

## EXPERIMENTAL SUBSTANTIATION OF THE USE OF HYDROXYAPATITE — TRICALCIUM PHOSPHATE BIOCERAMICS FOR REPLACING BONE DEFECTS AFTER TUMOR REMOVAL

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**Background:** Recently, bioactive ceramics based on hydroxyapatite (HAP) and tricalcium phosphate (TCP) have been preferred as implants in bone engineering. **Aim:** To study bone regeneration under conditions of filling metaphyseal defects with the original HAP-TCP composition. **Materials and Methods:** The experiment was carried out on inbred rats, the observation period was 2, 4, 8 weeks. Morphological studies were carried out using light and electron microscopy. **Results:** In the dynamics of observation at 2, 4 and 8 weeks, a gradual arrangement of osteoclasts and osteoblasts on the surface of HAP-TCP was recorded, which indicates its high biocompatibility with bone tissue. During the experiment, the processes of resorption of implants, mineralization, proliferation of cellular elements of collagen and osteogenesis (osteoblasts and osteoclasts) and the formation of mature bone tissue were recorded. **Conclusions.** Experimental studies of plastic bone defects proved the presence of the osteoinductive and osteointegrative effect of HAP-TCP composition, which contributes to a more dynamic uncomplicated course of reparative osteogenesis. **Key Words:** bone, HAP-TCP, implantation, reparative osteogenesis.

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Reparative osteogenesis management is one of the biggest challenges in traumas and diseases of locomotor system, especially in major bone defects. Osseous tissue has unique regenerative capacity, although it is remarkably limited in cases of three-dimensional bone defects, emerging after removal of benign tumors, primary malignant or metastatic tumors. All these surgeries cause bone defect replacement with various implants for targeted correction of regenerative osteogenesis, aiming at a timely and complete medical and social rehabilitation of the operated patients [1, 2].

In bone engineering, bioactive ceramics, bioglass, biositals, enamel, and collagen-glycosaminoglycan composites are supposed to be promising materials for bone defect replacement. Recently, the advantage is given to bioactive ceramics on the base of:

- hydroxyapatite (HAP) — complete chemical and crystallo-chemical analog of mammals bone mineral substance, possessing a complete immunologic compatibility and bioactivity — the property to stimulate osteogenesis, biointegrate with bone, serve as a plastic material for bone synthesis and become a part of bone tissue that is replaced by HAP;
- tricalcium phosphate (TCP) — complete chemical analog of bone tissue mineral substance, having a complete biocompatibility, however due to non-

conformity of crystal structure and increased solubility compared with HAP, it biodegrades in the body more rapidly

Based on HAP and TCP, the family of bioactive HAP-TCP ceramics for bone surgery was developed by the I.M. Frantsevich Institute for Problems of Material Science of the NAS of Ukraine [3]. HAP-TCP advantages are the following: high biological compatibility, apyrogenicity, implant integration with bone tissue without fibrous capsule development, gradual material replacement with a native osseous tissue, easy storage and possibility of repeated sterilization, absence of infection transmission risk, sufficient number and variety of forms, possibility of regulating its properties, porosity, and the absence of ethical difficulties and religious limitations [3].

According to the National Cancer Registry of Ukraine for 5 years (2014–2019), in Ukraine 15,546 patients with metastatic skeletal lesions were registered [4]. To date, it is established that the surgical treatment of this category of patients significantly increases the overall survival and quality of life [5, 6].

Personification of treatment leads to the results in creation of 3D-models of grafts with subsequent replacement of bone defects formed in individual patient to create the most favorable conditions for integration of the graft with the mother bone — lack of the impact, reliable graft fixation, and absence of clinical and radiological signs of its rejection [7–9].

The aim of the study was to explore bone regeneration under the conditions of metaphyseal defects filling with HAP-TCP in experimental setting.

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**Abbreviations used:** HAP — hydroxyapatite; TCP — tricalcium phosphate.

