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Properties of Calcium Phosphate/Hydrogel Bone Grafting Composite on the Model of Diaphyseal Rat Femur's Defect: Experimental Study

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Background. The problem of bone defects replacement is relevant nowadays, that is why many scientists create new synthetic bone substitutes, but the «ideal» material has not been found so far.
The aims of the study: 1) to determine the suitability of the monocortical defect model in the rat femur diaphysis with additional prophylactic reinforcement with a bone plate for assessing the biological properties of implanted materials using the commercially available ChronOS® material as an example; 2) to assess of the osteoconductive properties of composite materials based on poly(ethylene glycol)diacrylate and octacalcium phosphate with architecture Kelvin and gyroid types on the developed model.
Methods. A prospective study, level of evidence II. A monocortical defect of the rat femoral diaphysis (length 7 mm) was produced under anaesthesia in aseptic conditions and fixed with a polyetheretherketone plate and six titanium screws. In the control group, the defect was left empty. In other groups, blocks of one of three materials were implanted — chronOS and composites of poly(ethylene glycol)diacrylate and octacalcium phosphate with 3D-printed Kelvin and gyroid architectures. After 3 and 6 weeks, the rats were sacrificed, and histological examination of the defect zone was performed. The amount of newly formed bone tissue was histometricly assessed, followed by statistical processing of the results.
Results. All rats have reached the planned endpoint, and there were no infectious complications or loss of fixation. Histological examination of the defect zone revealed minimal bone growth in the Control group, rather slow bone formation in the Gyroid group, and statistically significantly more pronounced bone formation in the pores of the materials in the Kelvin and Chronos groups.
Conclusions. Bone defect in this model was not spontaneously filled with bone tissue and allowed us to study the biological properties of bone substitutes (the ability to biodegrade and osteoconductive properties). The osteoconductive properties of a composite material based on poly(ethylene glycol)diacrylate and octacalcium phosphate with a Kelvin architecture are higher than with a gyroid architecture and are comparable to that of the chronOS.

Keywords: bone repair, critical-size defect, hydrogel, bone substitutes, 3D-printing.

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Оценка свойств костнозамещающих материалов на основе полиэтиленгликоль диакрилата и октакальциевого фосфата на модели монокортикального диафизарного дефекта бедренной кости крысы: экспериментальное исследование

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Актуальность. Проблема замещения дефектов кости актуальна в настоящее время, постоянно ведутся поиски новых синтетических костнозамещающих материалов, однако идеальный материал не найден до сих пор.
***Цели исследования*:** 1) определение пригодности модели монокортикального дефекта диафиза бедренной кости крысы с дополнительным профилактическим армированием при помощи накостной пластины для оценки биологических свойств имплантируемых материалов на примере коммерчески доступного материала chronOS®; 2) оценка остеокондуктивных свойств композитных материалов на основе полиэтиленгликоль диакрилата и октакальциевого фосфата с архитектурой Кельвина и типа гироид на разработанной модели.
Материал и методы. Монокортикальный дефект диафиза бедренной кости крыс размером 7 мм в длину производили под наркозом в асептических условиях операционной и фиксировали полиэфирэфиркетоновой пластиной и шестью титановыми винтами. Крыс распределяли случайным образом на четыре группы по 12 особей в каждой. В группе «Контроль» у животных костный дефект не заполняли. У животных в группе «Хронос» дефект заполняли подготовленным материалом chronOS® в виде полуцилиндрического блока, в группе «Кельвин» — исследуемым материалом с архитектурой Кельвина, в группе «Гироид» — исследуемым материалом с архитектурой типа гироид. Через 3 и 6 нед. крыс выводили из эксперимента и производили гистологическое исследование зоны дефекта. Затем выполняли гистометрическую оценку количества новообразованной костной ткани с последующей статистической обработкой результатов.
Результаты. В ходе эксперимента все животные достигли планируемой конечной точки, инфекционные осложнения и потеря фиксации зафиксированы не были. При гистологическом исследовании зоны дефекта выявлен минимальный рост кости в группе «Контроль», достаточно медленное образование кости в материале группы «Гироид» и статистически значимо более выраженное образование костной ткани в порах материалов в группах «Кельвин» и «Хронос».
Заключение. Разработанная модель дефекта кости спонтанно не заполняется костной тканью и позволяет проводить изучение биологических свойств костнопластических материалов (способность к биодеградации и остеокондуктивные свойства). Остеокондуктивные свойства композитного материала на основе полиэтиленгликоль диакрилата и октакальциевого фосфата с архитектурой Кельвина выше, чем с архитектурой типа гироид, и сопоставимы с таковыми у материала chronOS.

