RESORBABLE SCAFFOLDS MODIFIED WITH COLLAGEN TYPE I OR HYDROXYAPATITE: IN VIVO STUDIES ON RABBITS

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Introduction

Aliphatic polyesters, for example copolymers of L-lactide and glycolide (PLGA), are very attractive materials to be processed into temporary scaffolds for bone and cartilage tissue engineering [1,2]. Several experiments performed in our group showed that the PLGA scaffolds support growth and differentiation of osteogenic cells [3,4] and are biocompatible with soft tissue in the animal model [5]. Besides, numerous studies confirmed that surface modification strategies with the use of inorganic phases (e.g. calcium phosphates) or biomolecules (e.g. collagens, glycosaminoglycans) may provide additional beneficial effect on bone tissue healing and regeneration [6]. However, in the case of 3D porous scaffolds it is difficult to depose homogenous layer of inorganic/organic phases on the pore walls within the whole volume of the scaffolds. It results from relatively high surface area, turtuosity of the pores and diffusion-limited phenomena. Therefore particular strategies have to be applied to modify the complete volume of the scaffolds. In our approach we use vacuum to remove air from the pores and thus to enhance the modification solution to penetrate all pores.

The aim of this study was to: i) modify the entire volume of the PLGA scaffolds with hydrohyapatite (HAP) or collagen, and ii) find out if applied modifications are beneficial for the healing process in critical-size osteochondral defects in rabbits.

Materials and methods

Scaffolds manufacturing, modification and characterization

PLGA with molar ratio of L-lactide to glycolide 85:15 (M_n=100 kDa, d=2.1) was synthesized according to a method described previously [7]. Cylindrical porous scaffolds (diameter 4 mm, height 5 mm) were obtained by an innovative solvent casting/salt particulate leaching method [4]. The size of the salt particles was 250-320 μm and their volume fraction was 85%. The scaffolds were modified with hydroxyapatite (HAP) by incubation in simulated body fluid (SBF) for 12 days [8] and with collagen (type I, bovine origin, Sigma) by soaking the scaffolds in collagen solution (40 μg/ml) for 24h. Modification media (i.e. SBF or collagen solution) were forced to penetrate the whole volume of the scaffolds by applying a vacuum. Afterwards the scaffolds were washed 3 times in tap water (in the case of SBF modification) and in UHQ water (in the case of collagen adsorption).

The scaffolds were characterized by scanning electron microscopy (Nova NanoSEM, FEI) equipped with EDS analyzer, FTIR spectroscopy (FTS Digilab, BioRad) and X-ray diffractometry (XRD, Panalytical X'Pert Pro system).

Scaffolds in vivo evaluation in New Zealand rabbit model

The research protocol was approved by Local Ethic Committee (University of Natural Science, Lublin, Poland, No 43/2008; 01 July 2008). Three types of scaffolds: without modification (PLGA), enriched with collagen (PLGA/coll) and enriched with hydroxyapatite (PLGA/HAp) were sterilized with oxygen peroxide plasma (Sterrad 120, ASP, J&J). Sham operation (defect created and but not filled with a scaffold) acted as control. Six animals were used in this study, i.e. both knees of the animals were operated.

General anesthesia

The mixture of xylazine and ketamine was administered by intramuscular injection (5 mg/kg xylazine and 35 mg/kg ketamine). The state of sedation and analgesia were achieved. The surgical site was prepared, the area surrounding the knee joint was shaved, and a venflon was placed into the marginal ear vein (v. auricularis marginalis). After 10 min general anesthesia was induced by intravenous administration of ketamine. To maintain the anesthesia ketamine was used as a continuous intravenous infusion using an infusion pump at a dose of 0.5 mg/kg/min.

Knee arthrotomy

Approach to the knee was obtained by making an incision on the side of the knee. The incision began at about L' distal part of femur to achieve a level 2 cm below the tibial tuberosity. The tissues were prepared along the same incision line, fascia lata was cut laterally on the block of the knee, then straight along the patellar ligament at a distance of about 7 mm from it, and then the capsule of the knee was cut. Patella with vastus lateralis muscle was moved medially – showing the surface of the femoral trochlea (FIG.1 A).

