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Bone regeneration after implantation of calcium phosphate cements based on metastable tricalcium phosphate

(*in vivo* experimental study)

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Calcium phosphate cement (CPC) is a material used to fill bone defects. Its advantages include being able to fill irregularly shaped spaces, its similarity to bone tissue, and ease of biodegradation. However, insufficient durability and unpredictable rate of resorption limit CPC use. Objective. Study the dynamics of morphological changes in rat femurs after implanting two types of CPC based on metastable α'–tricalcium phosphate (α'–TCP) into defects in the distal metaphysis. Methods. 42 male white rats were used in the study. In each rat, defects were created in the distal metaphysis of the left femur and filled with one of the two types of CPC. The animals were split into two groups: I (n = 21) — CPC based on α'–TCP powder; II (n = 21) — CPC based on α'–TCP powder reinforced with hydroxyapatite (HA) whiskers (4 % mass). Both varieties of CPC were developed and prepared at the Department of Solid-State Physics at the V. N. Karazin Kharkiv National University (Ukraine). 14, 30, and 60 days after the surgery, the animals were sacrificed, and histological analyses were performed. Results. For both types of CPC, inflammation was not observed in the region around the implant at 14, 30, or at 60 days. Bone tissue formed on the surface of the materials. The stages of bone repair were similar to the known stages of bone repair. As a result of the resorption of the CPC, 60 days after surgery the CPC comprised 26.83 % of the area of the defect in group I and 29.93 % in group II. The rest of the area was composed of lamellar bone. The two groups did not differ significantly in rate of CPC resorption or bone tissue formation. Conclusions. The two types of CPC studied, based on α'–TCP (group I) and α'–TCP reinforced with HA whiskers (group II), are biocompatible, osteoconductive, and osteoinductive. In addition, these materials are biodegradable and, with time, are replaced by bone tissue.

Кальцій-фосфатні цементи (КФЦ) є перспективним матеріалом для заповнення дефектів кісток завдяки рідкому стану, який дає змогу заповнювати порожнини неправильної конфігурації, та спорідненості з кістковою тканиною й здатністю до біорезорбції. Проте недостатня міцність і непрогнозована біодеградація обмежують їхнє використання. Мета. Дослідити динаміку морфологічних змін стегнових кісток щурів після імплантації в дефект у дистальному метафізі двох видів цементів на основі метастабільного α'–трикальційфосфату (α'–ТКФ). Методи. У дослідженні використано 42 самці білих лабораторних щурів, яким моделювали дефект у дистальному метафізі лівої стегнової кістки і заповнювали одним із двох видів цементів. Залежно від цього тварин розподілили на групи: I (n = 21) — твердою фазою КФЦ був порошок α'-ТКФ; II (n = 21) — порошок α'-ТКФ, зміцнений голчастими кристалами гідроксилапатиту (ГА) (4 мас%). Обидва КФЦ розроблені та виготовлені на кафедрі фізики твердого тіла фізичного факультету Харківського національного університету імені В. Н. Каразіна (Україна). Через 14, 30 і 60 діб після операції тварин виведено з експерименту та виконано гістологічні дослідження. Результати. Запальної реакції навколо обох видів КФЦ не зафіксовано на всі терміни спостереження. Кісткова тканина утворювалася безпосередньо на поверхні матеріалів, остеорепарація перебігала відповідно до загальновідомих стадій. Унаслідок резорбції КФЦ через 60 діб після введення в ділянці дефекту залишилось 26,83 % (група I) і 29,93 % (II) кераміки, решту території займала кісткова тканина пластинчастої структури. Не встановлено відмінностей між групами за швидкістю біодеградації чи утворення кісткової тканини. Висновки. Досліджувані КФЦ, тверда фаза яких складається з α'-ТКФ або α'-ТКФ, армованого голчастими кристалами ГА, є біосумісними, мають остеоіндукутивні й остеокондуктивні якості. Матеріали є біорезорбтивними — поступово заміщуються кістковою тканиною. Ключові слова. Дефект кістки, регенерація кістки, кальцій-фосфатний цемент, метастабільний α'-трикальційфосфат, гідроксилапатит, стегнова кістка, експеримент, щури