Ключевые слова: регенерация костной ткани, критический дефект, гидрогель, костнозамещающие материалы, 3D-печать.

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BACKGROUND

Development of new artificial bone substitute materials is rather relevant due to the high need for them, but so far, no universal material meeting all clinical requirements, nor a set of medications with clear indications for application in various situations have been created [1, 2, 3, 4]. Advances in materials sciences allow to develop a large number of compounds and composites with probable clinical properties (biodegradability with a given rate, osteoconductivity, osteoinductivity and osteogenecity). However, it is impossible to confirm or refute the presence of such properties only theoretically, without performing studies on animal models [5, 6, 7].

One of the promising directions in materials science is the development of bone substitute implants with three-dimensional pore architecture, which allows to improve the osteoconductive properties while maintaining the strength characteristics [8, 9, 10]. One of the possible materials for filling bone defects can be hydrogel-based composites with addition of calcium phosphates with the Ca/P<1.5 weight ratio (e.g., octacalcium phosphate (OCP) with the chemical formula $Ca_8(HPO_4)_2(PO_4)_4 \cdot 5H_2O$)), which have a higher resorption rate compared to hydroxyapatite and tricalcium phosphate [11, 12]. An increase of osteoconductive properties is typical for structures with a cohesive-porous architecture and a fraction of voids more than 75% of the volume [13]. But such parameters pose the problem of determining their optimal spatial arrangement without losing the mechanical strength of the whole system. One of the possible ways to solve this problem can be the application of modern methods of topological optimization [14, 15, 16]. Good combination of mechanical properties and physical permeability in calculations are shown by the Kelvin and gyroid-type architectures, but the actual advantages of each of them require experimental verification [17, 18].

Aims of the study:

1) determination of applicability of the rat monocortical femoral diaphyseal defect model with additional prophylactic reinforcement by plate to assess the biological properties of implantable materials with the use of commercially available ChronOS® material as an example;
2) evaluation of osteoconductive properties of composite materials based on polyethylene glycol diacrylate and OCP with Kelvin and gyroid-type architecture on the designed model.

METHODS

Materials

The new-generation bioresorbable macroporous 3D constructs under study represent a hydrogel composite filled with calcium phosphate particles. A polyethylene glycol diacrylate (PEG DA) hydrogel was used as the matrix of the composite. OCP was used as the second phase of the composite — $Ca_8(HPO_4)_2(PO_4)_4 \cdot 5H_2O$.

Photopolymerizable suspensions based on PEG DA, OCP, water and photoinitiator were used for the synthesis of hydrogel composites according to the procedure described in our previous article [19]. Stereolithographic 3D printing using an Ember DLP printer (Autodesk, USA) was chosen to form the above-mentioned constructs. Three-dimensional models of the structures were created using Monolith (Autodesk, USA) and Fusion 360 (Autodesk, USA) computer programs.

Total porosity of the created materials was about 70% of the volume, the main channels are 1250 μm in size and the transitions between them are 750 μm, which provides the maximum permeability of these architectures. During the process of computer-aided design, 3D models with Kelvin and gyroid-type structures were selected to achieve the aim of the study (Fig. 1) [17, 19].

The Kelvin structure is a close packing of truncated octahedrons with pass-through channels perpendicular to each face. The gyroid-type structure is a minimal periodic surface [20].

A widely used fully synthetic ceramic material based on $Ca_3(PO_4)_2$ (tricalcium phosphate) ChronOS® (DePuy Synthes, Johnson & Johnson, Switzerland), which chaotic porosity is formed by uncontrolled foaming and solidification of the initial material in industrial conditions, was used as a comparison material. The total porosity of this material is approximately 55% of volume, the size of the main pores is 300 μm and the transitions between them are 50 μm.

