Three-dimensional TCP scaffolds enriched with Erythropoietin for stimulation of vascularization and bone formation

N.V. Kharkova1, I.V. Reshetov1, A.S. Zelianin1, V.V. Philippov2, N.S. Sergeeva3, I.K. Sviridova4, V.S. Komlev5, U.U. Andreeva6, O.A. Kuznecova6

ABSTRACT
In our work, we conducted studies to evaluate the reconstruction of an artificially created critical radial defect in rats with the use of tri-calcium phosphate scaffold enriched with erythropoietin (EPO). A model of the bone defect on the radius forearm in rats of a critical size has been developed. This model allows to conduct the study without the use of osteosynthesis. EPO is a well-known hormone which regulates formation of the red blood cells (2, 3). EPO increases the expression of VEGF and promotes angiogenesis (3, 4). Therefore, EPO may have a great potential for use as a growth factor for angiogenesis and play its particular role in the bone tissue regeneration. There are no published studies that describe interactions between EPO and biomaterials for development of the bone tissue. Thus, the purpose of this study was to evaluate the interaction between EPO and tri-calcium phosphate scaffold (TCP) with a well-studied biocompatibility and the given porosity, as well as to determine whether EPO in the enriched TCP will promote the bone regeneration. The X-ray analysis was performed in 10 days, 28 days and 3 months after the surgery. The histological analysis was performed in 28 days and 3 months after the surgery. The results have demonstrated that the complex of TCP scaffold with erythropoietin is a promising growth factor for stimulating the development of the bone tissue, since the TCP scaffold enriched with erythropoietin is very easy to obtain in a non-invasive and simple procedure, as well as the complex promotes interaction with the surrounding tissues and induces the bone regeneration.

Keywords: scaffold, tri-calcium phosphate, erythropoietin, bone tissue engineering

INTRODUCTION
Treatment of bone defects of a critical size remains a serious challenge in surgery and orthopedics. Segment defects are the result of various injuries, infections, resections of tumors of various localization, as well as congenital deformity of bone development (1, 2, 4). Conceivably, bone defects of a critical size do not heal spontaneously, despite the surgical stabilization (3, 4). The method of using autologous bone grafts in reconstructive surgery for congenital malformations and defects caused by some trauma or disease, as well as for cosmetic procedures in certain parts of the body, have their disadvantages, such as a limited bone scraping, non-identity of bone shape, discomfort and pain in the area of the donor site. However, the elaborated synthetic bone graft materials, allografts or artificial bone substitutes may act like foreign bodies after they are introduced into the human body and cause complications, including inflammation and rejection of the implant. Currently, some researches are underway to develop and test bone-substituting materials, in order to minimize these shortcomings (5, 6).

Scaffold is an artificial bone substitute, a synthetic biomaterial in the form of a mesh and sponge, with predefined structures and shapes that are used to increase the area of cell adhesion to the implant, proliferation and tissue formation. An ideal scaffold must have biocompatibility, biodegradability and good mechanical strength to support the...
tissue in the area of the defect. In addition, it must have a suitable porosity, pore size and interconnected network for the penetration of cells, as well as it’s necessary to ensure an adequate ratio of calcium and phosphorus (6, 7).

Tri-calcium phosphate (TCP) is a bioabsorbable ceramics which quickly degrades in the body to calcium and phosphate. The pores in TCP scaffold allow the vascular network to penetrate, the bone tissue to grow into the transplant material after its placement inside the body what allows TCP to be used in various implantation procedures. There are two forms of TCP, α-TCP and β-TCP which have similar solubility, but differ in thermodynamic stability in a biological environment and in normal temperature ranges (6, 8).

In some publications, it was shown that a satisfactory bone regeneration can be achieved with the use of various stimulants of angiogenesis and neurotization of the bone tissue, so BMSC were combined with the particles of hydroxyapatite/tricalcium phosphate (TCP).

**Goal of this study:** study of the effect of 3D printed TCP frames with erythropoietin on the formation and increase of the vascular network around the scaffold embedded into the artificially created defect of the rat radius bone.