Key words. Bone defect, bone repair, calcium phosphate cement, metastable α′–tricalcium phosphate, hydroxyapatite, rat femur, experiment

Introduction

Today, there are many materials for replacing bone defects, and each has certain advantages and disadvantages. In particular, ceramics based on hydroxylapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$) and tricalcium phosphate (TCP, $Ca_3(PO_4)_2$) are used in orthopedics, maxillofacial surgery, dentistry due to their composition, which is close to the mineral component bone matrix, as well as such properties as biocompatibility, osteoconduction, osseointegration, ability to be replaced by bone tissue [1–3]. The prospect of using TCP (β-TCP) as an osteoplastic material is related to its proven osteoinduction, i.e., the ability to stimulate the development of cells in the osteogenic direction [4, 5]. Clinical observations over the course of a year even showed the identical efficiency of application for the reconstruction of bone defects of β-TCP granules (diameter 1 mm, macroporosity 400 µm, microporosity ((2.73 ± 1.0) µm) and autografts [6]. A recently published meta-analysis [7] determined that materials based on β-TCP in combination with the use of blocked plates can become a safe and effective alternative to the use of autografts during open wedge-shaped high tibial osteotomy (a gap of more than 10 mm) in patients with osteoarthritis of the knee joint, considering in particular, the absence of the need for additional surgical intervention, excessive blood loss, hematoma formation, and pain. However, the authors noted differences in the physicochemical properties of industrial samples of calcium phosphate ceramics from different manufacturers, which is reflected in their ability to biodegrade with replacement by bone tissue [4, 5, 7] In addition, a difficult task for the developers of calcium-phosphate ceramics is the controlled speed of their resorption and the strength and mechanical reliability necessary for the requirements of orthopedics, which is also determined by the physicochemical structure.

Since bioactive ceramics are used as a material for filling bone defects, which have a complex shape and arbitrary dimensions, the creation of calcium-phosphate cements (CPCs) with the necessary viscosity and setting time at body temperature, appropriate compressive strength, and an ideal dissolution rate for the formation of bone tissue has become relevant [8, 9]. Like any cement, CPC consists of solid (powder of one or a mixture of calcium phosphates) and liquid (aqueous solution) phases, which after mixing acquire a paste-like consistency, and then harden in a short time. According to the final product, CPCs are classified as brushite (dicalcium phosphate dihydrate, DCPD) and apatite [1, 10, 11]. Their use as bone substitutes is very promising, since a weakly crystalline apatite phase similar to bioapatite is formed as a result of hardening of apatite cements. The solid phase of such cements is produced by hydrolysis of α-TCP, but the powder obtained as a result of high-temperature transformation consists of relatively large particles with high crystallinity, which significantly reduces its bioactivity. The need for grinding in a ball mill and sieving significantly complicates the production procedure and increases the cost of CPC. Another way of obtaining nano-sized α-TCP powder is the annealing of an amorphous calcium phosphate precipitate with a Ca/P ratio of 1.5 at temperatures in the range of 500–700 °C [12]. During heating, such α-TCP turns into a stable β-TCP; therefore it is called metastable and denoted as α′-TCF. It is assumed that apatite CPCs based on metastable α′-TCF has the same biological properties as CPCs based on high-temperature α-TCP, but is much simpler to obtain and, accordingly, more economically beneficial.

Due to their plasticity, CPCs are convenient to use, however, despite numerous studies, there are still some limitations that prevent their widespread clinical use. Firstly, it is an unpredicted biodegradation, and secondly, low mechanical qualities [9, 13]. Research on improving the properties of the solid component of CPCs is related to changing the size and shape of particles, their interaction, increasing macroporosity, etc. [14]. Various impurities are also used, for example: strontium — to provide anti-osteoporotic properties and increase compressive strength [15]; polylactic glycolic acid (PLGA) nanofibers with carboxymethyl cellulose (CMC) to increase porosity, durability and osseointegration [16], etc. One of the approaches to improve the strength characteristics of metastable α′-TCP without losing its biodegradability is the addition of HA filamentous fibers to the solid component of TCP [1].

Purpose: to investigate the time course of morphological changes in rat femurs after implantation into a defect in the distal metaphysis of two types of cements based on metastable α′–tricalcium phosphate.

