant acid phosphatase (TRAcP) were assessed on the 7th, 14th, 30th, and 60th days. Animals were removed from the experiment on the 14th, 30th, and 60th days of the research. A large number of capillaries, vascular channels, and close contact of the regenerate with the implant were detected in the histological samples after HA/β-TCP/l-Ge–700 osteoreplacement on the 14th day. On the 30th day, formation of lamellar bone tissue well-integrated with the parent bone as well as partial resorption of ceramic granules was noted. When using HA/β-TCP–700, similar results were observed 2 weeks later. A significant difference was established between the levels of bone biochemical markers in the control and experimental groups. In the experimental group, the activity of AP and BAP reached peak values on the 14th day. In the control group, they increased gradually with a peak on the 60th day. Therefore, osteoreplacement with germanium-doped ceramics was accompanied by an early osteoblastic reaction. TRAcP activity was higher in the experimental group on the 14th day, while in the control group, it was elevated on the 30th and 60th days. This indicates a lower level of osteoresorptive processes in the experimental group. It was concluded that HA/β-TCP/l-Ge–700 has osteoinductive and osteointegration properties. It also provides early neoangiogenesis and osteoblastic reaction.

Key words: Bioceramics, germanium, hydroxyapatite with β-tricalcium phosphate, bone fractures, rabbits

1. Introduction

Reparative osteogenesis is a cascade of complex molecular and cellular reactions that cause, thanks to the cellular type of regeneration, the formation of bone tissue in the area of the fracture that is identical to the original one, with the restoration of its anatomical shape, histological structure, and functional properties [1,2,3]. At the same time, despite the dynamic improvement of technical means and methods of conservative or operative treatment of fractures, the frequency of complications of their consolidation remains quite significant. Thus, among dogs, according to retrospective analyses, the frequency of long tubular bones' fractures is 82%–85%, among which fragmental fractures make up 16%–25%. The frequency of pathological fractures due to neoplasia reaches 3%–5%. The complicated course of reparative osteogenesis in the form of nonunions, pseudojoints, and infection can be 16%–20%, regardless of the fixation method [4–10].

Most commonly, complications of reparative osteogenesis in animals arise from fragment fractures leading to the formation of bone defects and the regenerative potential's loss of bone tissue as a result. Along with this, bone defects arise due to bone infections, neoplasias, surgical correction of abnormal skeletal development, bone plastic surgery in osteoarthritis due to the ligamentous apparatus's rupture of the joints. That is, in all the mentioned cases, osteoreplacement is necessary.

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The known disadvantages of autoosteosubstitution (limited graft volume, pain at the harvesting site, risks of infection, and ethical and financial problems) are even more significant in veterinary patients than in human medicine.

In recent years, a fairly significant number of biological and synthetic bone replacement materials for bone tissue restoration have been proposed [11–14]. These include synthetic polymers (polyethylene, polyurethane, polylactides, etc.), biological polymers (collagen, chitosan, fibrin, etc.), bioglass and biosieves, bioinert (aluminum and zirconium oxides), and bioactive calcium phosphate ceramics.

All bone substitute materials differ significantly in osteoinductive, osteointegration, osteoconductive, and osteogenic properties, as well as in the degree of biodegradation [15]. Among them, calcium phosphate ceramics are advancing most dynamically from the experimental stage to preclinical testing and clinical approval.

Mainly, calcium phosphate ceramics include hydroxyapatite and α- or β-tricalcium phosphate, which are compositionally related to the mineral component of bone and possess adequate osteoconductive properties for osteosynthesis conditions. Moreover, the presence of pores in calcium phosphate ceramics gives it matrix properties for adhesion, and proliferation of progenitor and endothelial cells, which imparts a certain osteoinductivity of this material [11,12,16].

To enhance the osteoinductive properties, calcium phosphate ceramics are alloyed with microelements that play a significant role in bone metabolism, such as Zn, Si, Sr, Cu, and Mg [17–20].

Previously, we [21–24] showed in rabbits with bone defects and in dogs with fragment fractures that two-phase granules comprising hydroxyapatite (72%) and β-tricalcium phosphate doped with silicon (1.3 wt.%) with an adsorption activity of about 230 mg/g induce an early reaction of endothelial cells, leading to rapid and high-quality formation of spongy-type trabeculae. This occurs due to an early osteoblastic reaction with accelerated formation of new compact bone tissue integrated with the parent bone tissue.

Germanium has antiinflammatory, hematopoietic, immunomodulating, antioxidant properties. Therefore, doping calcium phosphate ceramics with germanium ions can enhance its osteointegration and osteoinductive properties [25, 26]. In this regard, germanium-doped calcium phosphate ceramics is the basis for further research. The obtained histological and biochemical results make it possible to evaluate its osteointegrative and osteoinductive properties, as well as its influence on the healing of model bone fractures in rabbits.

The purpose of the work is bone regenerates' histological evaluation and the dynamics of bone metabolism markers after osteoreplacement with germanium-doped calcium phosphate ceramics in rabbits.

2. Materials and methods

2.1. Experiment design

The study was conducted on clinically healthy California white rabbits, 3 months old, weighing approximately 2.5 kg, which were kept in the conditions of Bila Tserkva National University's vivarium. The animals were housed in individual cages in a room with forced ventilation, combined lighting, and daily cleaning. Feed was provided with specialized certified compound feed for rabbits of the "Selevana" trade mark (Ukraine) at the rate of 200 g per head per day with free access to water.

Experimental (n = 12) and control (n = 12) groups of animals were formed. The following anesthetic support was used for the reproduction of model fractures: 2% acepromazine solution intramuscularly (0.7 mg/kg), thiopenate solution at a rate of 6 mg/kg intravenously, and local infiltration anesthesia with a 0.5% lidocaine solution (3 mg/kg).