**MATERIALS AND METHODS**

**Description of the Model and Experiment. Surgical Research Method**

In this work we used the laboratory rats provided. All animals were 5 to 6 months old of Vister breed, both the does weighing approximately 300-350 g at the time of the operation, and males whose weight reached 450 to 500 g. The use of different sex rats in the experiment is determined by determining the biocompatibility and resorbability index in the males and females.

All rats were divided into three groups: a control group with a critical defect not filled with the implant; a group of animals with TCP scaffold; a group with TCP scaffold enriched with erythropoietin.

Each of these groups was subdivided into three subgroups with control for the periods of 10 days (no histology was performed on this term), 28 days and 3 months after surgery.

**Stages and Features of the Introduction of Scaffolds in the Experimental Animals (Models)**

All surgical interventions were performed under aseptic operating conditions. For anesthesia, an intramuscular injection of Zoletil-50 was used at a dose of 2 mg/kg of the animal body weight and intramuscular injection of Xyla in a 2 % solution at a dose of 1.5 mg/kg of the animal body weight.

Taking into account the rat anatomy, the radius bone was chosen for introduction of the scaffold. Since between the radial and ulnar bones there is a bone membrane which makes it possible to implant scaffold into one of the bones without using osteosynthesis on the bone.

A skin incision was made with the scalpel in the membrane between the ulna and radius in the length of up to 15 mm. The superficial fascia was dissected, the ventral vascular bundle was separated and taken outwards, the muscles were elevated with the help of the dissectors (the muscles were not cut) exposing the radial bone in the projection of the distal end. At the distal end of the radial bone, a fragment of the bone corresponding to the critical bone size and identical to the implant (corresponding to the critical size) was removed. The defect was formed with the help of the sterile electrical burr in the diameter of 2 mm with removal of the bone and fragments and also bone dust accompanying it all with a constant irrigation.

The scaffold created at the Institute of Problems of Laser and Information Technologies of the Russian Academy of Sciences, in accordance with the scaffold creation technologies described above, was installed in the place of the removed bone (**Figure 1 a, b, c**). The radial bone was stabilized with the help of the ulnar bone, in order to restrain the defect and to fix the scaffold inside the defect accurately during the entire study period.
Figure 1: а, b – Preparation of the operative field to create a critical defect in the radial bone in rats. c – Installing the scaffold in an artificially created defect

Hemostasis. A layered wound closure, prolene 5-0. The postoperative sutures were treated with 70 % ethanol solution. During the first three days after the operation, the animals were administered with lincomycin at the dose of 10 mg/kg of the animal body weight and paracetamol at the dose of 10 mg/kg of the animal body weight once a day as an anesthetic and antibacterial therapy. In 2 to 3 days after the surgery, the animals resumed the normal movement of the right forepaw and showed no signs of pain or discomfort.

Bringing the animals out of the experiment was carried out according to a predetermined schedule. All experiments and bringing the animals out of the experiments were carried out under anesthesia using formalin.

RESULTS
X-ray Examination

The radiography was performed on a DM apparatus. In the end, the matrix disappears and is completely replaced by 100 P in the terms of 10 days, 28 days and 3 months after the surgery. The focal distance was equal to 70 cm with 60kVp mode and 30 mA. The exposure time made 0.05 s.

On Day 10

On the radiographs in the control group of the rats without an implant, the bone defect remains unchanged, as after the surgery. On the radiographs in the second group, the whole (unfragmented) implant is found at the site of the bone defect. The implant retains its size and shape. The ends of the implant and the bones are consolidated. No signs of the implant replacement by the bone tissue are observed. On the radiographs in the third group, the whole (unfragmented) implant is found at the site of the bone defect. The implant retains its size and shape. The ends of the implant and the bones are consolidated. No signs of the implant replacement by the bone tissue are observed.