## MATERIALS AND METHODS

The assessment of the bioactive ceramics HAP-TCP ( $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ , RAM I Kyiv) as a bone matrix and stimulator of reparative osteogenesis at implantation into the tibial metaphyseal region was provided in the experiment in 16 animals (white outbred rats, body weigh 300–350 g) from the vivarium of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of the NAS of Ukraine. Introduction of animals into the experiment, surgical intervention and withdrawal of animals from the experiment were performed under general thiopental anesthesia. Periods of observation were 2, 4, and 8 weeks. All manipulations in animals were performed according to the requirements of General Ethical Principles of Experiments on Animals (Ukraine, 2001) in accordance with the European convention for the protection of vertebrate animals used for experimental and other scientific purpose. The protocol of animal experiments was approved by the Bioethical Committee (protocol № 5 of 08.03.2018).

For morphological studies by light microscopy, the fragment or whole rat tibia was taken together with the implanted HAP-TCP and fixed in 10% solution of neutral formaldehyde, decalcified in 4% nitric acid solution, dehydrated in ascending concentrations of alcohols, and immersed into paraffin. Serial histological sections 7–9  $\mu\text{m}$  thick were made on a microtome, and stained with Weigert's iron hematoxylin and eosin, as well as van Lawn picrofuxin. The stained sections were analyzed by OlympusBX-63 microscope with CellSenceDimension 1.8.1 morphometric program. The estimation of tissue areas formed at the region of the implanted HAP-TCP was performed between two fragments of the cortex adjacent to the implanted HAP-TCP.

For morphological studies by electron microscopy, the tissue obtained from the biopsy of the implantation zone was dissected into pieces of approximately 1  $\text{mm}^3$  and fixed with 2% solution of glutaraldehyde buffered with 0.1 molar Serensen's mixture, pH 7.3, for 1.5 h. After washing for 20 h in the same buffer, the tissue was decalcified in 5% nitric acid for 2–8 h or with ethylenediamine tetraacetate for 20 days and additionally fixed in 2% osmium tetroxide solution in Serensen's buffer for 1.5 h. Further, the tissue was dehydrated in ascending concentrations of alcohols (70–100°) and absolute acetone, and immersed in a complex of epoxy resins "Epon". Ultrathin sections were made by the ultramicrotome LKB 8800 111 (Sweden) and contrasted with uranium acetate. The preparations were examined using the electron microscope JEM-100B (Japan) at an acceleration voltage of 60 kV.

Statistical analysis was performed using Microsoft Excel XP. To determine the differences between the obtained indicators Student's *t*-test was used. Differences at  $p < 0.05$  were considered statistically significant.

## RESULTS

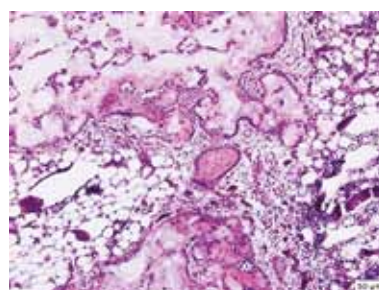
**Light microscopy.** At 2 weeks post-implantation: directly on the HAP and TCP particles single osteo-

clasts were determined, which formed resorption cavities into which osteoblasts were ingrown. Osteoblasts were located on the surface of HAH and TCP particles, forming an osteoid. The location of osteoclasts and osteoblasts on the surface of HAP-TCP indicates its high biocompatibility with bone tissue.

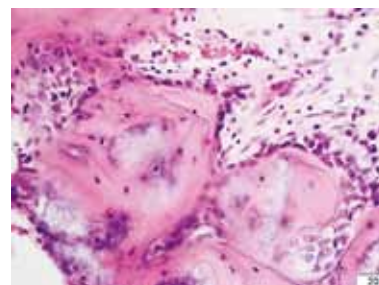
At 4 weeks post-implantation: in the cortex zone, HAP and TCP particles were interconnected by newly formed bone tissue with the maternal bone adjacent to the implantation site. Osteocytes were located on the surface of the bone trabeculae in narrow and dilated lacunae. The intertrabecular spaces were filled with fibroreticular tissue. The osseous trabeculae that form the intertrabecular spaces had a high density of osteoblasts in the marginal compartments, and their accumulation was observed in the areas between HAP and TCP particles. The cortex of the maternal bone was characterized with the signs of post-traumatic reactive remodeling associated with the expansion of vascular bone canals (Fig. 1).