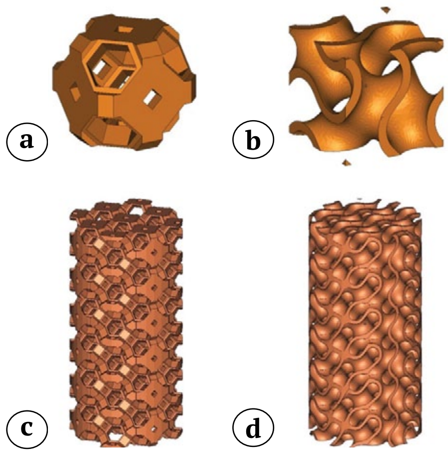

Fig. 1. Isometric projection of computer models of three-dimensional structures with different architectures: a — an elementary cell of a Kelvin type structure; b — an elementary cell of a Gyroid type structure; c — a cylindrical structure with a Kelvin type architecture; d — a cylindrical structure with a Gyroid type architecture

Animals

The study was performed on 53 male Rattus norvegicus Wistar rats aged 25-30 weeks from the Research Equipment Sharing Center "SPF-vivarium" of the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences. Animals were kept in the vivarium in cages, 3 animals in each. Access to water and food was ad libitum.

Experiment design, surgical procedure, laboratory tests

Type of the study – prospective, evidence level II.

To induce anesthesia in animals, 6.25/6.25 mg/kg of Zoletil 50 (Zoletil, Virbac Sante Animale, France) and 4 mg/kg of Xyla (Xyla, Interchemie werken "De Adelaar B.V.", Netherlands) were injected intraperitoneally.

In aseptic conditions of the operating room, after reaching the surgical stage of anesthesia, the rat was positioned on the right side on the surgery table. Surgical area was cleared of hair and a 6.0-cm skin incision was made in the projection of the femur. Then, using sharp and blunt tissue dissection, we made an approach to the femur along the lateral intermuscular septum. After performing the approach in the middle third of the diaphysis of the femur, a semicylindrical defect of 7 mm length and 4 mm radius was created along the dorsal surface with a diamond-coated conical burr 2.3 mm in diameter using a Marathon-N2 machine (Saeyang Microtech, Republic of Korea) (Fig. 2 a).

Rats were randomly allocated into four groups of 12 animals in each. In the "Control" group of animals the bone defect was not filled. In the "Chronos" group the defect was filled with the prepared ChronOS® material in the form of a semi-cylindrical block, in the "Kelvin" group - with the studied material with Kelvin architecture, in the "Gyroid" group – with the studied material with gyroid-type architecture.

The bone was reinforced with a 30.0×4.0×4.5 mm polyetheretherketone plate. It was placed on the posterolateral surface of the femur and fixed with 6 titanium screws 1.2 mm in diameter and 10 mm long (Konmet, Russia), 3 screws on each side of the defect (Fig. 2 b). After fixation, the wound was sutured layer-by-layer, no dressings were applied, and no immobilization was used. The average duration of surgery was 39±8 min (23 to 54 min). Blood loss did not exceed 1.0 ml.

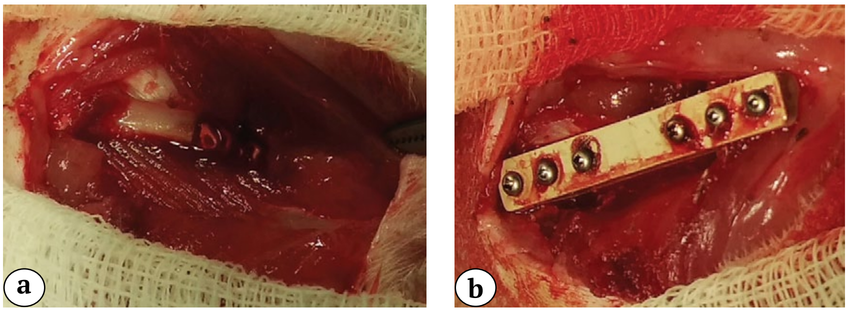

Fig. 2. The view of surgical wound:
a — bone defect with implanted material;
b — bone augmentation by plate and screws

Three and six weeks after the surgery, the animals were withdrawn from the experiment according to the standard procedure using a CO2 chamber with subsequent material sampling (femur segment with a defect) for histological examination.