On Day 28

On the radiographs in the control group, the bone defect remains unchanged, as after the surgery. On the radiographs in the second group, the implant with a partial resorption is found at the site of the bone defect. The ends of the implant and the bones are consolidated. Some rudiments of the implant replacement by the bone tissue are viewed. On the radiographs in the third group, the whole (unfragmented) implant is found at the site of the bone defect. The implant retains its size and shape. The ends of the implant and the bones are consolidated. There are no signs of the implant replacement by the bone tissue.

In 3 Months

On the radiographs in the control group, the bone defect remains unchanged, as after the surgery with a partial growth of the ends of the radius. On the radiographs in the second group, in place of the bone defect remains of the implant are detected with the growth of the radial bone ends. On the radiographs in the third group, at the site of the bone defect the implant is detected with a partial degradation. However, the implant retains its size and shape.
Figure 2: 3-month radiograph with the implant and erythropoietin

Histologic Examination

The bones of the forearm with the soft tissues taken from the area of the operation were fixed in a 10 % solution of neutral formalin and decalcified in an Electrolytic decalcifying solution (Bio Optica, Italy). The paraffin modulator HISTOMIX was added to paraffin to improve the quality of the sections (BioVitrum, Russia). Transverse and longitudinal serial sections of a thickness of 4–5 μm were made of all the samples, then, they were stained with hematoxylin and eosin, picrosirius red and toluidine blue on acidic glycosaminoglycans (GAG). The study, analysis and microphotography of the histological preparations was performed with the help of a LEICA DM4000 B LED microscope equipped with a LEICA DFC7000 T digital video camera and LAS V4.8 software (Leica Microsystems, Switzerland). The preparations were studied with a light, phase-contrast, dark-field and polarization microscopy.

Results of Morphological Examination

Control-1 (without implant)

28 days (10 animals)

In the preparations, fragments of the forearm bones, of the humerus and elbow joint with the surrounding muscles were found in all animals. In the proximal parts of the radial bones, defects with the signs of consolidation were observed, due to the formation of corns of varying maturity degrees located on the edges of the defect and filling the bone marrow cavities adjacent to the fracture sections of the radial bones. In all the animals, the corn consists of three components: fibro- reticular tissue, fibrous cartilage and forming bone consisting of trabeculae between which the full-blooded, thin-walled vessels are located (Figure 3).

Figure 3: The formation of callus with foci of the fibroblast proliferation and an abundance of capillary type vessels filled with red blood cells (indicated by arrows) is observed. Around the emerging bone beams the connective tissue with the fibroblast proliferation is visualized
The bone component prevails over the others and is formed, due to an endochondral ossification. A moderate fibrosis is observed in the tissues surrounding the corn, with no inflammation. In some preparations, the surrounding soft tissues are slightly soldered to the corn.

At high magnifications, in the preparations of the individual animals a moderate osteoclastic reaction is distinguished indicating the remodeling of the newly formed corn bone tissue. When stained with pyrosirius red with the use of a simple light microscopy, as well as phase-contrast, dark-field and polarization microscopy, the structural organization of the corn collagen fibers and intact bone is visible. The intact bone tissue consists of the organized collagen fibers stained red with a sirius dye which are located in the picrinophilic matrix. When using a polarization microscopy, the matrix is anisotropic and gives predominantly a green glow. Unlike the intact bone, numerous collagen fibers are seen in the corn mostly found in the fibro-reticular and cartilage components. The collagen fibers form thicker and coarse multidirectional bundles what is especially clearly seen when using a phase-contrast, dark-field and polarization microscopy. The fibers are anisotropic, but unlike the intact bone, they give not only a green, but also orange and red glow what indirectly indicates their greater thickness. When staining with toluidine blue, a metachromasy is seen in the intact corn cartilage, indicating the presence of glycosaminoglycans (GAG) in the cartilage matrix. The presence of the cartilage and fibro-reticular tissue in the area of the corn indicates its immaturity what corresponds to the period of the 1st month. In other bones, the bone tissue has no signs of destruction, the bone marrow with the signs of an active hemopoiesis, the articular cartilage is intact.