At 8 weeks post-implantation: in some places, osteocytes were located on the surface of the bone trabeculae, and osteoblasts were located on the marginal surface (Fig. 2). Most of the surface of the bone trabeculae was covered with active osteoblasts, which have a weakly basophilic stained nucleus surrounded by a rich cytoplasm, which indicates the activation of biosynthetic processes in such cells. An accumulation of osteoblasts on which an osteoid is formed was observed.

**Morphometric study** was conducted for assessing the ratio of tissues formed in the cortex with implanted HAP-TCP. The ratio of tissues (%) in the area of the defect between fragments of the cortex with implanted HAP-TCP was determined as follows: bone



**Fig. 1.** HAP and TCP particles surrounded by trabeculae of the newly formed bone and fibroreticular tissue in the bone canal. H&E,  $\times 200$



**Fig. 2.** Bone trabeculae with high density of osteoblasts on the marginal surface. The clusters of the osteoblasts, HAP and TCP particles. H&E,  $\times 400$

tissue — 23%, fibroreticular tissue — 15%, HAP-TCP — 62%; the ratio of tissues in the area of the spongy bone tissue: bone tissue — 32%, fibroreticular tissue — 14%, HAP-TCP — 54%. Based on morphometry data, it was found that the area of bone tissue was by 8% larger than the area of fibroreticular tissue, and the area of tissues in the zone of the defect located in the spongy bone tissue was by 18% larger compared to the area of fibroreticular tissue ( $p < 0.001$ ), these results suggest that HAP-TCP is a stimulator of osteogenesis at the site of implantation.

**Electron microscopy study** provides the evidence of active, synchronous processes of implant resorption with chaotic replacement by collagen masses, which are subject to mineralization (ossification), the proliferation of cellular elements of collagenogenesis and osteogenesis (osteoblasts and osteoclasts), as well as saturation of newly formed tissue with blood cells and formation of mature bone tissue with canals of varying width inside.

At 2 weeks post-operation, the main difference is the presence of mature functionally active osteoblasts in the area of osteoreparation (Fig. 3). Most of them are located in the perivascular spaces. This pattern indicates a high level of biosynthetic activity during this period. The newly formed bone tissue has a delicate fluffy pattern, a wide profile of cellular lacunae

The term of 4 weeks post-operation is specified by non-uniformity of the osteoreparation zone pattern, which is expressed in the mosaic structure of the newly formed bone tissue (Fig. 4, 5). This is due to the completion of the processes described for the earlier stages because the area of bone maturation is adjacent to the response of fibrovascular tissue to the implant material. The structure of the newly formed bone becomes coarser. The areas of bone directly adjacent to the implant regenerate faster.

At 8 weeks post-operation, there was a normal remodeling of bone tissue (Fig. 6, 7). However, in some cases, it should be mentioned that in the study of bone with implanted HAP-TCP, the processes of HAP resorption are slower and after the 8-week period, a signifi-

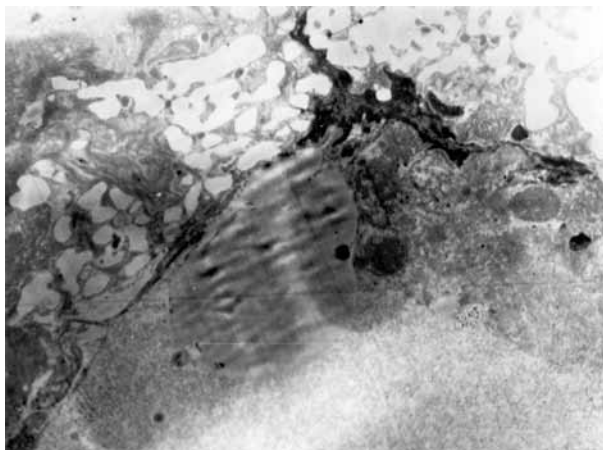
cant amount of HAP is visible, although the pores are filled with bone tissue.

Therefore, the data obtained from EM studies indicate that HAP-TCP stimulates reparative osteogenesis, especially at the periphery at the site of contact with bone. Closer to the center, reparative osteogenesis is slowed down due to the formation of connective tissue, which prevents further HAP-TCP remodeling.