The samples were fixed in 10% neutral buffered formalin for 24-48 h. After this time, the materials were decalcified in 25% neutral Trilon B solution at room temperature on an Orbital Shaker OS20 machine (Biosan, Latvia). After decalcification according to the standard procedure, the materials were washed in distilled water. Then histological processing and embedding were performed. Next, we made 3-4 µm thick cross sections of paraffin blocks and stained them with hematoxylin and eosin.

Histometric analysis

Blind histometric analysis was performed to evaluate the results. Images of histological specimens were obtained using a Leica DM LB2 light microscope (Carl Zeiss, Germany) and an AxioCam ICc3 digital camera (Carl Zeiss, Germany). Digitized images were converted to JPEG format. Histometric evaluation was performed using Fiji program [21] at 20x magnification with measurement of the area of newly-formed bone tissue in the material lacunae in mm^2. Newly-formed bone tissue was identified by its specific structure and staining. We took into account the areas that were not connected with the endosteum or cortical bone in order to avoid the inclusion of overgrowths. For each sample, a calculation was performed on three specimens, giving a total of 18 values for each group (only for "Chronos 3 weeks" and "Control 6 weeks" groups – 15 values).

Statistical analysis

The results of each group were presented as mean, maximum and minimum values. Nonparametric Kruskal-Wallis test was used to determine the statistical significance of differences. It was calculated separately for the groups with withdrawal periods of 3 and 6 weeks using StatSoft Statistica 10.0 software (2011).

RESULTS

During the experiment, 2 animals died from anesthesia-related complications. Four animals underwent iatrogenic femoral fracture in the area of the defect during the surgery, and therefore euthanasia was performed using a CO2 chamber. One animal from the "Chronos" group (No. 12) had a postoperative wound dehiscence with a large soft tissue defect the next day after the surgery due to aggression of its cage neighbors. As a result, euthanasia was performed for ethical reasons. The rest of the animals underwent surgical intervention, survived and were withdrawn from the experiment without any peculiarities.

Animals' body weight increased during observation. The average weight of the rats at the time of enrollment in the experiment was 347 g, at the time of withdrawal 3 weeks later was 398 g, 6 weeks later – 445 g. No significant difference between the groups concerning the weight and the weight gain was observed.

No changes in animals' behavior during the experiment were observed. In the postoperative period, all animals moved on four legs without restrictions. Postoperative wounds healed with primary intention in all animals. No external signs of infectious complications were recorded. There were also no signs of purulence, damage or migration of fixators, or bone fractures during the sampling and examination of the implantation area.

The defect area in the "Control" group was filled on the histological sections with granulation connective tissue. No formation of bone tissue trabeculae not associated with the endosteum was observed neither 3 nor 6 weeks later, that allows us to consider this defect as critical (Fig. 3).

In the "Chronos" group, all the specimens showed a transverse section of rectangular-shaped material, the external linear dimensions of which did not change 3 and 6 weeks later. Inorganic base of the block was identified as fields of cellular pale gray structure containing no cellular or fibrous elements. Almost all blocks were surrounded outside by a thin layer of granulation connective tissue, and no marked inflammatory reaction (large number of polymorphonuclear neutrophils, macrophages or giant cells of foreign bodies) was noted 3 and 6 weeks later.

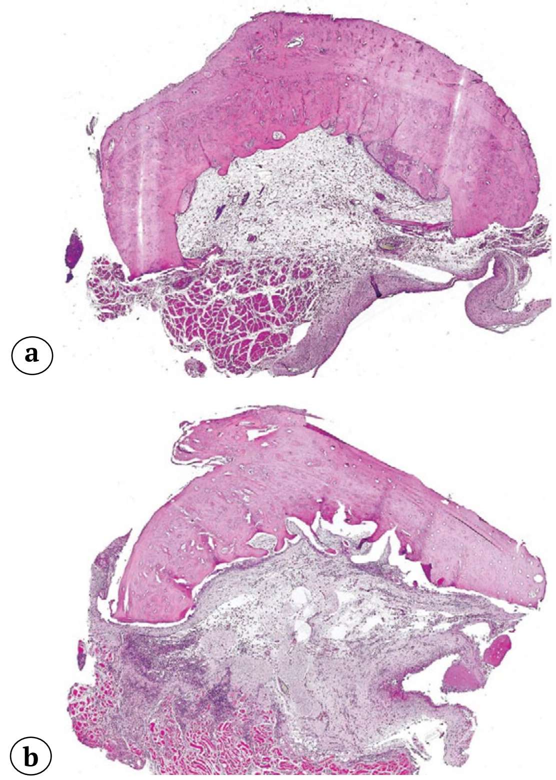

Fig. 3. The cross sections of femur at the defect level (Control group):
a — after 3 weeks; b — after 6 weeks. Staining with hematoxylin and eosin. Mag. ×20