Scaffolds' implantation

In the middle of the trochlear groove of the femur a round hole in the shape of a cylinder with a diameter of 4 mm and a depth of 5 mm, covering the subchondral layer was created for the implant (FIG.1B). The hole was flushed with isotonic solution of NaCl 0.9%. In the prepared place the scaffold was inserted (FIG.1C,D). Tissues were apposed in layers anastomosed with absorbable sutures (Polyglactine 910, 3/0) according to anatomical alignment (FIG.1E). Non-absorbable suture material (Polyamide, 3/0) was used on the apposed skin (FIG.1F).

Postoperative management

After recovery rabbits were able to freely move in the cages. Medication with analgesic was administered (butorphanol at a dose of 0.1 mg/kg). Sul-Tridin 24% was also

administered at a dose of 30 mg/kg once a day for five days. After ten days skin sutures were removed.

Postoperative observation

During the post operative time most of the animals revealed no signs of knee dysfunction. Within 2-3 days after surgery individual animals showed lameness of the first degree, which had no significant effect on the general condition of the animals. One rabbit in the study group (without any scaffold) showed a significant swelling of the knee sustained in a week after surgery. This rabbit showed a first degree lameness with limited range of motion at the stifle joint. After the euthanasia it was determined that the joint had inflammation, an increased amount of synovial fluid, and degenerative changes that significantly deformed the surface of the joint and were the cause of the clinical changes described in the functioning of the joint.

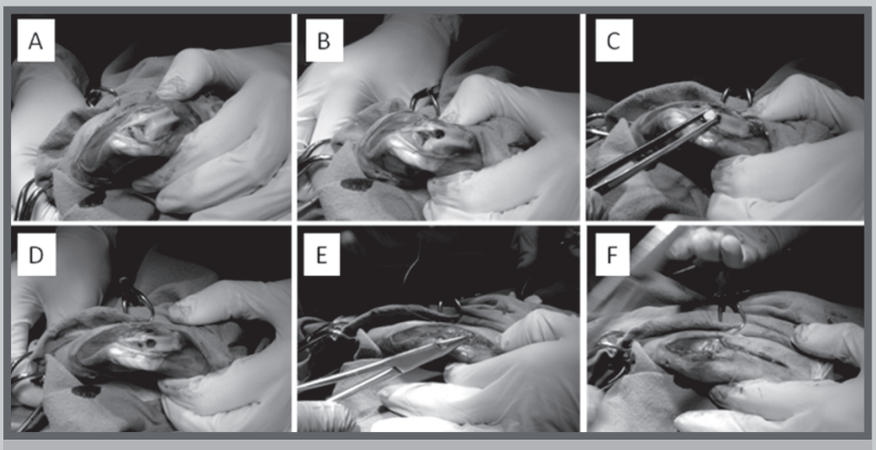

FIG. 1. Surgical procedure of scaffolds implantation into rabbit femoral trochlea: A – exposed surface of femoral trochlea, B – drilled hole in the middle of the trochlear groove, C– placing the scaffold, D – osteochondral defect filled with the scaffold, E – tissues apposed with absorbable sutures, F – skin apposed with non-absorbable sutures.

Euthanasia

After four weeks from the operation the rabbits were introduced into a state of general anesthesia with xylazine and ketamine. After reaching deep anesthesia euthanasia was performed by intracardiac injection of sodium pentobarbital. Then, the distal femoral epiphyses were excised, placed in 4% formalin and collected for further testing.

Cone Beam Computed tomography (CBCT)

The CBCT evaluations of the femora were carried out on GXCB-500/i-CAT (Gendex Dental System, Italy) volumetric tomograph at an isometric voxel size of 125x125x125 μm^3 and total scanning time of 23 s. Data acquisition and treatment was performed on iCAT Vision program working in DICOM standard.

