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Induction of bactericidal activity by degradable implants

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Abstract

Introduction The problem of implant-associated infections is far from being solved in arthroplasty, osteosynthesis of fractures, and spinal pathology. The development of biodegradable implants with bioactive properties is a promising direction. **The purpose** of this study was to evaluate the *in vitro* bactericidal activity of implants made from a degradable material polycaprolactone (PCL) impregnated with hydroxyapatite and an antibiotic. **Material and methods** To study antibiotic availability, antibiotic-impregnated PCL cylindrical samples (n = 6) were incubated in distilled water at 37 °C. To evaluate the antibacterial properties, samples in the form of porous disks were used: control samples from PCL; 1) PCL samples coated with antibiotic and hydroxyapatite; 2) PCL samples coated only with antibiotic; 3) PCL samples coated only with hydroxyapatite; (n = 6 for each type of tested samples). The disk diffusion method was used to determine the sensitivity of microorganisms to antibiotics. The microbial strains used were *S. aureus* ATCC 25923, P. aeruginosa ATCC 27853 and E. coli ATCC 25922. Test microorganisms were cultivated on beef peptone agar (MPA) at 37 °C for 24 hours. Quantitative data were subjected to statistical processing. **Results** It was determined that 82.6 % of the antibiotic was released during the first day of incubation and 8.2 % on the second day. Control samples did not show a bactericidal effect. Samples 3 showed an antibacterial effect against E. coli culture. Samples 1 and 2 equally demonstrated significant inhibition of the growth of *S. aureus*, *P. aeruginosa*, and *E. coli*. **Discussion** Most of the antibiotic is released into the hydrolyzate during the first two days of incubation. Porous implants made of PCL and impregnated with an antibiotic have pronounced antimicrobial activity against the most common gram-negative and gram-positive bacteria that cause purulent complications in surgical practice. Nanostructured hydroxyapatite on the surface of the implant does not reduce bactericidal activity. **Conclusions** Porous polycaprolactone implants filled with hydroxyapatite and antibiotics are targeted to stimulate bone regeneration and simultaneously ensure antimicrobial activity. Nanostructured hydroxyapatite on the implant surface does not decrease bactericidal activity.

Keywords: bioactive implant, polycaprolactone, hydroxyapatite, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, antimicrobial activity, hydrolytic degradation

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INTRODUCTION

Over the last ten years remarkable progress has been made in the development of surgical techniques for bone reconstruction using bioresorbable implants having osteoinductive activity. The first fixation devices fabricated from biodegradable materials have become available since the early 1980s [1, 2]. They are still used in traumatology as pins and screws and do not need surgery for their removal [3-7]. Such pins are mostly made of polylactic acid and have no osteogenic activity; therefore fracture healing occurs in the usual terms [4, 8].

The risks of septic complications following internal osteosynthesis should not be negligible. The studies aimed at enhancing the bioactivity of polymer implants filled with antibiotics (Biomatrix, Allomatrix-implant, Osteomatrix, CollaPan G, CollaPan L) [9, 10] demonstrated efficiency of such approach. However, the matrix of such implants is shaped as a fine-grained material or a thin fibrous film. Thus, they do not enable stable osteosynthesis. Fused deposition modeling [11] for printing of implants with 3D-structure currently uses a filament made of linear bioresorbable polyesters, such as polylactic acid (PLLA), polycaprolactone (PCL), polyglycolic acid (PGA) and their copolymers. Saturation of these implants with antibiotics could provide antimicrobial activity associated with structural integrity and controlled resorption of an implant.

The aim of this study was to study the *in vitro* accessibility of antibiotics during hydrolytic degradation of polycaprolactone (PCL) products and bactericidal activity.