Material and methods

The experiments were performed in compliance with the requirements of the Law of Ukraine «On the Protection of Animals from Cruelty Treatment» (Articles 26, 31) [17], the European Convention on the Protection of Vertebrate Animals Used for Research and Other Scientific Purposes (Strasbourg, 1986) [18] and Directive 2010/63/EU. Design of the experiment was discussed and approved at the meeting of the Bioethics Committee at the State Institution Professor M. I. Sytenko Institute of Spine and Joint Pathology of the National Academy of Medical Sciences of Ukraine (Protocol No. 194 dated 08.07.2019).

Calcium-phosphate cements

The powder of metastable α′-TCP was obtained by rapid nitrate synthesis, washing, lyophilization, and subsequent heat treatment of sediments with a Ca/P ratio of 1:1. Washing of sediments under certain conditions made it possible to increase the Ca/P ratio to 1.5. Single-phase metastable α′-TCP was processed by thermal treatment of hydrolyzed sediments [19]. A 2.5 % solution of sodium hydrogen phosphate, Na2HPO4, was used as a liquid phase for cement preparation, which was added to a portion of α′-TCP powder, which ensured a ratio of solid phase / liquid phase — 1/1.25, immediately before use. Next, the mass was thoroughly mixed with a spatula until a homogeneous paste was obtained and it was kept for 3–5 min [20]. The bone defect was sealed with the obtained paste, sealing new portions of the paste with a spatula, until the defect was completely filled and the release of body fluid from it ended.

The procedure for preparing reinforced cement was identical to the one described, but 4 wt% of needle-like HA crystals were added to the solid phase in advance. They were obtained by hydrothermal synthesis according to the original technology developed at the Department of Solid State Physics of the Faculty of Physics of V. N. Karazin Kharkiv National University (temperature 235 °C, pressure 20 atm, time 1 h) [21].

Animals

The study involved 42 male white laboratory rats from the population of the Experimental Biological Clinic of the State Institution Professor M. I. Sytenko Institute of Spine and Joint Pathology of the National Academy of Medical Sciences of Ukraine (at the beginning of the experiment, the animals were 5–6 months old, body weight 220–400 g), which were kept 5 in a cage with free access to food and water, on a standard diet with a temperature of indoors (22 ± 2) °C.

All rats underwent surgical interventions to simulate a hole defect in the distal metaphysis of the left femur, which was filled with the investigated CPC. According to its composition, the animals were randomly divided into groups:

– 1 (n = 21) — the solid phase of CPC was α′-TCP powder;

– 2 (n = 21) — the solid phase of CPC was α′-TCP powder strengthened by needle crystals of HA.

Animals were removed from the experiment by administering a lethal dose of anesthetic (ketamine, 120 mg/kg, intramuscularly) 14, 30, and 60 days after surgery.

Surgical interventions

Surgical interventions were performed under aseptic and antiseptic conditions under general anesthesia (ketamine, 50 mg/kg body weight, intravenously). After preparation of the operating field (shaving the hair on the left knee and thigh, treatment with Kodan® forte antiseptic (Schülke & Mayr GmbH, Germany)), the distal metaphysis of the femur was opened through an anterior-lateral approach. Defects (depth 3 mm, diameter 2 mm) were modeled with a dental bur, washed with an antiseptic, dried and filled with ceramic material, which at that time had a paste-like consistency and was prepared in advance (1 g of ceramic powder was mixed with 1 ml of sealing liquid for 5 minutes before entering the defect) (Fig. 1). The material was expected to harden within 5 minutes. Then the wound was treated locally with the antibiotic Bicilin®-3 (PJSC Kyivmedpreparat, Ukraine), the muscles and the skin wound were sutured. The skin in the area of surgical intervention was treated with Kodan® forte antiseptic.

Histologic study

For histological study, operated femurs were isolated, cleaned of soft tissues and placed for fixation for 4 days in a 10 % solution of neutral formalin. Decalcification was carried out in a 10 % formic acid solution. After that, the distal metaphyses with the implantation area were cut off, dehydrated in alcohols of increasing concentration, soaked in paraffin with xylene and embedded in paraffin. The prepared frontal histological sections 5–6 μm thick were stained with hematoxylin and eosin and analyzed under a BX63 light microscope (Olympus, Japan).