Bone defects were formed from the lateral surface of the femur metaphysis distal part (spongy bone tissue) and the radius diaphysis dorso-lateral surface (compact bone tissue). Operative access was carried out in compliance with the rules of asepsis and antiseptics. After dissecting the periosteum, perforated bone defects were created using a 3 mm diameter drill in the radius and a 4.2 mm diameter drill in the femur. In animals of the experimental group, these defects were replaced with granules of hydroxyapatite ceramics doped with germanium; and in control animals, with granules of unalloyed hydroxyapatite ceramics (Figure 1).

Knotted sutures made of synthetic polypropylene suture material were placed on soft tissue wounds. Twice a day for 7 days, the sutures were treated with the antiseptic agent "Ioddicerin," containing 100 g of iodine (0.5 g), dimethyl sulfoxide (30.0 g), and glycerin (69.5 g).

In the postoperative period, general clinical examination and visual assessment of the wound process were performed daily in rabbits. Wound healing took place according to the initial tension, so the sutures were removed on the 7th day.

2.2. Material for bone replacement

For bone replacement, bioactive ceramics synthesized at the Institute of Materials Science Problems named after I. M. Frantsevich, National Academy of Sciences of Ukraine (Kyiv), were used.

Two-phase calcium phosphate ceramic (HA/β-TCP–700) granules, comprising 65 wt.% hydroxyapatite phase (HAP) and 35 wt.% β-tricalcium phosphate (β-TCP), were prepared for the research. The granules had a size range of 600–800 microns. The material was obtained through the decomposition of nonstoichiometric hydroxyapatite at a temperature of 800 °C.

Nonstoichiometric hydroxyapatite, $Ca_{(10-x)}(HPO_4) \times (PO_4)_{(6-x)}(OH)_{(2-x)}$, $(0 < x < 1)$, was synthesized using the traditional method of chemical precipitation by mixing a solution of nitric acid hydrate $Ca(NO_3)_2{\cdot}4H_2O$ calcium salts and disubstituted ammonium phosphate $(NH)_2HPO_4$. After washing, treating with a foaming agent, and drying, the resulting sediment was crushed and dispersed to obtain the required fraction.

Doping of calcium phosphate ceramics with germanium (HA/β-TCP/l-Ge–700) was carried out by introducing 1.0 wt.% of germanium metaphosphate $(Ge(PO_3)_4)$ in the form of a colloidal solution into a freshly precipitated gel of stoichiometric hydroxyapatite (HAP). The synthesis of stoichiometric HAP $(Ca_{10}(PO_4)_6(OH)_2)$ was carried out by the traditional method of chemical precipitation by mixing a solution of nitric acid hydrate $Ca(NO_3)_2{\cdot}4H_2O$ calcium salts and disubstituted ammonium phosphate $(NH)_2HPO_4$ with an estimated Ca/P ratio of 1.67.

Figure 2a shows the diffractogram of undoped annealed stoichiometric hydroxyapatite, which indicates the hydroxyapatite's presence of 100% phase. After doping, adding pore formers, drying, and annealing at 800 °C, phase changes occurred in the material due to the addition of phosphate ions from germanium metaphosphate. The diffractogram of stoichiometric hydroxyapatite annealed granules doped with 1.0 wt.% germanium is presented in Figure 2b, which indicates the phase composition of the 65% hydroxyapatite finished granules and 35% β-tricalcium phosphate.

Granules with a size of 600–800 μm were used for implantation. After annealing, the germanium's concentration in the material is 0.8 wt.%.

Based on the research findings regarding the microstructure of unalloyed samples (Figures 3a and 3b) and germanium-doped (Figures 3c and 3d) two-phase ceramic samples, it can be seen that the HA/β-TCP/l-Ge–700 structure is thinner and more uniform with the presence of nano-, meso-, and macropores, which increases the solubility and adsorption activity of the granules.

Figure 1. Technique of filling model bone defects in rabbits with hydroxyapatite ceramics.

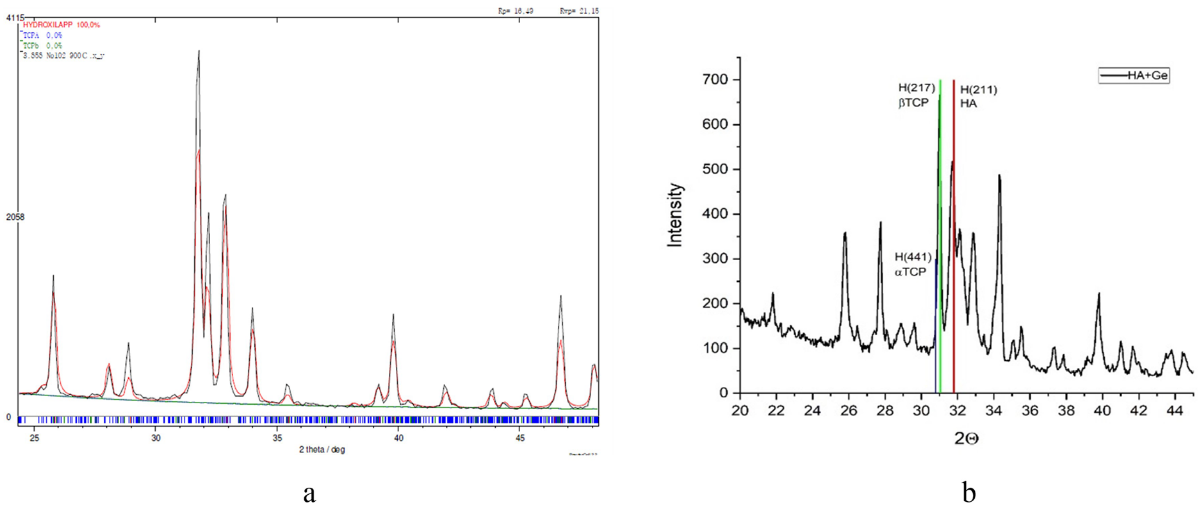

Figure 2. Diffraction pattern of undoped stoichiometric hydroxyapatite after annealing (a) and doped hydroxyapatite 1.0 wt.% germanium after annealing (b).