MATERIAL AND METHODS

Two types of implant samples were studied *in vitro*. Type 1 was shaped as a nail used in orthopedic surgery (cylindrical PCL samples, 10.0 mm long and 2.4 mm wide). The accessibility of antibiotics was assessed. To study bactericidal activity, disks made from polycaprolactone (PCL) using 3D printing technology, 10 mm in diameter and 1 mm thick, were used. The disks had cells with a diameter of 1-1.5 mm, limited by crossbars of 1 mm (like implants designed to treat bone defects). The surface of all implants was impregnated with cefotaxime, a broad-spectrum antibiotic.

The implants were designed and manufactured at Tomsk Polytechnic University. The components for the preparation of composite materials were ε-polycaprolactone (Sigma-Aldrich, United States; Mn 80000) and hydroxyapatite (Fluidinova, Portugal; 10 ± 5 μm). For preparation of the composite, PCL was dissolved in high purity acetone (EKOS-1, Russia) with a concentration of 15 wt %. Hydroxyapatite (HA) was pre-ground in a ball mill in a ceramic chamber with ceramic grinding media with the addition of acetone in a mass ratio of 1.5: 1 at a rotation speed of 72 rpm for 12 hours. After HA grinding, the PCL solution was added to the chamber and mixed in the ball mill. After drying, the obtained composite was crushed in a low-speed polymer crusher (Shini SG-1621N, Taiwan). The ground composites were extruded using a Filabot EX2 single screw extruder (Filabot, USA) to obtain 4-mm filaments. Additionally, HA particles were applied to the implant surface by dipping into a suspension of HA powder and antibiotic cefotaxime in a solvent, and then dried to remove residual solvent.

To study hydrolytic degradation, each cylindrical PCL sample (n = 6), impregnated with an antibiotic, was placed in a separate measuring cell filled with distilled water, the volume of which was determined at 4 ml per 1 cm^2 of the sample surface. Next, the samples were incubated in a thermostat at a temperature of 37 °C. The incubation medium was changed daily. The hydrolysate was subjected to a chemical analysis for the content of the antibiotic, which was determined on a spectrophotometer by the absorption intensity at a wavelength of 243 nm, relative to the standard calibration curve. The duration of incubation was 7 days.

The disk-diffusion method for determining the antibiotic sensitivity of microorganisms was applied to reveal the bactericidal activity [12].

The following strains of microbes were used to evaluate the antibacterial properties: *Staphylococcus aureus* ATCC 25923 (gram-positive bacteria), *Pseudomonas aeruginosa* ATCC 27853 (gram-negative bacteria) and *Escherichia coli*, *E. Coli* ATCC 25922 (intestinal bacteria).

Conditions for cultivating test microorganisms. Test microorganisms were cultivated on beef-peptone agar (MPA) at 37°C for 24 hours. A working suspension of test cultures was prepared from a culture of this test strain grown on dense nutrient medium (MPA) at 37 °C for 24 hours. Nutrient medium for evaluating the bactericidal properties of products was Muller – Hinton agar. The method of direct suspension in a sterile isotonic solution of colonies of a pure 18-24-hour culture of bacteria grown on a dense non-selective nutrient medium (MPA) was used for the preparation of the inoculum. The density of the suspension is 0.5 McFarland turbidity standard.

The discs of the test products were applied on a day-old fresh medium of microbial test culture. The time between the preparation of the microbial culture lawn and the application of disks on it was no more than 15 minutes.

Four types of products were studied for antibacterial activity. Discs without calcium phosphate coating and without antibiotics served as control. The other types were discs coated with hydroxyapatite and antibiotic (1), discs coated only with antibiotic without hydroxyapatite (2), discs coated only with hydroxyapatite (3).

Incubation after application of the discs was performed at 35 ± 1 °C, and lasted for 18 hours. A total of 36 studied tests were conducted (n = 6 for each type of tested samples).

The bactericidal activity of the implant was assessed by the zone of growth inhibition of the tested microorganisms around the disks. The checking was carried out in reflected light. For measuring the zone of growth inhibition, we were guided by the zone of complete suppression of visible growth. The bactericidal activity of the products was considered significant if the zone of growth inhibition around the disks was more than 1 mm.