3 months (10 animals)

In 3 months, nine out of ten animals showed no signs of fusion of the radial bone defect: the corns were observed only at the edges of the radial bones adjacent to the defects filling the adjacent areas of the bone marrow cavities, and the spaces between the fragments of the radial bones were filled with soft tissues. The corns' tissue had a structure similar to the previous one and consisted of a bone, fibro-reticular and cartilaginous components what is especially clearly seen at the use of a standard light microscopy, as well as a polarization, phase-contrast and dark-field microscopy at staining with pyrosirius red.

The ratio of the corn components was somewhat different for different animals. One of the animals with consolidation of the defect showed formation of the most mature callus: the bone component prevailed over the fibro-reticular and cartilage one. In all cases, an enchondral ossification of the corn cartilaginous component was noted. The corn tissue in the defect area of all the animals ensured the fusion of fragments of the radial and ulnar bones what probably contributed to immobilization of the mobile bone fragments. The osteoclastic reaction into the corn tissues indicating the bone remodeling was absent in all the animals. A moderate perifocal fibrosis without inflammatory changes was observed in the tissues surrounding the defect. Outside the defect, the bone tissue of the upper limb and the articular cartilage, similarly to the previous term, did not have any pathological changes and were richly supplied with the blood (Figure 4).

Figure 4: The newly formed trabeculae are firmly attached to the bone matrix of the fragment. There are osteoclasts present between the fibers of the connective tissue. There are also identified multiple blood capillaries with endothelial cells bulging into the lumen (indicated by the arrow)
Control-2 (implant without additives)

28 days (10 animals)

In the preparations, the implant fragments are determined that are represented by the basophilic and picrinophilic isotropic masses that are not stained with toluidine blue. In 10 animals, the implant is located in the area of the defect. In animals, a thin mature capsule is formed around the implant which consists of thick collagen fibers. The signs of consolidation of the bone defect with the implant are visible in all the animals (Figure 5).

![Image](image1.png)

**Figure 5:** Implant introduction zone (encircled in green). The blue arrows indicate the proliferative connective tissue. The red arrow indicates the osteoclasts

In animals, in the defect area the corn like a cuff covers the fragments of the radial bones, and in its composition a cartilage and bone tissue predominate. At the same time, around the large implant fragments, there are areas marked with a reticulo-fibrous or fibrous tissue in the capsule. Most of the animals show fusion of the fragments of the radial bones with the ulnar ones. All the animals showed the surrounding soft tissues round defects to have fibrosis of a varying severity. The bones with the bone marrow and joints outside the defect are intact. The inflammatory reaction was detected only in one of the animals in the corpus callosum in the area of the false joint and is represented by lymphoid infiltration.

3 months (10 animals)

In the period of 3 months one portion of the preparations demonstrate an availability of an implant similar on the structure, optical and tinctorial properties to the previous term one. The implants are located in the area of the defect and surrounded by a thin mature fibrous capsule. Some animals show the signs of consolidation of the defect, due to formation of the corn consisting of a reticular fibrosis, cartilage and bone tissue, and replacing the areas of the bone marrow cavity adjacent to the defect. Ossification of the cartilage tissue has an enchondral character. Fibrosis of the surrounding tissues in all the animals is moderate and there is no inflammatory reaction. Outside of the defect – the bone tissue with the bone marrow, as well as the cartilage tissue are unchanged.

![Image](image2.png)

**Figure 6:** The group of rats with an implant without additives, 3 months. Implant introduction area. Observed formation of the connective tissue on its periphery. The presence of a young loose fibrous connective tissue with an abundance of the blood capillaries and a cluster of multicore macrophages. The fibrous tissue is abundantly supplied with blood (indicated by the arrow)
Implant with erythropoietin

1 month (10 animals)
In the preparations, the implants are detected integer (non-fragmented). In 10 animals, the implant is located in the area of the defect. In animals, a thin, mature capsule is formed around the implant and it consists of thick collagen fibers. The signs of consolidation of the bone defect with the implant are observed in all the animals.