Connective tissue ingrowth with cellular elements and vessels into the pores throughout the entire thickness of the material with bone trabecular formation in the periphery of the block (25-35 trabeculae per section on average) was observed in 3 weeks. In 6 weeks, bone trabecular formation in the pores throughout the entire material was observed (Fig. 4).

In the "Gyroid" group, the continuous presence of rounded cavities repeating the outlines of the implanted material blocks, which contained brown-red colored structures without cellular elements and fibers of various irregular shapes with the presence of permanent ribbon structure elements (Fig. 5) was noticed on the histological sections. These ribbon structures were considered to be residues of the hydrogel used to produce the blocks of material. Histological specimens showed complete filling of the material pores with granulation connective tissue and bone trabeculae formation 3 weeks after implantation. However, 6 weeks after implantation, the area of the newly-formed bone was smaller than that in the "Chronos" and "Kelvin" groups. At the same time, the material itself and its structure could be seen on the specimens of that time with great difficulty.

In the "Kelvin" group, the histological specimens also revealed the zones of rounded voids following the contours of the implanted material blocks and containing smaller brown-red colored structures without cellular elements and fibers (Fig. 6). The shape of these voids differs from those in the specimens with implanted material with gyroid-type architecture. Osteoconductive properties of material with the Kelvin architecture were better expressed, as evidenced by the greater mass of bone tissue around the material and in its pores. Compared to the histological specimens of the "Chronos" group, the areas of bone tissue were larger and followed the pore architecture of material.

The results of calculating the area of the newly-formed bone tissue in all groups are presented in Table 1.

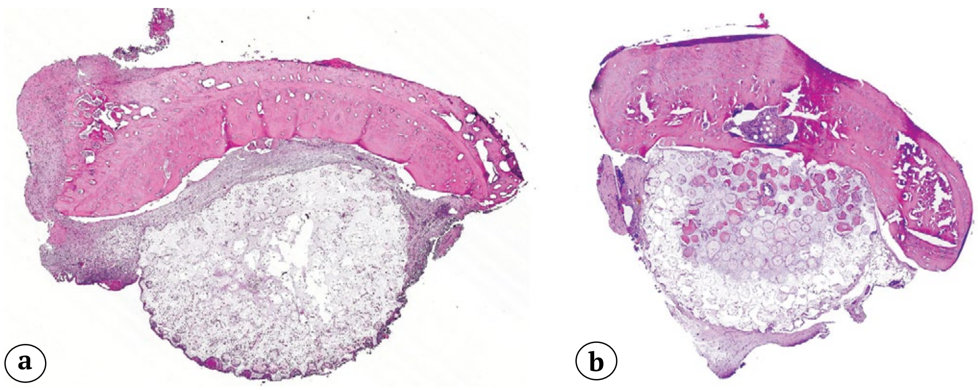

Fig. 4. The cross sections of femur at the defect level (Chronos group):
a — after 3 weeks; b — after 6 weeks. Staining with hematoxylin and eosin. Mag. ×20