Statistical analysis was performed using AtteStat 13.1 program (Russia): median values (Me), standard deviation (SD) and the lower and upper quartiles (Q1-Q3). The evaluation of the normal distribution of samples was performed using the Shapiro-Wilk test.

RESULTS

The study showed that 82.6 % of the antibiotic was released on the first day of incubation (Table 1). During that period, the mass of the samples increased slightly what can be explained by the absorption of water by the polymer. On the remaining days of the observation period, there was no significant change in the sample weight. The integrity of all samples was maintained throughout the entire incubation period.

Table 1

Average values of cefotaxime in hydrolysate and average weight of samples

Days of incubation	CANT, mg/cm^2, M ± SD	Release of antibiotic*; %	Weight, mg	% of weight change from initial level
0	0	0	66.2 ± 2.9	0
1	0.534 ± 0.074	82.6	66.5 ± 3.0	100.5
2	0.044 ± 0.009	8.2	66.4 ± 2.8	99.8
3	0.019 ± 0.012	3.6	66.3 ± 2.9	99.8
4	0.022 ± 0.008	4.0	66.0 ± 3.0	99.6
5	0.004 ± 0.001	0.8	66.0 ± 3.0	99.6
6	0.002 ± 0.001	0.4	65.9 ± 3.0	99.6
7	0.002 ± 0.001	0.4	65.5 ± 2.9	99.3
Total for 7 days	0.627 ± 0.050	100	–	–

Notes: CANT – is the concentration of the antibiotic in the hydrolysate; * – % release of antibiotic relative to the final value.

Control discs applied to lawns against all types of bacteria did not show a bactericidal effect. A continuous growth of microbial cultures was observed around the disks (Fig. 1). In all samples, the zone of growth inhibition around the discs was not determined (Table 2). This series confirmed that the implant matrix of pure polycaprolactone does not have a bactericidal effect.

Disks of product 3, coated only with hydroxyapatite (without antibiotic), showed a bactericidal effect only against the *Escherichia coli* culture (Fig. 2 c). The average zone of growth retardation in these samples exceeded 4 mm (Table 2).

Discs with antibiotic or antibiotic combined with hydroxyapatite demonstrated significant inhibition of bacterial growth (Fig. 3).

This experimental study revealed high activity of the products with the antibiotic against *P. aeruginosa*, *S. aureus* and *E. coli* cultures. The zone of complete inhibition of bacterial growth was 15.5-23.0 mm in sample 2 and 15.8-25.7 mm in sample 1 (Table 2). It should be emphasized that the application of a nanostructured hydroxyapatite on the surface (product 2) does not decrease the bactericidal effect of the antibiotic.

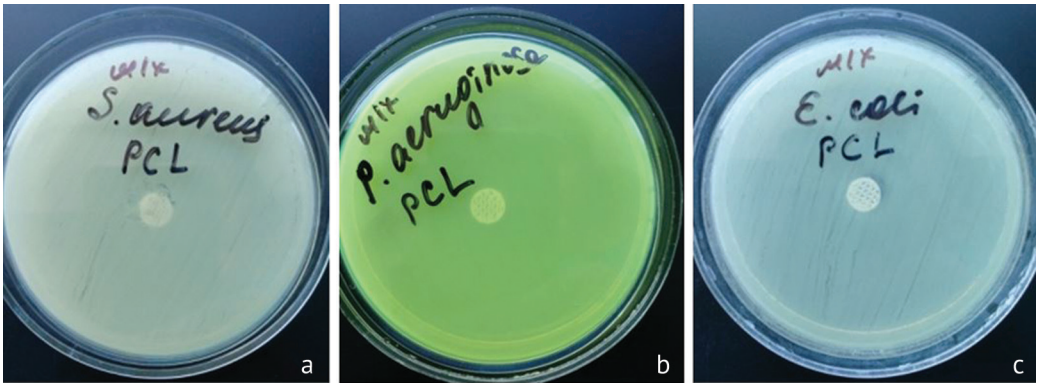

Fig. 1 Modified disk-diffusion method for determining the antibiotic sensitivity of microorganisms, absence of a zone of inhibition of the growth: a – *S. aureus*; b – *P. Aeruginosa*; c – *E. coli*