3 months (10 animals)
In the preparations, fragments of an implant similar in structure, optical and tinctorial properties to the implants of other groups, are detected. In all the animals, the implant fragment is detected in the place of the bone defect. In all the animals, a capsule is formed around the implant: in some places it is thin, mature, in other areas it is represented by a granulation tissue with numerous macrophages and giant cells performing an implant resorption. The callus, as well as in other groups, consists of reticulofibrosis, cartilage and bone components, in some places it solders the fragments of the radius to the ulna. The bone tissue is formed, due to a mesenchymal ossification. The signs of consolidation are present in all the animals. In the defect surrounding soft tissues, fibrosis of a varying severity (from mild to moderate) is observed. Outside the defect, the bone marrow, bone and cartilage tissues do not differ from those in other groups.

Figure 7: Implant introduction area. Green arrows – vessels of the newly formed tissue, Blue arrow – a capsule of the connective tissue with the initial formation of a fibrotic fold inside the implant

Figure 8: The group of rats with an implant with EPO, 3 months. Implant introduction area. The implant is highlighted by green

The implant is used as a matrix to form a young connective tissue that penetrates it (blue arrow).

Statistical Analysis
The data were analyzed with the use of SPSS software (version 19, SPSS, Inc., Chicago, Illinois, USA). The statistical analysis was performed with the use of the Mann-Whitney U-test or Student’s T-test (others). The value of p ≤ 0.05 was statistically significant.
DISCUSSION

Achievements in the field of bioengineering suggest a possibility of producing bone tissue with a biological variability which serves to replace segments of the resected or lost bones. Continuing to study biotechnology of scaffolds is necessary, as for the reconstruction of a bone defect of a critical size the implants provide two main functions: firstly, restoration of the shape of the defect of a critical size, and secondly, they act as a biological conductive structure for the cells and/or growth factors promoting bone formation (osteoconduction), some may also have osteoinductive properties (9). The difficulties associated with restoration of the vascular perfusion and, thus, oxygenation and nutrient delivery to the inside of the scaffold, are significant limiting factors for the formation of bones in the center of the scaffolds (9). Scientists have conducted studies describing recommendations for the induction of angiogenesis (9,10). These include: introduction of a large dose of BMP (approximately 600 mg) and a platelet-rich plasma (PRP) or by incubating seed scaffolds in the vascular bioreactor (9, 11-15). Several studies have been conducted on different models of animals to study the possibility of regenerating a bone defect of a critical size using autologous MSC in both areas of orthopedics and in maxillofacial area (9, 20-22). It has also been demonstrated that the mouse or human bone marrow stromal cells seeded on calcium phosphate (CaP) stimulate the bone formation implanted subcutaneously in the immunocompromised mice (22,23).

The results of our study confirmed the possibility of using our developed model of a radius bone defect of a critical size in rats. Restoration of the functions of the rats' paws, without any side effects, was observed during several days after the operation which also had a positive effect on bone healing and remodeling. For the reconstruction of the radial bone in rats, TCP scaffolds with a given defined pore size were used what helps stimulate vascularization and oxygen content for the growth of the tissue cells, including the vascular structure. The surgical experimental model and TCP scaffold frame were found to be satisfactory for conduction of the experiment.

In the studies on an experimental model with the use of TCP scaffold enriched with erythropoietin, an active formation of the vascular system was observed what suggests that the problem of increasing the level of oxygen and nutrients in the implant was solved, and this indicates an improvement in bone regeneration at a critical-size defect. A limited formation of the vascular system in the group of rats with TCP scaffold without a bioreactive agent, respectively, led to a slower bone formation.

CONCLUSION

The TCP scaffold complex with erythropoietin suggests a promising alternative growth factor to stimulate development of the bone tissue, since TCP scaffold enriched with erythropoietin is very easy to obtain in a non-invasive and simple procedure, and also the complex promotes interaction with the surrounding tissues and induces a bone regeneration.

ACKNOWLEDGEMENT

“The authors are grateful for the financial support to the Russian Foundation for Basic Research in carrying out this study in the framework of a scientific project № 15-29-04796 ofi_m.”

REFERENCES