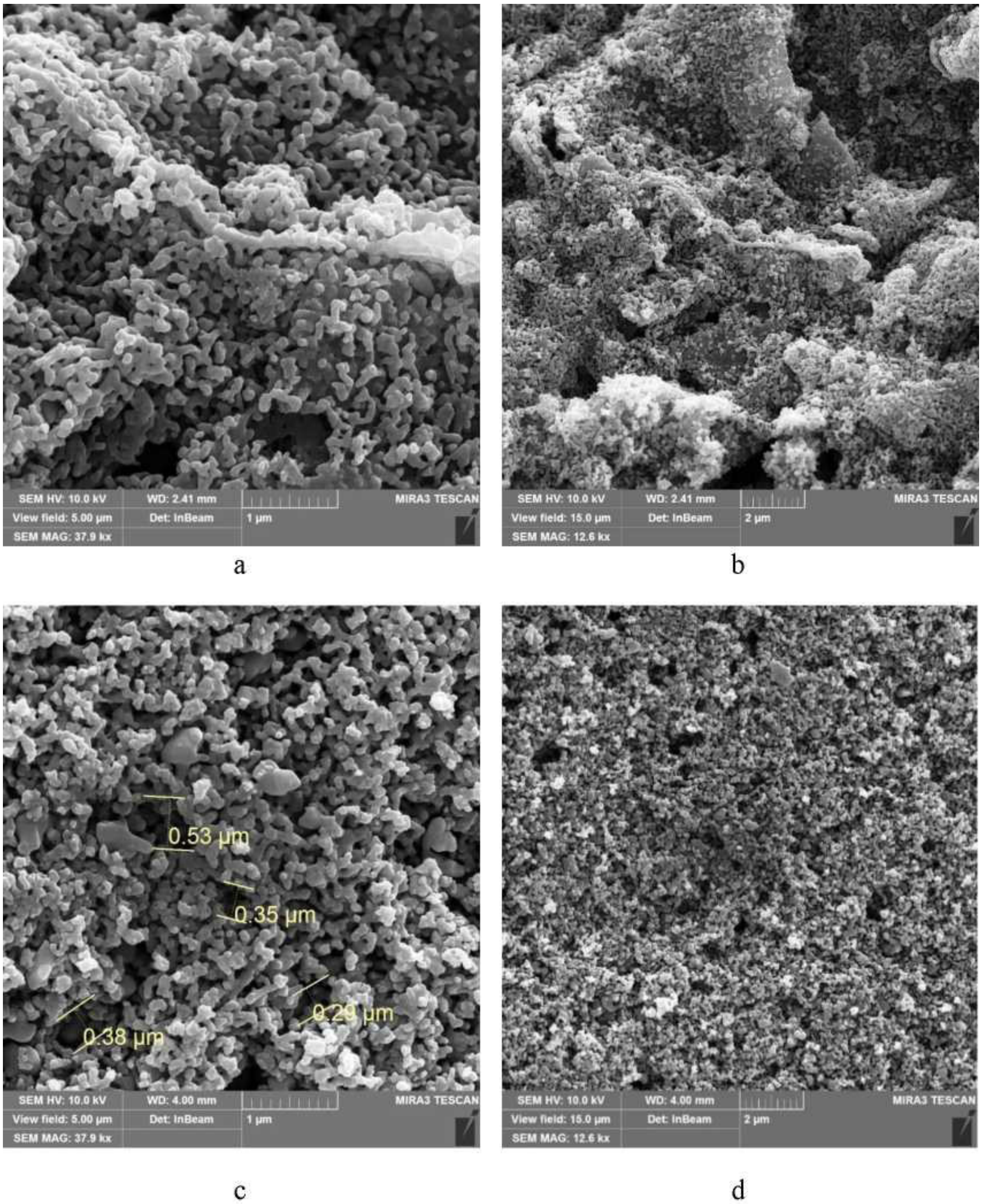

Figure 3. Microstructure of HA/β-TCP–700 granules (a, b), magnification 37.9 × 103 and 12.6 × 103; HA/β-TCP/l-Ge–700 granules (c, d), magnification 37.9 × 103 and 12.6 × 103.

2.3. Biochemical studies

Blood samples were collected from the external jugular vein before anesthesia and on the 7th, 14th, 30th, and 60th days following the implantation of calcium phosphate materials.

The total activity of alkaline phosphatase (AP) and its bone isoenzyme (BAP) was determined in blood serum according to Vagner et al. [27] using reagent kits from Granum Laboratory (Ukraine).

The level of tartrate-resistant acid phosphatase (TRAcP) activity in blood serum was determined with reagent kits of "Granum Laboratory" (Ukraine). Measurements were made with a Stat Fax 4500 spectrophotometer.

2.4. Histomorphological study

Animals were removed from the experiment on the 14th, 30th, and 60th days of the study by intravenous administration of thiopenat (sodium thiopental, Brovafarma LLC, Ukraine) at a dose of 50 mg/kg.