Table 2

Bactericidal properties of products in relation to microbial test-cultures (Me (Q1-Q3))

Microbes	Zone of growth inhibition, mm			
	Product 1	Product 2	Product 3	Product 4
	Pure PCL	Antibiotic	HA + antibiotic	HA
Staphylococcus aureus	0	21.25 (20.54-21.56)	23.11 (22.77-23.41)	0
Pseudomonas aeruginosa	0	15.85 (15.33-16.0)	17.23 (15.88-17.47)	0
Escherichia colli	0	22.37 (21.12-23.08)	23.80 (23.10-25.77)	4.27 (2.89-4.94)

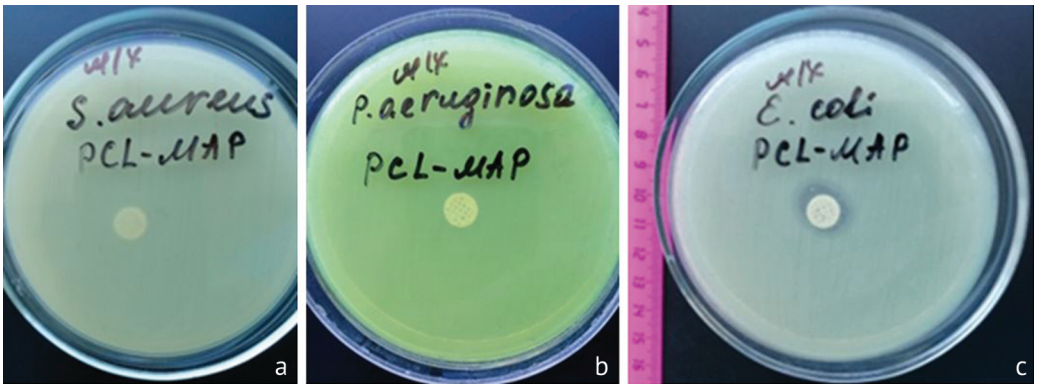

Fig. 2 Modified disk-diffusion method for determining the antibiotic sensitivity of microorganisms: a – no growth inhibition of *S. aureus* culture; b – no growth inhibition of the *P. aeruginosa* culture; c – zone of significant growth inhibition of *E. coli* is 4.27 (2.89 – 4.94) mm