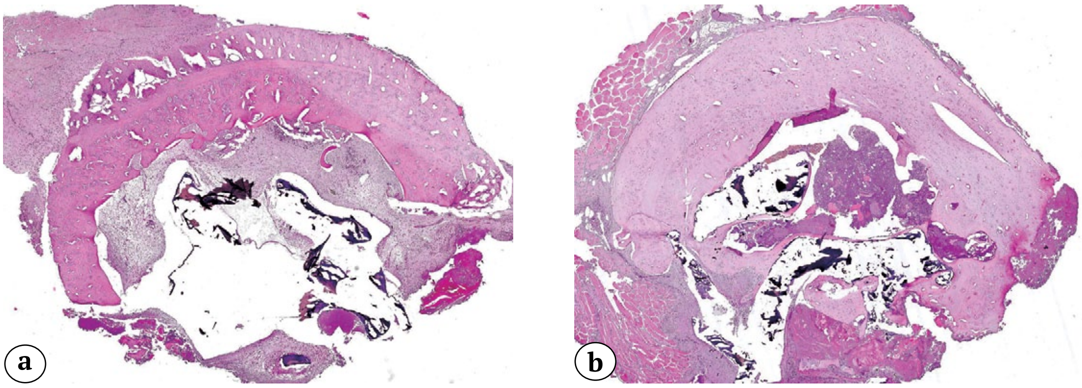

Fig. 5. The cross sections of femur at the defect level (Gyroid group):
a — after 3 weeks; b — 6 after weeks. Staining with hematoxylin and eosin. Mag. ×20

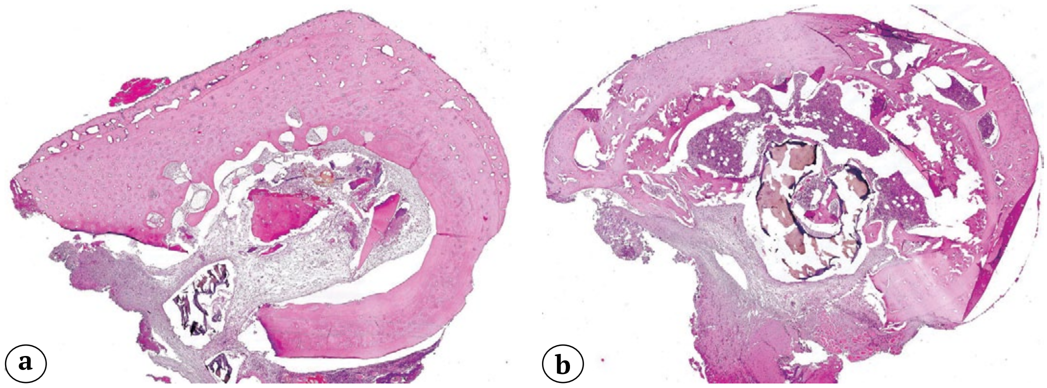

Fig. 6. The cross sections of femur at the defect level (Kelvin group):
a — after 3 weeks; b — after 6 weeks. Staining with hematoxylin and eosin. Mag. ×20

Table 1

Results of calculating the area of the newly-formed bone tissue in all groups

Group	Observation period, weeks	The area of the newly-formed bone tissue, mm ^{2}		
		Average value	Min. value	Max. value
«Control»	3	0.0515	0	0.7070
	6	0.1462	0	0.8350
«Chronos»	3	0.3694	0.0920	0.7760
	6	1.1686	0.1570	3.1230
«Kelvin»	3	0.9107	0	1.8270
	6	1.2650	0.0870	2.9780
«Gyroid»	3	0.0497	0	0.6060
	6	0.0853	0	0.6710

The differences between the groups in the area of the newly-formed bone tissue were statistically significant in both cases — 3 weeks (Kruskal-Wallis H-criterion is 57.4399, which corresponds to $p<0.01$) and 6 weeks after implantation (Kruskal-Wallis H-criterion is 38.4702, which corresponds to $p<0.01$).

DISCUSSION

Various models are used to study the biological properties of materials in vivo, both in terms of the animal choice (mice [22], rats [23, 24, 25, 26, 27], rabbits [28], dogs [29]), and in terms of the implantation technique. It is obvious that the defect must be critical to study the properties of materials, i.e., it must not have the possibility of spontaneous replacement. Otherwise, the presence of material will even decelerate the replacement by bone tissue and reflect only the rate of its biodegradation.

Critical defects described in the literature can be conventionally divided into two types. The first type includes the defects that due to their stability do not require additional augmentation preventing pathological fractures of the operated bone (rounded burr cranial defects [25, 30, 31], rounded burr defects of the femoral and tibial epiphyses [28]). The second type includes the defects requiring stabilization due to the high risk of bone fractures under normal loading — complete segmental defects of the femur, fixed with a nail [22], plate [32] or external fixator [33].