Fixation of bone regenerate samples, which were selected for histomorphological examination, was carried out in a solution of neutral formalin (10%). Subsequently, decalcification was carried out with a specialized Rapid decalcifier solution (manufactured by Kaltek, Italy), followed by dehydration in alcohols of increasing concentration, clarification in xylene, and embedding of samples in paraffin Plasti Wax by KALTEK (Italy). Histological sections of the samples were prepared using a rotary microtome, with a thickness ranging from 5 to 10 µm. These histosections were then stained with Weigert's iron hematoxylin and a 1% alcohol-based eosin solution (manufactured by Diapath, Italy). Microscopy of the samples was performed with a Fusion FS 75–30 trinocular microscope (manufactured by Micromed, China) and visualized using a Microscope digital eyepiece MDS-500 camera and Vividia Able Scope software.

2.5. Statistics

Statistical processing of the results was carried out using the Statistica 10 program (StatSoft Inc, USA, 2011), and the data are presented in Table in the form of x ± SD (x ± deviation). The reliability of the differences between the digital indicators of the control and experimental groups and the indicators of clinically healthy rabbits before the formation of a bone defect was determined using ANOVA. Values of $p < 0.05–0.001$ were considered reliable, taking into account the Bonferroni correction.

3. Results

Clinically, animals of both groups showed a slight increase in local temperature and moderate swelling of tissues the next day after modeling bone defects, which indicated a postoperative inflammatory reaction. No disturbances in the function of the injured limb were observed. On the 3rd day of the postoperative period, the local temperature was within the physiological norm. On the 7th day, tissue swelling and pain reaction were absent, and the wound edges exhibited connective tissue adhesion with signs of epithelization, indicating the need for suture removal.

On the 14th day of reparative osteogenesis, palpation of the defect area revealed nonpainful periosteal thickening, which completely disappeared in the animals of the experimental group by the 30th day. In control rabbits, on the 14th day, a more pronounced densification of tissues was noted both in the area of the radius diaphysis and the metaphysis of the femur, which was verified by palpation by the 30th day, and disappeared completely only by the 60th day of the study.

3.1. Histological studies

3.1.1. Compact bone tissue

On the 14th day of reparative osteogenesis in the experimental group for osteoreplacement with doped ceramics (Figure 4a), bone regenerates were histomorphologically characterized by the presence of capillaries and vascular channels, chaotically formed beams of coarse bone tissue, which tightly surrounded the ceramic granules with newly formed beams. The surface

Table. Dynamics of bone metabolism's biochemical markers in rabbit blood serum for doped and nondoped implantation of calcium phosphate ceramics.

Day	Group	**AP,** u/L	**BAP**, u/L	**TRAcP,** u/L
0	Norm, (n = 24)	65.75 ± 0.44	41.18 ± 0.42	29.78 ± 0.6
7	HA/β-TCP/l-Ge–700, (n = 12)	73.0 ± 0.56** •••	52.0 ± 0.41*** •••	30.08 ± 0.75
	HA/β-TCP–700, (n = 12)	69.74 ± 0.76 ••	44.38 ± 0.63 ••	29.69 ± 0.51
14	HA/β-TCP/l-Ge–700, (n = 12)	89.54 ± 0.43***•••	57.54 ± 0.48***•••	39.63 ± 0.59***•••
	HA/β-TCP–700, (n = 12)	70.47 ± 0.37 •••	46.25 ± 0.47 •••	34.29 ± 0.48 •••
30	HA/β-TCP/l-Ge–700, (n = 9)	79.19 ± 0.44* •••	45.23 ± 0.61* •••	35.42 ± 0.59***•••
	HA/β-TCP–700, (n = 9)	76.93 ± 0.66 •••	46.94 ± 0.34 •••	42.29 ± 0.24 •••
60	HA/β-TCP/l-Ge–700, (n = 6)	68.92 ± 0.34*** •••	44.7 ± 0.35*** •••	33.55 ± 0.76***••
	HA/β-TCP–700, (n = 6)	80.52 ± 0.36 •••	51.13 ± 0.42 •••	38.13 ± 0.48 •••

Note: 1) p value: * – < 0.05; ** – < 0.01; *** – < 0.001, compared to the indicators of the control group; 2) p-value: • – < 0.05; •• – < 0.01; ••• – < 0.001, compared to indicators of clinically healthy animals.

layers of ceramics and adjacent trabeculae included a fairly large number of the osteoblastic series' cells, osteoblasts, and a smaller number of osteoclasts.

In the control group (Figure 4b), on the 14th day, the samples of the regenerates were poor in osteogenic cells, and their coarse-fibrous, loosely located formations did not have close contact with the granules of calcium phosphate ceramics. Individual formations of blood capillaries were also present.

On the 30th day of reparative osteogenesis, in the histosection samples of the research group (Figure 4c), the remains of bone replacement material's granules are tightly surrounded by massive lamellar beams with the remains of rough fibrous bone and a significant number of cells in osteocytic lacunae. The surface of lamellar trabeculae contained rows of osteoblasts, which is a sign of strengthening and thickening of bone beams. In the contact areas of the bone regenerate with the mother bone, islands of newly formed bone tissue were noted on the surface of the formed trabeculae, as signs of organotypic remodeling.

In the control samples (Figure 4d), on the 30th day, the bone defect of the compact bone was mostly filled with coarse fibrous bone tissue, although spongy-type trabeculae with a small number of osteogenic cells were also present. The contact area with the parent bone is in a state of moderate osteoresorption, without close contact of the ceramic granules with the regenerate.

On the 60th day of reparative osteogenesis, almost the entire area of the radial bone's diaphysis model defect in the animals of the experimental group (Figure 4e) was filled with compact bone tissue, with the exception of small point areas filled with spongy bone tissue, the coarse fibrous elements of which penetrated the remnants of resorbed granules osteoclasts and were replaced by areas of coarse bone tissue.