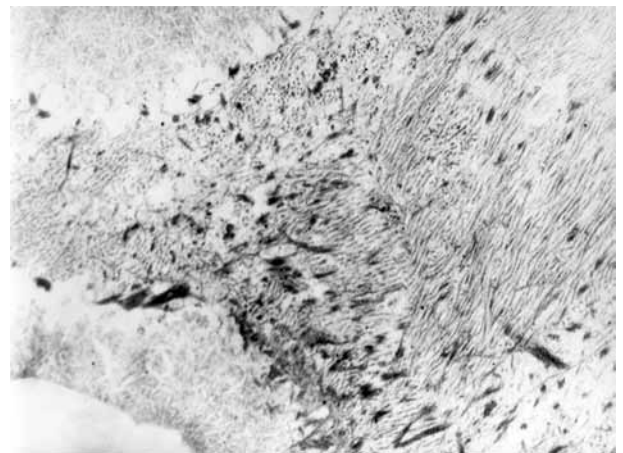
## DISCUSSION

The progression of the tumor is characterized by its spreading to sites distant from the primary focus, and as far bones are the most numerous organs in the anatomical structure of a human, the frequency of their affection with cancers of different localizations reaches 95% [10]. Therefore, treatment modalities aimed at the prevention of bone lesions and the treatment of the consequences of these lesions have recently become increasingly important. Moreover, the use of the drugs that affect bone metabolism to prevent the development of bone metastases in complex treatment of cancer is recognized by many researchers [3, 8, 11].

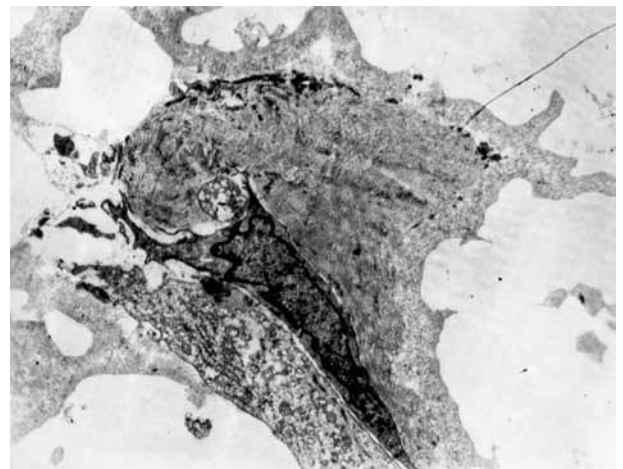
In the last 2 decades, in the literature on treatment of patients with solid tumors much attention has been



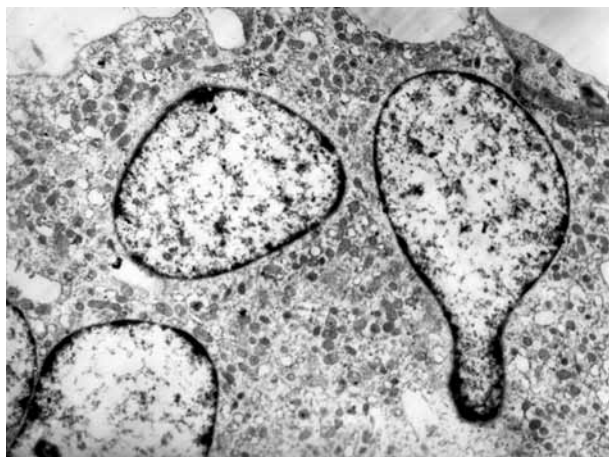
**Fig. 3.** Electronogram. Osteoblasts near HAP-TCP, with podosomes penetrating the material.  $\times 10\,000$