A DP73 camera (Olympus) and Cell Sens Dimension 1.8.1 software (Olympus, 2013) were used to obtain digital images.

Histomorphometry

In the implantation area, using the Cell Sens Dimension 1.8.1 software (Olympus, 2013), the areas of newly formed tissues (bone and connective) and ceramic material (on 5 central sections of each distal metaphysis) were measured, then their relative content was calculated (bone tissue — B %, fibrous tissue — F %, Ceramics — C %) of the total defect area. Newly formed bone trabeculae together with bone marrow were considered as bone tissue.

Statistics

The obtained numerical values are given as mean and standard deviation. The Student's t-test for independent samples was used to compare the indicators of different samples for the same study period and

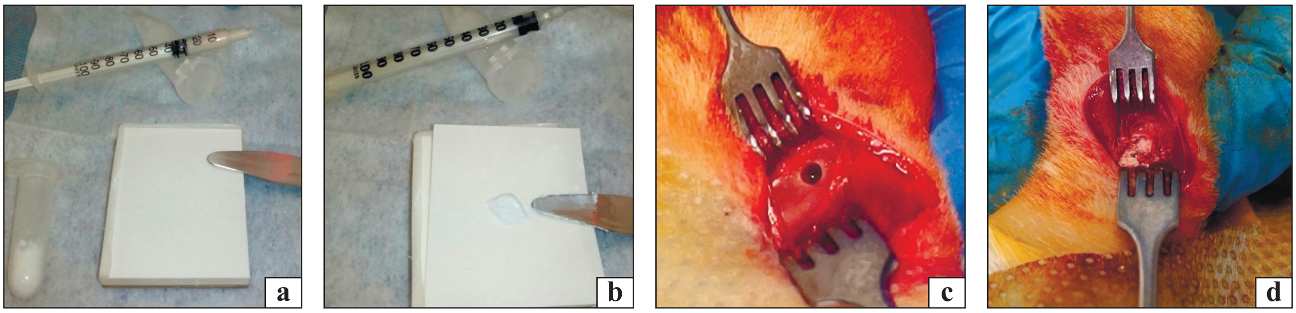

Fig. 1. Stages of surgical intervention: material preparation (a, b), defect in the distal metaphysis of the rat femur (c), filled with a paste-like ceramic material (d)

the area of connective tissue for different periods; differences were considered significant at $p < 0.05$. To compare the parameters of the same sample for the three observation periods, one-factor analysis of variance ANOVA with Bonferroni's correction was used; it was considered significant at $p < 0.017$.

Results and their discussion

Animals of both groups fully loaded the operated limb during all observation periods, were characterized by normal motor activity, food and water consumption. No complications were observed in the postoperative period.

Macroscopically, the area of the defect was detected only 14 days after the operation by the presence of a cavity in the cortex filled with whitish content.

During histological examination 14 days after implantation, the ceramic material in rats of both groups was clearly defined in the defect and almost completely filled it (Fig. 2). Around the perimeter of the material, connective and bone tissues were formed in different proportions. Newly formed bone trabeculae were characterized by a significant number of osteocytes. In the connective tissue, the cell density was high. Osteoblastic and fibroblastic diferon cells predominated. On the surface of the ceramic material, multinucleated cells of foreign bodies of the osteoclast type were found (Fig. 2, b; 2, d; indicated by arrows), which participated in its bioresorption.

According to the results of the histomorphological study, C % and B % in femoral bone defects did not differ statistically significantly in the studied groups of rats, and F % was 2.04 times greater ($p = 0.004$) in group 2 (Table).

30 days after implantation, as in the previous period of the study, the ceramic material was well defined on histological specimens in animals of both groups; its relative area did not change statistically significantly compared to the 14th day (Table). However, signs of reconstruction were recorded along its entire perimeter: sprouting of blood vessels (Fig. 3, b, indicated by an arrow) and newly formed bone tissue (Fig. 3, d, nB).

B % was not statistically significantly different compared to the 14th day in rats of both groups and between groups (Table). However, it is necessary to consider the formation of bone tissue in them in the area of the cortical layer defect. At this observation period, it was spongy bone tissue with a lamellar structure (Fig. 3, a, marked nCx; 3, c — nB).