In the control group, on the 60th day (Figure 4f), filling of the defect with both coarse-fiber and lamellar trabecular bone tissue was noted. Bone beams with rows of osteoblasts on the surface were clearly visualized, and osteocytes walled in lacunae in their thickness. The granules of the composite material were in loose contact with the newly formed bone tissue.

3.1.2. Spongy bone tissue

On the 14th day of reparative osteogenesis, histomorphological samples from the research group (Figure 5a) revealed the formation of thin bony beams around the ceramic granules, tightly covering the surface. Well-formed vascular tubules were observed between these bony beams. At the border with the parent bone, there is a significant number of poorly differentiated osteogenic cells, bone marrow elements, and a small number of fat cells.

In the samples of the control group (Figure 5b), at the same time, the bone defect was filled with regenerated fibrous structure with a moderate density of cellular elements around the ceramic granules and single vascular elements, compared to the experimental group. The contact between the regenerated mass and the ceramic granules was incomplete.

On the 30th day, in the samples of the experimental group (Figure 5 c), spongy bone tissue in the form of massive beams tightly adhering to the granules of ceramic material was noted. The remains of coarse-fiber bone tissue were focally located in the superficial and deep layers of the composite. Vascular channels had clearly formed walls. Rows of osteoblasts were visualized on the periphery of trabeculae, and osteocytes walled in lacunae were visible in their thickness. The contact of the newly formed bone tissue with the ceramics was noted along the entire perimeter of the granules' remains.

In the samples of the control group, on the 30th day (Figure 5d) of osteoreplacement, formation centers of coarse fibrous bone tissue and single vascular channels were noted. Granules of the composite material were located between trabeculae of cancellous bone; their close contact was not observed.

On the 60th day, in the samples of the experimental group (Figure 5e), the spongy bone tissue formed was in close contact with the remains of the composite granules, which had completely lost their initial structure.

The regenerate in the control group, in which the defects were replaced by nonalloyed ceramics (Figure 5f), was visualized in the form of spongy-type trabeculae on the 60th day, but the granules of the composite material still retained their structure and, in the vast majority, did not have close contact with the regenerate or were freely located in the intertrabecular spaces.

3.2. Biochemical research

Dynamic changes in the bone metabolism biochemical markers and the activity of total alkaline phosphatase in the rabbits' blood serum were established both for osteoreplacement with unalloyed calcium phosphate ceramics and for the inclusion of germanium ions in its structure (Table). These changes turned out to be reliable in terms of quantitative values and at different times of the study.

General alkaline phosphatase is involved in the regulation of the blood phosphate buffer system and the level of extracellular phosphates, reciprocally ensures the level of extracellular calcium, and affects the level of glucose in the blood plasma and its transport in tissues. It is not specific enough, as it includes bone, liver, and intestinal isozymes. When blood is taken on an empty stomach, its specificity for reflecting the state of bone metabolism increases [28].