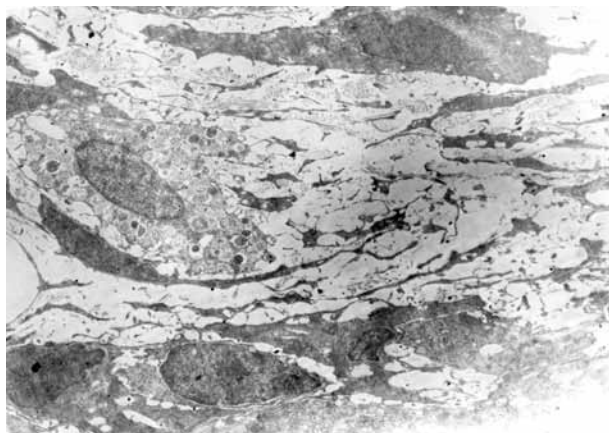
**Fig. 4.** Electronogram. Randomly oriented masses (bundles) of newly formed collagen fibers on the border with HAP granules. Numerous foci of collagen tissue mineralization.  $\times 6500$



**Fig. 5.** Electronogram. Active collagen fiber production by osteoblasts with mineralization foci on the border with HAP masses. Dense osteoblasts of the 3<sup>rd</sup> order along with the «young» osteoblast. GAP resorption.  $\times 6500$



**Fig. 6.** Electronogram. “Young” multinucleated osteoclast in newly formed bone tissue.  $\times 6500$



**Fig. 7.** Electronogram. Rat tibia. HAP granules and fibroblasts between them. In the study of tissue biopsies taken from the central areas of implantation after 8 weeks, granules of ceramic material are noted, and between them, the connective tissue was located.  $\times 6500$

paid to the possible prevention and treatment of metastatic skeletal lesions to prevent severe complications of the clinical course of the disease and significant worsening the quality of life of treated patients [8, 10, 12–14].

However, most importantly, the introduction of techniques aimed at restoring bone tissue in case of tumor damage or after surgery into clinical practice assume ever greater importance [7, 8, 15, 16]. Such techniques include the use of bioactive ceramics, in particular the agent HAP-TCP, which in tactical terms due to the synergy of its components provides full integration of the implant with bone tissue and subsequent induction of osteogenesis [17], and in strategic perspective — significant improvement of patients' quality of life.

Our experimental studies of bone defect plasticity in rats proved the presence of HAP-TCP osteoinductive and osteointegration effect. The absence of inflammatory and allergic reactions in the area of implantation indicates the biocompatibility of HAP-TCP with bone tissue and bone marrow. HAP-TCP composite material induces the formation of bone-ceramic composite in the defect zone, which contributes

to a more dynamic uncomplicated course of reparative osteogenesis.