It is technically easier to perform studies on the defects without augmentation, however, such defects are less relevant to real clinical cases in terms of their biological properties. The study of segmental diaphyseal defects is dictated by the clinical request to restore the length of a limb shortened for various reasons without the use of long-term distraction osteogenesis techniques or extremely difficult transfers of vascularized bone grafts. But these models are technically difficult to be implemented and may not be suitable as screening models. In the present study, we used a model of a monocortical defect of the femoral diaphysis with plate and screw fixation. This choice was determined by the necessity of combining a critical diaphyseal bone defect with reliable fixation of the material in the defect and ensuring the possibility of adequate locomotion of the animal during the observation period without increasing the risk of fixator and bone fracture.

The suggested model of defect creation showed its capabilities as indicated by the absence of infectious and mechanical complications during the observation period of up to 6 weeks under the conditions of preserved locomotor function in rats. Among the advantages of this model over other fixation methods are fixator placement in the tissues and the absence of external fixator elements that can potentially injure the animal itself or its cage neighbors [33]. The proposed model differs from the intramedullary fixator in the fact that the medullary canal is free of fixator and there is no need to place the material near the bone, which brings our model closer to the clinical case [22]. Simplicity of surgical technique and possibility of reliable material fixation in the defect area due to the congruence of shapes and plate pressing distinguish our model from the complete defect [32].

The present study showed that the monocortical defect, like the complete one, was critical, as indicated by the absence of spontaneous bone tissue replacement of the resected area within 6 weeks. In addition, the proposed biological model is suitable for the study of potential bone substitute materials, as evidenced by the marked infiltration of the ChronOS® material with proven osteoconductive properties by the bone tissue.

The second objective of our study was to determine the effect of two types of three-dimensional material architecture on the osteoconductive properties of OCP-loaded hydrogels based on polyethylene glycol diacrylate. The impact of the porous material structure on bone ingrowth has been described in the literature [18]. The results of the study suggest that in terms of osteoconductive properties, Kelvin architecture is superior to gyroid-type architecture in PEG DA-based composites. One of the reasons for this may be the biological interaction of tissues with three-dimensional pores, resulting in triggering of certain mechanotransductive signals and the orientation of precursor cell differentiation along the osteoblast pathway, which is better expressed in the Kelvin structure. Additional causes may be more rapid degradation and loss of strength of the mechanical structure, which made it difficult to form a new bone at a later period. In terms of properties, this was probably comparable to the conditions in the defect of the "Control" group.

CONCLUSION

The developed bone defect model is critical and enables to study biological properties of osteoplastic materials (biodegradability and osteoconductive properties). Osteoconductive properties of the composite material based on polyethylene glycol diacrylate and octacalcium phosphate with Kelvin architecture are higher than those of the gyroid type.

DISCLAIMERS

Author contribution

Shcherbakov I.M. — concept and design of the study, collection and processing of material, writing the draft, statistical analysis.

Klimashina E.S. — concept and design of the study, collection and processing of material, text editing.

Evdokimov P.V. — concept and design of the study, collection and processing of material, writing the draft.

Tikhonov A.A. — collection and processing of material, text editing.

Putlayev V.I. — concept and design of the study, collection and processing of material, text editing.

Shipunov G.A. — collection and processing of material.

Zatsepin V.A. — collection and processing of material.

Dubrov V.E. — concept and design of the study, text editing.

Danilova N.V. — collection and processing of material, writing the draft.

Malkov P.G. — collection and processing of material, text editing.

All authors have read and approved the final version of the manuscript of the article. All authors agree to bear responsibility for all aspects of the study to ensure proper consideration and resolution of all possible issues related to the correctness and reliability of any part of the work.

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Competing interests. The authors declare that they have no competing interests.

Ethics approval. The study was approved by the local Ethics Committee of the Lomonosov Moscow State University (protocol No 82-zh, 25.10.2018). The study was conducted in compliance with the principles of humane treatment of laboratory animals in accordance with the requirements of the European Convention for the Protection of Vertebrate Animals used for Experiments and other Scientific Purposes and Directive 2010/63/EU of the European Parliament and the Council of the European Union of September 22, 2010 on the protection of animals used for scientific purposes.

Consent for publication. Not required.

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