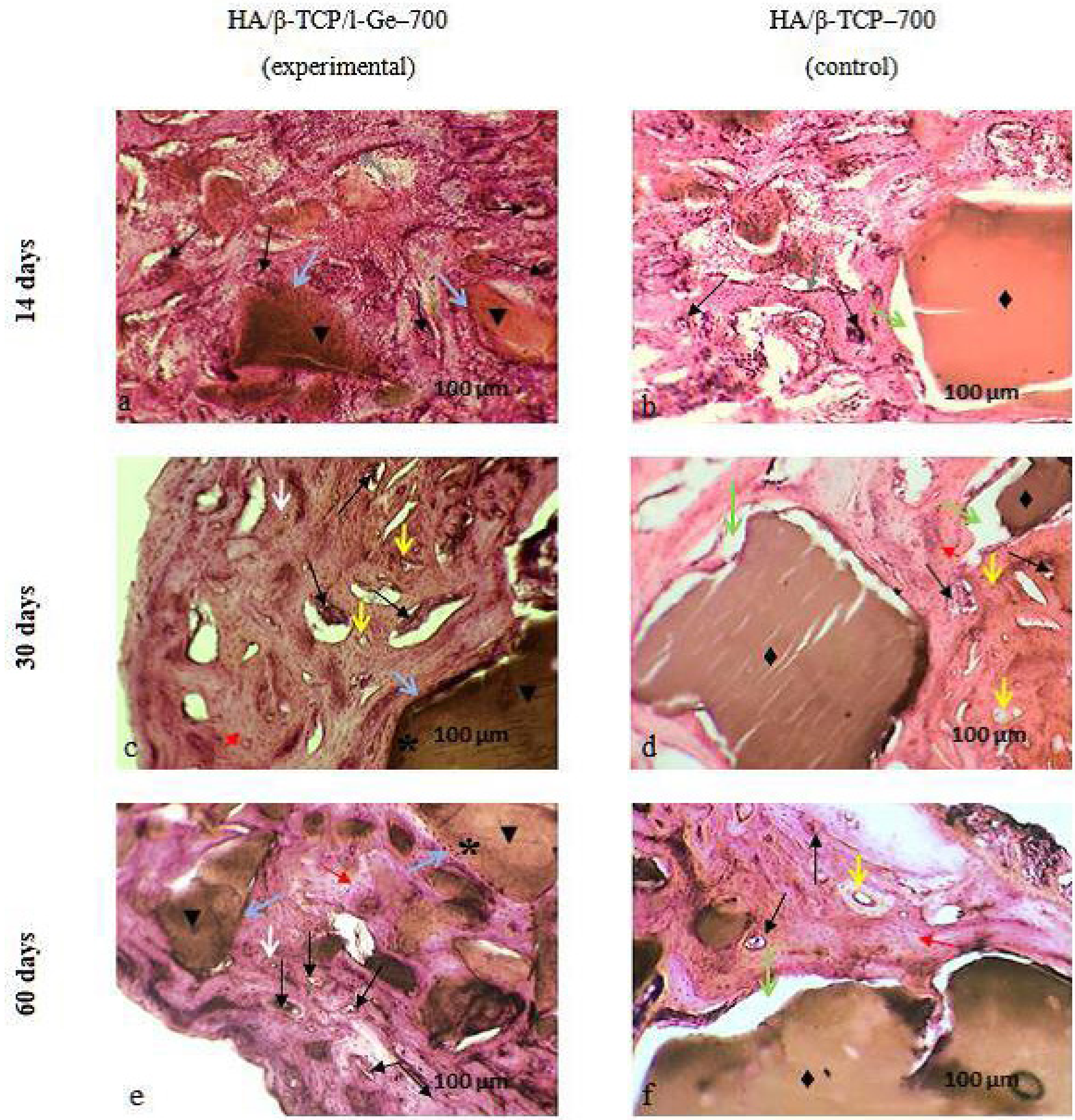

Figure 4. Histological picture of the radius' bone regenerate during reparative osteogenesis in rabbits : ▼ – granule of germanium-doped calcium phosphate ceramics; ♦ – granule of unalloyed ceramics; * – the granule is integrated into the bone tissue; vascular channels (thin arrow); dense contact of granules with bone regenerate (blue arrow); lack of granule's contact with the bone regenerate (green arrow); osteons at the stage of formation (yellow arrow); formed osteons (white arrow); nuclei of osteoblasts (gray arrow); osteocytic lacunae (red arrow).

In the dynamics of changes in the activity of total alkaline phosphatase, significant differences were observed between the control and experimental groups at all times of the study from the 7th to the 60th day. At the same time, in the control group, a gradual increase in the activity of this enzyme was noted with a high level of reliability ($p < 0.001$) on the 14th day, reaching its peak on the 60th day of the study (80.52 ± 0.36 u/L) ($p < 0.001$). In the experimental group, the peak value was 89.54 ± 0.43 u/L ($p < 0.001$), recorded on the 14th day of reparative osteogenesis. Subsequently, total alkaline phosphatase activity decreased dynamically towards the level of clinically healthy rabbits, but the difference from them was highly reliable in all respects ($p < 0.001$). The