Histology

The formalin from rabbit distal femoral epiphyses was removed by washing the specimens in tap water for two days. As a decalcification agent hydrochloric acid (TBD-1 Rapid decalcifier, Thermo Shandon Ltd, UK) was used for 5 days. After that the specimens were immersed in graded ethanol series for 24h each and twice for 1h in xylene. Finally, the samples were bathed in liquid Histoplast paraffin at 56°C for 3 days, and then embedded in a fresh portion. The embedded paraffin blocks were cut by rotary microtome (RM 2145, Leica Microsystems, Germany) for 9-µm thick sections. After deparafinization of tissue slices in xylene and rehydratation in ethanol series the specimens were stained with Masson-Goldner trichrome according to standard procedure, dehydrated again and mounted in universal histological mounting medium.

Results and discussion

The goal of this study was to modify entire volume of the PLGA scaffolds with natural bone extracellular matrix components, i.e. collagen type I and hydroxyapatite with the aim to enhance healing/regeneration of tissues in experimentally created osteochondral defects in rabbits.

FIG. 2 shows the microstructure of the scaffolds registered under scanning electron microscope. The scaffolds had interconnected pores of a size close to the size of porogen particles used in the process of their preparation, i.e. 250–320 µm. PLGA/coll scaffolds looked similar to the scaffolds without modification, but EDS analysis revealed presence of 2.5% of nitrogen originating from collagen. In PLGA/HAp scaffolds pore walls were more rough due to mineral deposits. Under higher magnifications, i.e. 4000x, round cauliflower-shape forms on the pore walls were visible in the scaffolds submitted to incubation in SBF (FIG. 1C, insert). EDS analysis confirmed that on PLGA scaffolds after contact with SBF calcium and phosphorus were detected and Ca/P ratio was 1.6, which is similar to that of hydroxyapatite. The FTIR and XRD examinations also confirmed presence of fine-crystalline hydroxyapatite phase (data not presented). The same results were observed on the scaffolds surfaces as well as in the cross-sections, what proves that the entire volume of the scaffolds was modified with collagen or hydroxyapatite.

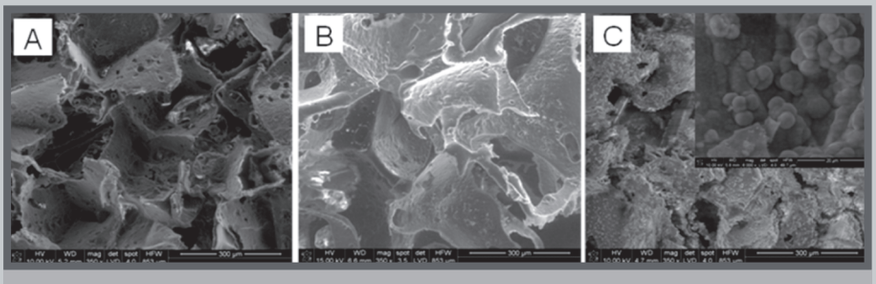

FIG. 2. SEM microphotographs of PLGA scaffolds non-modified (A), collagen- (B) and HAP-modified (C), original magnification 350x; insert 4000x.

In the first column of FIG. 3A digital camera pictures of intact femoral trochlea, trochleas with experimental defects filled with the scaffolds (PLGA, PLGA/coll, PLGA/HAP) as well as empty defect after 4 weeks from the surgery are presented. In all specimens the area where the defect was created was clearly distinguishable and hyaline cartilage was not healed. Cone beam computed tomography examinations in 3D projection (FIG. 3B) show that the defects were not totally filled with mineralized tissue, however 2D cross-