In some places, cells of connective tissue remained in the area of the defect. F % decreased compared to the 14th day by 9.38 times ($p < 0.001$) in rats of group 2 and was smaller compared to the indicator of group 1 by 5.77 times ($p = 0.004$). In the animals of group 2, no significant differences in this indicator were found (Table).

After *60 days after* implantation, in two rats of group 1 and three of group 2, the ceramic material was completely rebuilt, the cortex was formed by compact bone tissue, and the area of the defect could be determined only by the disordered arrangement of bone trabeculae.

Remains of ceramics were found in the remaining animals, the relative area of which decreased compared to the 30th day of observation by 1.94 times in group 1 ($p < 0.001$) and by 1.82 times ($p < 0.001$) in group 2. Significant differences in this the indicator between the groups has not been established. In both groups, bone tissue with a lamellar structure formed around the CPC. It also sprouted inside the ceramics. The cortex was formed by compact bone tissue (Fig. 4).

Discussion

The focus of the study was the analysis of bone tissue remodeling and two CPCs implanted in hole defects made in the distal metaphyses of rat femurs. CPCs differed in the composition of the solid phase: one contained α′-TCF powder (group 1), the other contained α′-TCP powder reinforced with needle-shaped HA crystals (4 wt%). α′-TCP powder was produced using the same technology [20], needle crystals