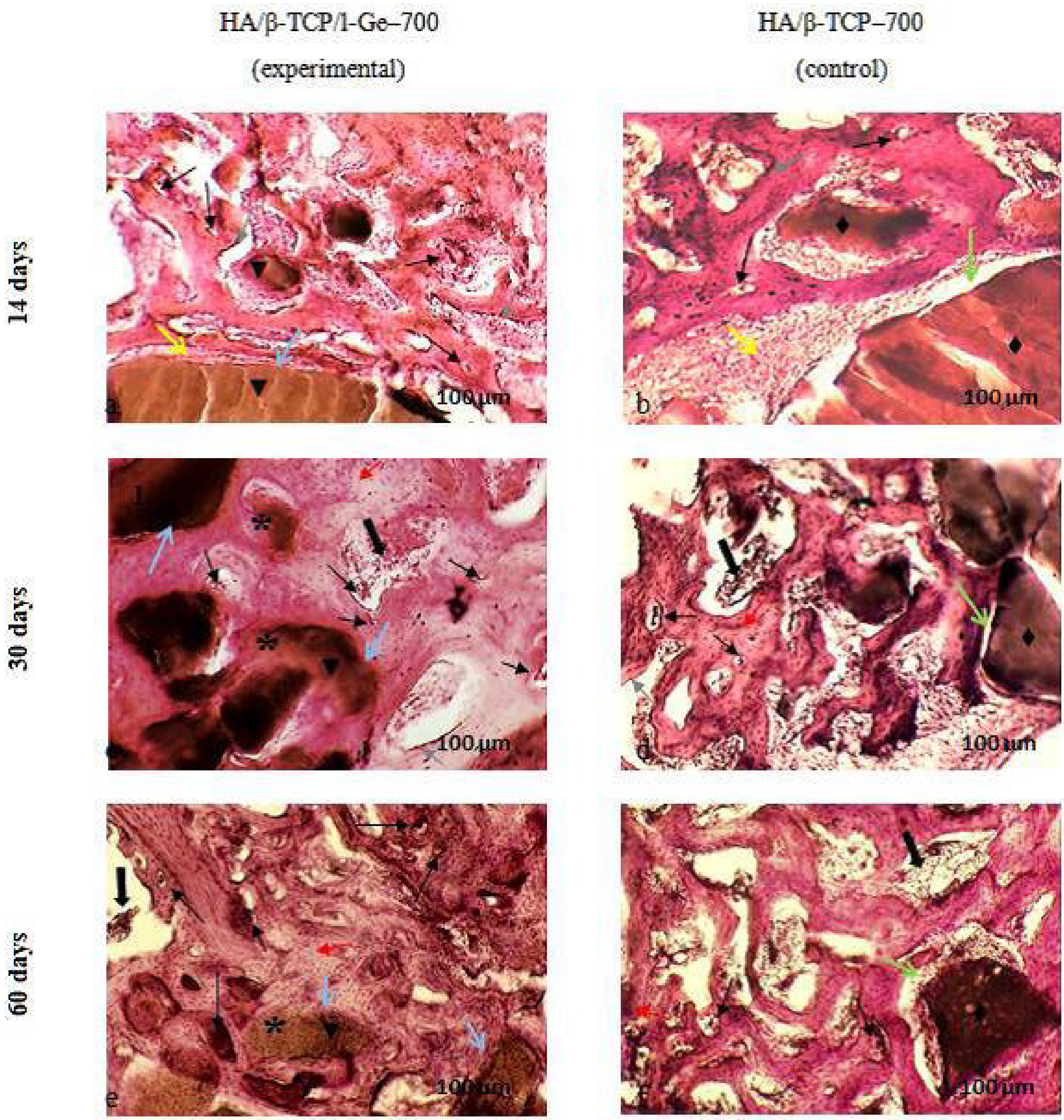

Figure 5. Histological picture of the femur's bone regenerate during reparative osteogenesis in rabbits: ▼ – granule of germanium-doped calcium phosphate ceramics; ♦ – granule of unalloyed ceramics; * – the granule is integrated into the bone tissue; cells of red bone marrow and adipose tissue in interbeam spaces (thick arrow) arrow); coarse bone tissue (yellow arrow); vascular channels (thin arrow); dense contact of granules with bone regenerate (blue arrow); lack of granule's contact with the bone regenerate (green arrow); nuclei of osteoblasts (gray arrow); osteocytic lacunae (red arrow).

level of significant difference between the control and experimental groups was the highest ($p < 0.001$) on the 14th and 60th day of the study.

Bone alkaline phosphatase (BAP) is localized in the cytoplasmic membrane of osteoblasts, generates extracellular inorganic phosphate, and is therefore considered a biochemical marker of osteogenesis [29].

In all study periods, the activity of bone AP in the control and experimental groups was significantly higher ($p < 0.01$–0.001) than in the clinically healthy rabbits.

In the control group, a moderate increase ($p < 0.01$–0.001) of BAP activity was observed within 30 days, but its peak indicator ($p < 0.001$) was detected only on the 60th day (51.13 ± 0.42 u/L) of reparative osteogenesis. However, for HA/β-TCP/l-Ge–700 bone replacement in experimental rabbits, the peak activity of BAP was already reached on the 14th day (57.54 ± 0.48 u/L) ($p < 0.001$). At the same time, intensive growth of this enzymatic activity was detected already from the 7th day, which was 1.2 times ($p < 0.001$) higher than in the control animals (Figure 6).

Therefore, the dynamics of BAP activity indicates an earlier and more intense osteoblastic reaction in the case of osteoreplacement with germanium-doped calcium phosphate ceramics.

Tartrate-resistant acid phosphatase (TRAcP) is excreted by macrophages and is a lysosomal enzyme of osteoclasts, and therefore belongs to specific markers of bone resorption [30]. In the control and experimental groups, its activity was significantly increased during the 14th, 30th, and 60th days of reparative osteogenesis. Moreover, in the experimental group, the peak of TRAcP activity occurred on the 14th day (39.63 ± 0.59 u/L) ($p < 0.001$), and in the control group on the 30th day (42.29 ± 0.24 u/L) ($p < 0.001$) and on the 60th day remained quite high (38.13 ± 0.48 u/L) ($p < 0.001$). In all periods of increasing TRAcP activity, the reliability level between its indicators in groups was high ($p < 0.001$) (Figure 7).

Since TRAcP activity reflects the level of osteoclast activity and, accordingly, the degree of bone tissue resorption, the established dynamics of TRAcP indicators indicates earlier and less intense osteoresorption in the experimental group for osteoreplacement with germanium-doped bioactive ceramics.

4. Discussion

Calcium phosphate ceramics is the most widely used material for bone replacement [31]. In particular, it was established [23, 24] that increasing the porosity of the two-phase ceramic with a composition of 72% hydroxyapatite and 28% β-tricalcium phosphate increases its adsorption activity and, accordingly, enhances its osseointegration properties.

Alloying of calcium phosphate ceramics with Zn, Mg ions determine their osteoinductive properties [32, 33]. Previously, we [22] established that doping with Si ions of two-phase ceramics in the composition of hydroxyapatite and β-tricalcium phosphate ensured an early endothelial reaction with the induction of angiogenesis and an early osteoblastic reaction. This made it possible to accelerate the healing of model hole fractures in rabbits and traumatic fragment fractures in dogs by 1.5 times.

According to the results of histomorphological evaluation of the bone regenerates, it was found that, regardless of the type of the bone tissue, calcium phosphate ceramics doped with germanium acquires pronounced osteoinductive properties with a corresponding increase in its osseointegration. This is manifested by an earlier and more intense neoangiogenesis, an early osteoblastic reaction with the formation of regenerate islands between and within the granules of the bone substitute material.

At the same time, osteoresorption is less pronounced (in terms of the osteoclasts' number) both during the inflammatory-resorptive stage of reparative osteogenesis (14th day) and the stage of bone regenerate remodeling (30th–60th day). All these processes took place more dynamically during the regeneration of bone defects in spongy bone, which is due to its naturally more intensive vascularization.

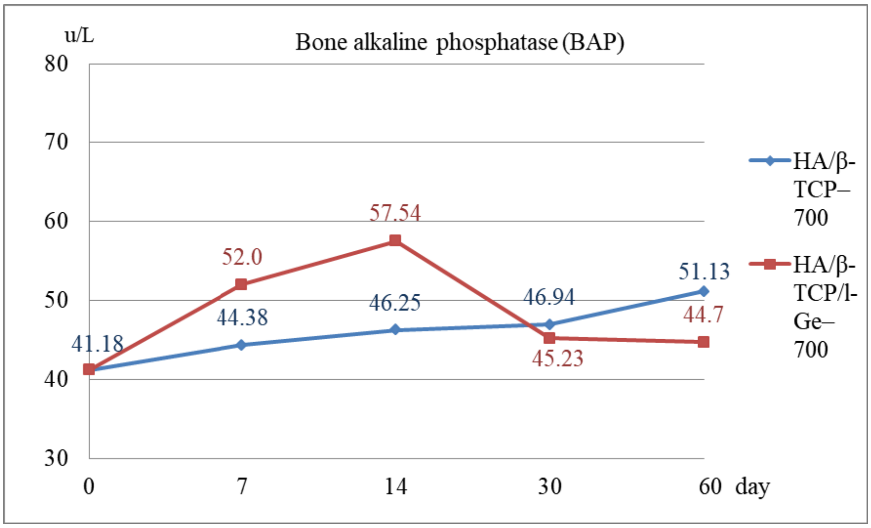

Figure 6. Dynamics of BAP activity in rabbits' blood serum during reparative osteogenesis using different types of ceramics.