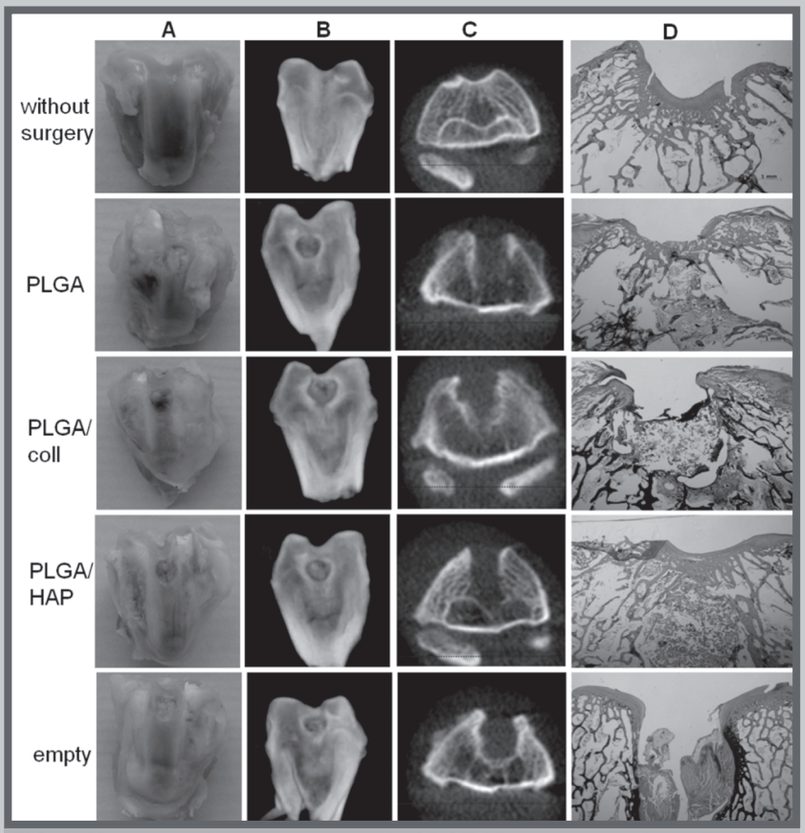

FIG. 3. Macroscopic appearance – A, CBCT-3D reconstruction – B, CBCT cross-section – C and histological examination – D of rabbit femoral trochlea without the surgery and after 4 weeks from the surgery treated with scaffolds: PLGA, PLGA/coll and PLGA/HAP as well as sham operation – empty defect; Masson-Goldner staining.

sections (FIG. 3C) show that the widths of the defect treated with PLGA/HAP and PLGA were much smaller than those of empty defect and treated with PLGA/coll scaffold. It suggests that PLGA scaffolds were beneficial for bone ingrowth and presence of hydroxyapatite enhanced this process.

Histological picture (FIG. 3D, first panel) shows typical structure of intact femoral trochlea. Thanks to trichrome Masson-Goldner staining the light green hyaline cartilage was visible on the top. Below it there was trabecular bone: stained in light green mature bone matrix, whereas the osteoid was coloured red. In the empty defect only fibrous tissue was visible (FIG. 3D, last panel). Interestingly, presence of scaffolds clearly enhanced bone/cartilage tissue regeneration. The best results were achieved for PLGA/HAP and PLGA scaffolds, where the ingrowth of newly formed trabeculae started from the peripheries toward center of the defects. The more developed hyaline cartilage was visible for PLGA/HAP scaffolds. Bone and cartilage healing seemed to be retarded on PLGA/coll, probably due to higher inflammatory response to the collagen of bovine origin.

Conclusions

In summary, the results show that PLGA scaffolds with defined microstructure (porosity and pore size) can be modified with a thin layer of collagen type I. Moreover in the entire volume of the scaffolds hydroxyapatite deposits can be created via biomimetic method. The in vivo experiments in rabbit model showed that bone formation was more advanced in the case of the scaffolds enriched with HAP. In the case of PLGA/coll scaffolds the extent of inflammation in surrounding tissues was much higher and healing was delayed as compared to non-modified PLGA scaffolds. Histological observations show that the presence of PLGA scaffolds is critical for the regeneration of osteochondral defects made in the knee of rabbits. In a sham operated knees the regeneration process was limited and the defects were filled with fibrous connective tissue instead of hyaline cartilage and bone tissue. CBCT examinations showed that bone mineralization was more advanced in the defects treated with PLGA/HAP scaffolds.

In brief, the results demonstrate that the PLGA scaffolds enriched with HAP have the highest potential for the treatment of critical-size osteochondral defects in rabbits.

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