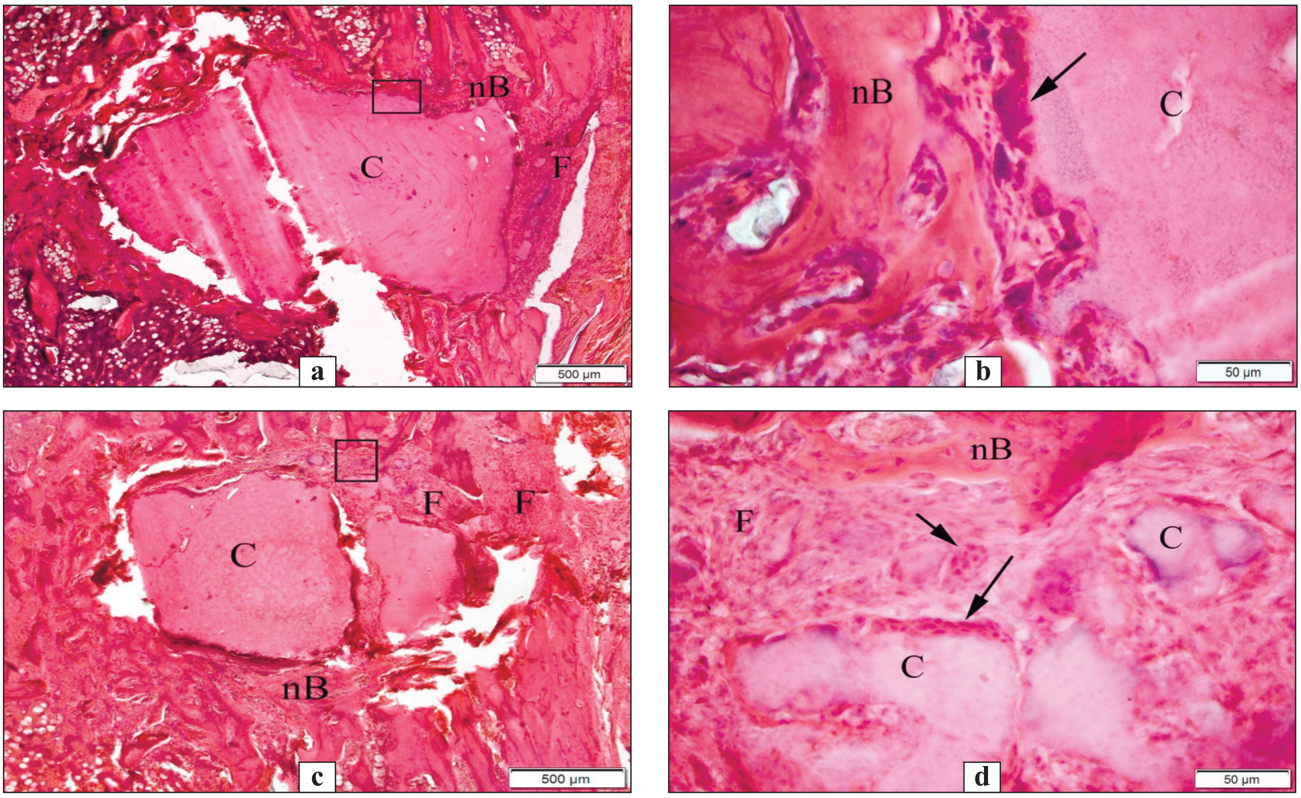

Fig. 2. Fragments of the distal metaphyses of the femurs of rats 14 days after the implantation of CPC, which contains α′-TCP (a, b) and α′-TCP, reinforced with needle HA (c, d). C - ceramics, nB - newly formed bone tissue, F - connective tissue, arrows - multinucleated cells of foreign bodies of the osteoclast type. Fig. 2, b is a fragment of Fig. 2, a; Fig. 2, d — Fig. 12, H&E stain