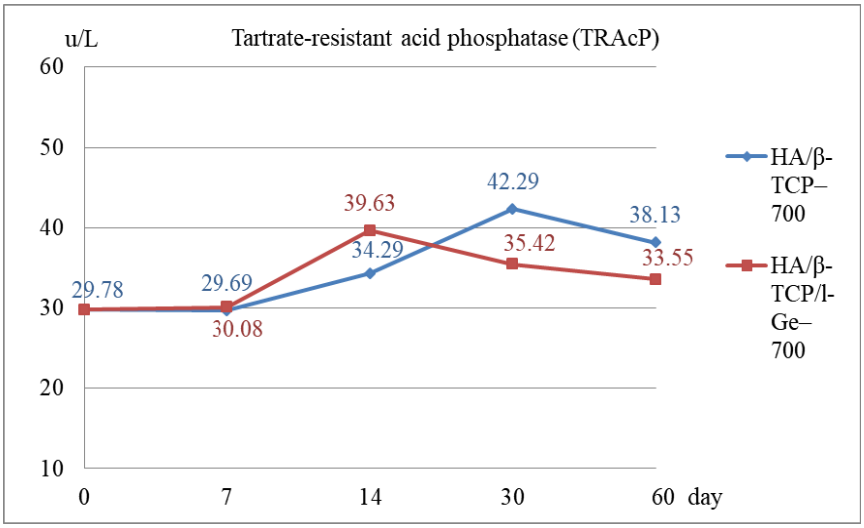

Figure 7. Dynamics of the TRAcP level in the rabbits' blood serum during reparative osteogenesis using germanium-doped calcium phosphate ceramics.

In general, osteoreplacement with germanium-doped calcium phosphate ceramics as a bioactive scaffold provides conditions for and induces adhesion, proliferation, and differentiation of endothelial cells and mesenchymal stem cells in interfragmentary diastasis. This ultimately leads to the formation of coarse-fiber bone tissue in the entire volume of the bone defect with its early replacement by lamellar bone tissue.

That is, reparative regeneration (healing of a bone defect by secondary tension) is as close as possible to physiological regeneration by primary tension. The pathochemical confirmation of this lies in the dynamics of biochemical markers of bone metabolism [28–30]. Thus, in the case of using HA/β-TCP/l-Ge–700, the increase in the activity of BAP reached peak values in the period of 7–14 days, and the activity of TRAcP on the 14th day, with a gradual decrease in the following periods of the study. That is, such dynamics of phosphatase activity were consistent with the histomorphological picture of reparative osteogenesis and reflected an early and intense osteoblastic reaction and a rapid inflammatory-resorptive phase.

Although germanium, like silicon, belongs to group 4 in the periodic table of chemical elements and is similar to it in terms of chemical properties, little is known about the ability of germanium to osteoinductivity. Separate works [34–36] showed that calcium phosphate composite coatings with the addition of germanium optimized the osseointegration of titanium alloy implants.

Osteoinductive properties of germanium could be attributed to its ability to induce interferonogenesis, and interferon itself inhibits osteoresorption mediated by IL-1 and α-TNF and stimulates the osteoblastic reaction [25].

At the same time, alloying calcium phosphate ceramics with germanium can give it new properties, and given its antibacterial, immunomodulating, and antitumor properties, bone replacement with such bioactive ceramics is promising in solving the problems of infected complications in traumatology and orthopedics, and in oncoorthopedics.

5. Conclusions

The histological picture of the bone regenerates indicates pronounced osteoinductive and osteointegrative properties of HA/β-TCP/l-Ge–700. Signs of this are an early osteoblastic reaction, faster formation at the site of a bone defect of full-fledged lamellar bone tissue, well-integrated with the parent bone. At the same time, HA/β-TCP/l-Ge–700 has moderate biodegradation. The dynamics of biochemical markers of bone metabolism confirms the optimized course of reparative osteogenesis with HA/β-TCP/l-Ge–700 osteosubstitution. In particular, the peaks of BAP activity on the 7th–14th days indicate an early osteoblastic reaction. The peak of TRAcP activity on the 14th day indicates a lower level of inflammatory and resorptive processes. The peak activity of BAP and TRAcP on the 60th day indicates the remodeling process of bone regenerate. Based on the research results, we believe that germanium-doped calcium phosphate ceramics can be promising for the needs of bone replacement in veterinary orthopedics.

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Conflict of interest

The authors declare that they have no conflicts of interest.

Compliance with standards for working with animals

The studies were carried out in accordance with the Law of Ukraine "On the Protection of Animals from Cruelty" of March 28, 2006, the rules of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes of November 13, 1987, and the Order of the Ministry of Education and Science No. 416/2072 dated March 16, 2012 "On approval of the procedure for conducting research, experiments on animals by scientific institutions".

Informed consent

The experiment protocol was approved by the Ethics Committee of the Belotserkovsky National Agrarian University on the treatment of animals in scientific research and the educational process (protocol No. 1 dated January 23, 2019).

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