## REFERENCES

1. Burianov AA, Chornyi VS, Dedukh NV, *et al.* Bone regeneration study after osseous defect filling with bioglass powdered form. *Annals Traumatol Orthoped* 2018; **3–4**: 10–4 (in Russian).
2. Fernandez de Grado G, Keller L, Idoux-Gillet Y, *et al.* Bone substitutes: a review of their characteristics, clinical use, and perspectives for large bone defects management. *J Tissue Eng* 2018; **9**: 2041731418776819. doi: 10.1177/2041731418776819.
3. Ulianchych N, Mishchenko O, Kondratets I, Zaitseva N. Controlled properties of osteotropic biomimetic implant material for various clinical applications (literature review and own results). *Rus J Biol Res* 2014; **2**: 100–12 (in Russian).
4. National Cancer Registry of Ukraine. <http://www.ncru.inf.ua/publications>. Accessed December 26, 2020.
5. Cappellari A, Trovarelli G, Crimi A, *et al.* New concepts in the surgical treatment of actual and impending pathological fractures in metastatic disease. *Injury* 2020; **11**: S0020-1383(20)30952-9. doi: 10.1016/j.injury.2020.11.025.
6. Cardoso P, Rodrigues-Pinto R. Surgical management of bone and soft tissue sarcomas and skeletal metastases during the COVID-19 pandemic. *Eur J Surg Oncol* 2020; **46**: 1178–9. doi: 10.1016/j.ejso.2020.04.027.
7. Jariwala SH, Lewis GS, Bushman ZJ, *et al.* 3D Printing of personalized artificial bone scaffolds. *3D Print Addit Manuf* 2015; **2**: 56–64. doi: 10.1089/3dp.2015.0001.
8. Agarwal R, Garcia AJ. Biomaterial strategies for engineering implants for enhanced osseointegration and bone repair. *Adv Drug Deliv Rev* 2015; **94**: 53–62. doi: 10.1016/j.addr.2015.03.013.
9. Turpin A, Duterque-Coquillaud M, Vieillard MH. Bone metastasis: current state of play. *Transl Oncol* 2020; **13**: 308–20. doi: 10.1016/j.tranon.2019.10.012.
10. Ryan C, Stoltzfus KC, Horn S, *et al.* Epidemiology of bone metastases. *Bone* 2020; 115783. doi: 10.1016/j.bone.2020.115783.
11. Koren'kov OV. Morphological peculiarities of healing of experimental defects of a long bone cortex under the condition of  $\beta$ -tricalcium phosphate implantation. *Orthopaed Traumatol Prosthet* 2015; **3**: 46–50. (in Ukrainian).
12. Bandini M, Pompe RS, Marchioni M, *et al.* Improved cancer-specific free survival and overall free survival in contemporary metastatic prostate cancer patients: A population-based study. *Int Urol Nephrol* 2018; **50**: 71–8. doi: 10.1007/s11255-017-1744-2.
13. Wang Z, Cheng Y, Chen S, *et al.* Novel prognostic nomograms for female patients with breast cancer and bone metastasis at presentation. *Ann Transl Med* 2020; **8**: 197. doi: 10.21037/atm.2020.01.37.
14. D'Oronzo S, Silvestris E, Paradiso A, *et al.* Role of bone targeting agents in the prevention of bone metastases from breast cancer. *Int J Mol Sci* 2020; **21**: 3022. doi: 10.3390/ijms21083022.
15. O'Keefe RJ, Mao J. Bone tissue engineering and regeneration: from discovery to the clinic — an overview. *Tissue Eng Part B Rev* 2011; **17**: 389–92. doi: 10.1089/ten.TEB.2011.0475.
16. Kon E, Filardo G, Roffi A, *et al.* Bone regeneration with mesenchymal stem cells. *Clin Cases Miner Bone Metab* 2012; **9**: 24–7.
17. Shimon VM, Alfeldiy SP, Shimon MV, *et al.* Rationale for tricalcium phosphate preparation application in treatment of long bones fractures and defects. *Annals Traumatol Orthoped* 2018; **39–40**: 37–40 (in Ukrainian).

# ЕКСПЕРИМЕНТАЛЬНЕ ОБҐРУНТУВАННЯ ЗАСТОСУВАННЯ БІОКЕРАМІКИ НА ОСНОВІ ГІДРОКСИПАТИТУ ТА ТРИКАЛЬЦІЮ ФОСФАТУ ДЛЯ ЗАМІЩЕННЯ ДЕФЕКТІВ КІСТКИ ПІСЛЯ ВИДАЛЕННЯ ПУХЛИНИ

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**Стан проблеми:** Останнім часом у якості імплантів у кістковій інженерії перевага віддається біоактивній кераміці на основі гідроксиapatиту (ГАП) та трикальцію фосфату (ТКФ). **Мета:** Вивчення регенерації кістки в умовах за-

повнення метафізарних дефектів композицією на основі ГАП-ТКФ. **Матеріали та методи:** Експеримент проводили на неінbredних щурах, терміни спостереження — 2, 4, 8 тиж. Морфологічні дослідження проводилися за допомогою світлової та електронної мікроскопії. **Результати:** У динаміці спостереження зафіксоване поступове розташування остеокластів і остеобластів на поверхні композиції ГАП-ТКФ, що свідчить про її високу біосумісність з кістковою тканиною. Впродовж експерименту відбувалися синхронні процеси резорбції імплантів, мінералізації, проліферації клітинних елементів колагено- і остеогенезу (остеобластів і остеокластів) та формування зрілої кісткової тканини. **Висновки:** Експериментальні дослідження пластики дефектів кісток довели наявність остеіндуктивного та остеінтеграційного ефекту композиції ГАП-ТКФ, що сприяє більш динамічному неускладненому перебігу репаративного остеогенезу.

**Ключові слова:** кістка, гідроксиapatит, трикальцій фосфат, імплантація, репаративний остеогенез.