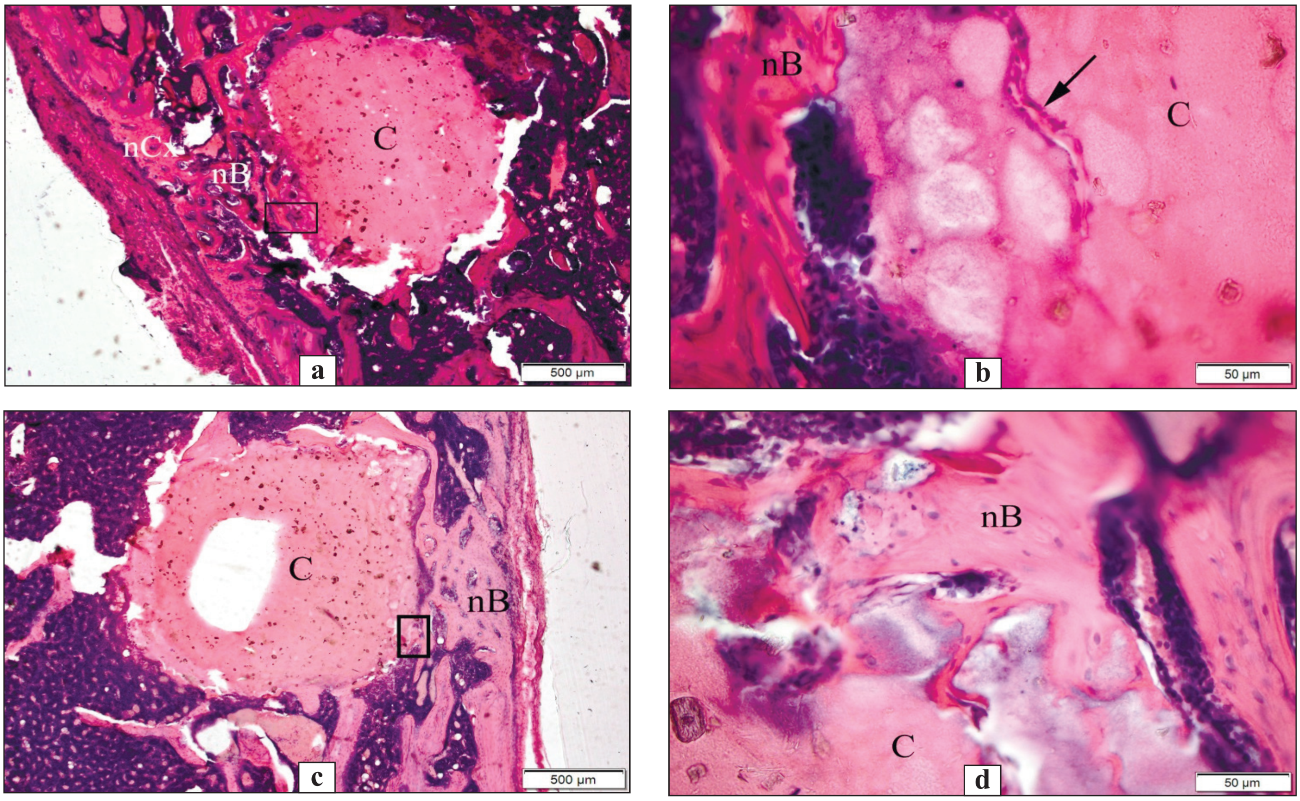

Fig. 3. Fragments of the distal metaphyses of the femurs of rats 30 days after the implantation of CPC, which contains α′-TCP (a, b) or α′-TCP reinforced with needle HA (c, d). C — ceramics, nB — newly formed bone tissue, F — connective tissue, arrow — sprouting of a blood vessel into the CPC. Fig. 3, b is a fragment of Fig. 3, a; Fig. 3, d — Fig. 3, H&E stain

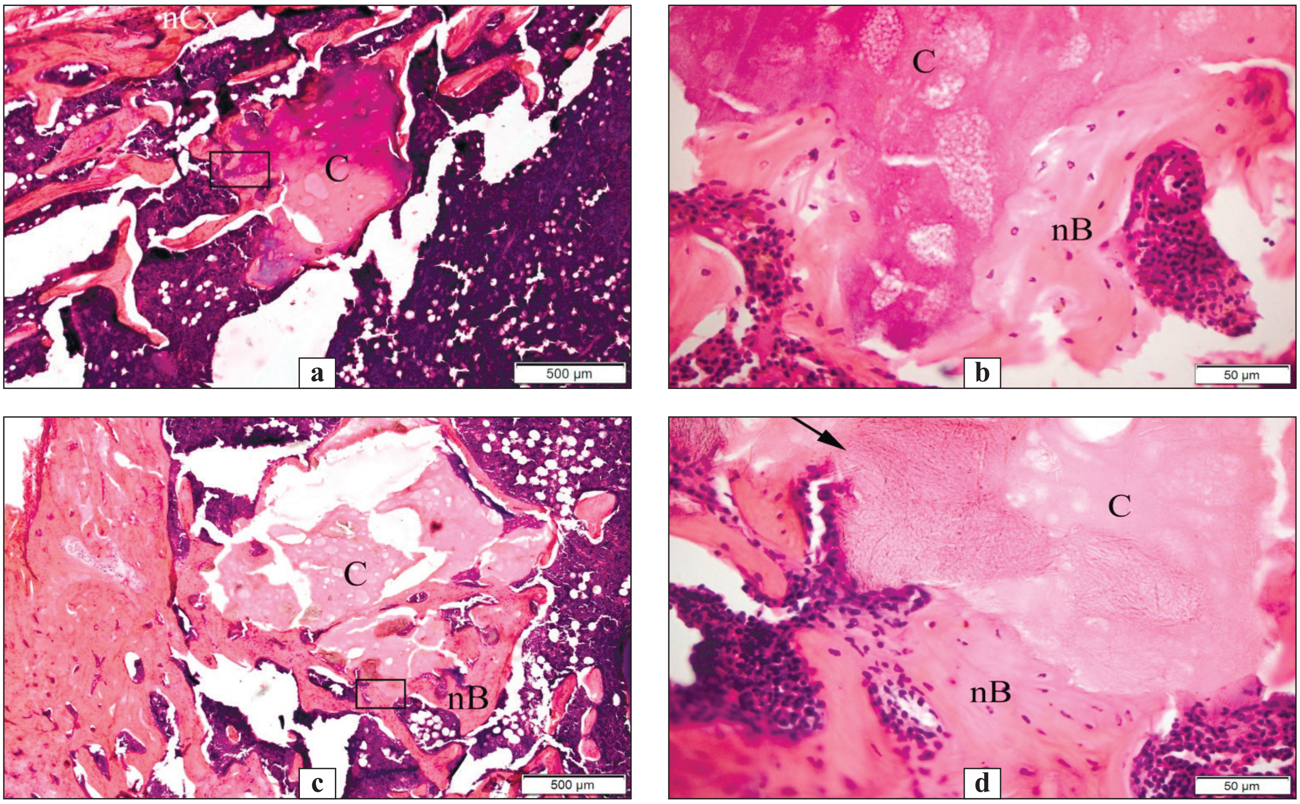

Fig. 4. Fragments of the distal metaphyses of the femurs of rats 60 days after the implantation of CPC, which contains α′-TCP (a, b) or α′-TCP reinforced with needle HA (c, d). C — ceramics, nB — newly formed bone tissue, arrow — acicular HA. Fig. 4, b is a fragment of Fig. 4, a; Fig. 4, d — Fig. 4, H&E stain

Table

Relative areas of tissues in defects of the distal metaphysis of the femur of rats according to the observation period

Area, %	Rat group	Term of observation, days		
		14	30	60
Ceramics	1	52.77 ± 14.74	51.93 ± 11.60 $p_2 = 1.000$	26.83 ± 7.07 $p_2 < 0.001$ $p_3 < 0.001$
	2	50.22 ± 11.13 $p_1 = 0.605$	54.39 ± 9.04 $p_1 = 0.473$ $p_2 = 0.621$	29.93 ± 8.37 $p_1 = 0.214$ $p_2 < 0.001$ $p_3 < 0.001$
Connective tissue	1	7.30 ± 4.07	9.18 ± 8.64 $p_2 = 0.448$	—
	2	14.91 ± 7.49 $p_1 = 0.004$	1.59 ± 2.22 $p_1 = 0.004$ $p_2 < 0.000$	—
Bone tissue	1	39.93 ± 14.41	38.89 ± 10.52 $p_2 = 1.000$	73.16 ± 7.07 $p_2 < 0.000$ $p_3 < 0.000$
	2	34.86 ± 11.67 $p_1 = 0.306$	44.01 ± 9.66 $p_1 = 0.135$ $p_2 = 0.029$	70.06 ± 8.37 $p_1 = 0.214$ $p_2 < 0.000$ $p_3 < 0.000$

Notes: p_1 – comparison of the relative plane of one type of fabric or ceramics for the same period between groups of animals; p_2 – comparison of the relative plane of one type of fabric or ceramic with the 14th day of observation within one group of animals; p_3 – comparison of the relative plane of one type of fabric or ceramic with the 30th day of observation within one group of animals.

of HA were added to it in advance. Both CPCs were developed and provided for research by the same manufacturer (Department of Solid State Physics, Faculty of Physics, V. N. Karazin Kharkiv National University, Ukraine, headed by Professor Z. Z. Zyman). This made it possible to avoid the difference in the physical and chemical characteristics of the material, which could affect its behavior in the biological environment in vivo, namely the bone [4, 5, 7].

CPC is a promising material for filling bone defects: firstly, the liquid form makes it possible to fill cavities of irregular configuration, and secondly, they are related to bone tissue, capable of bioresorption [1, 9, 14, 16]. We established that both investigated CPCs (α′-TCP and α′-TCP reinforced with needle-like crystals of HA) are biocompatible, as evidenced by the absence of an inflammatory reaction for all observation periods. The materials also have osteoinductive qualities: bone tissue was formed directly on their surface; osteoreparation proceeded according to well-known stages [22] with the formation of bone tissue of a lamellar structure by the end of the study (60 days). Both CPCs were subject to resorption with replacement by bone tissue: 60 days after insertion, 26.83 and 29.93 % of ceramics remained in the defect area in group 1 and group 2, respectively. Despite the fact that α-TCP is characterized by a higher rate of resorption than HA [23, 24], we did not establish a significant difference between the groups in terms of the content of ceramic material in the implantation area. There were also no significant differences between the groups regarding the relative area of bone tissue 60 days after surgery.

Conclusions

An *in vivo* experimental study using histology methods established that the studied calcium phosphate cements, the solid phase of which consists of α′-TCP or α′-TCP reinforced with needle-like HA crystals, are biocompatible, have osteoinductive and osteoconductive qualities. Both materials are bioresorbable — they are gradually replaced by bone tissue. No differences were found between them in the rate of biodegradation or bone formation.

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Conflict of interest. The authors declare no conflict of interest.

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BONE REGENERATION AFTER IMPLANTATION OF CALCIUM PHOSPHATE CEMENTS BASED ON METASTABLE TRICALCIUM PHOSPHATE

(*IN VIVO* EXPERIMENTAL STUDY)

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