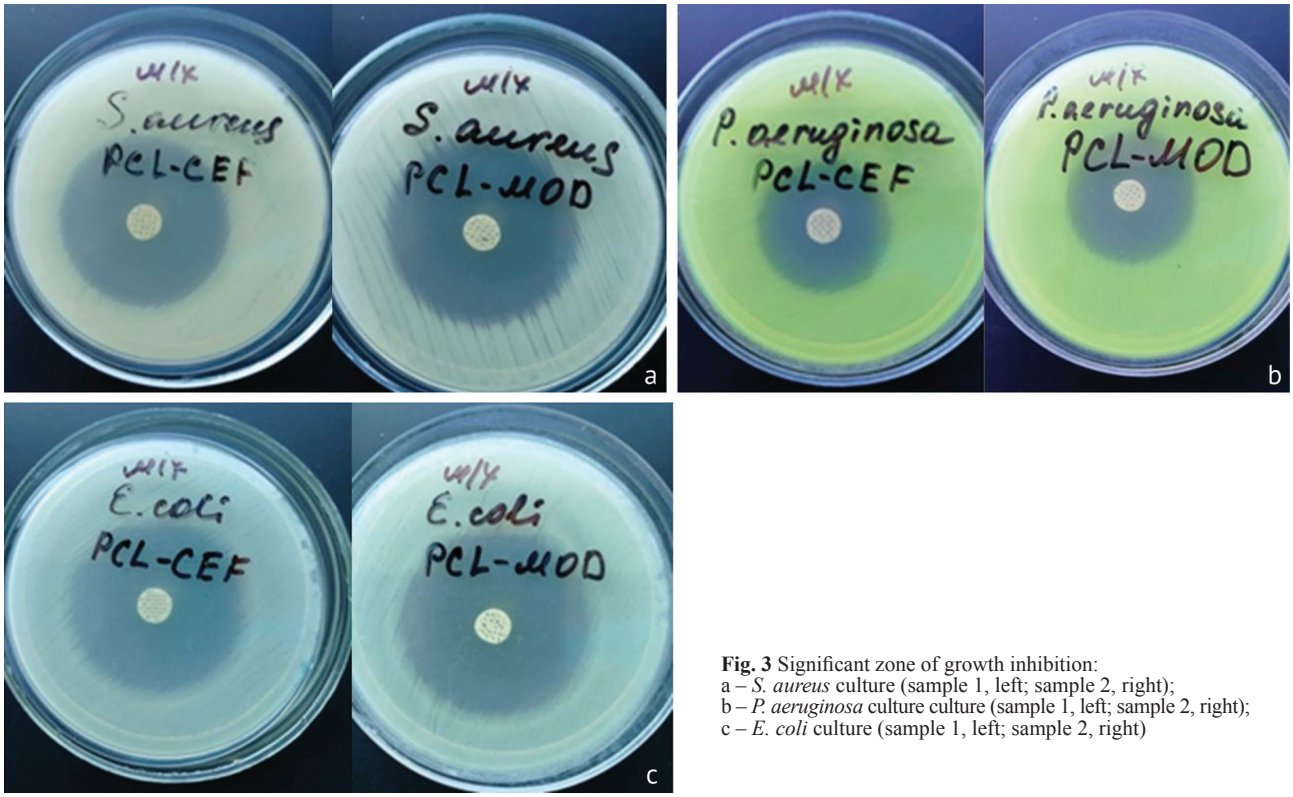

Fig. 3 Significant zone of growth inhibition:
a – *S. aureus* culture (sample 1, left; sample 2, right);
b – *P. aeruginosa* culture culture (sample 1, left; sample 2, right);
c – *E. coli* culture (sample 1, left; sample 2, right)

DISCUSSION

The most promising synthetic polymers for medical use are aliphatic polyesters based on hydroxyalkanocarboxylic acids: polylactide, polyglycolide, polyhydroxybutyrate. Their degradability under the influence of biological factors can be used in new devices and implants. Bioinert implants should be distinguished from bioresorbable polymers used in reconstructive surgery. Resorbable polymers ensure required function and structure over the period of host tissue regeneration. They must be able to decompose under the influence of body fluids with the formation of non-toxic products. The rate of decomposition of a solid bioresorbable implant polymers into liquid products should be controllable and not exceed the rate of tissue regeneration (in bone regeneration process, it is a period of months) [13, 14].

Currently, products made from polylactic acid and polycaprolactone have been frequently used in medicine. Introduction of inorganic substances into the composition of the polymer matrix enables the control over the physicochemical and mechanical properties of polylactide [15-17]. However, their application for bone fragments fixation has not been widely used as they do not meet the requirements of the AO/ASIF principles [13, 18].

New research trends are aimed to develop materials and implants enabling osseointegration of an implant followed by controlled matrix resorption [19, 20]. However, new generation of implants does not exclude risks of septic complications related to surgery [21, 22].

The relationship of bacterial agent with polymers or with the ceramic surface of implants remains unclear.

Nevertheless, a large clinical material on the use of intraosseous implants shaped as wires with a bioactive hydroxyapatite surface revealed complete absence of inflammatory complications [23]. The technology of intramedullary reinforcement with HA-coated wires is applied for treatment of fractures and pseurarthrosis [24, 25].

We chose polycaprolactone (PCL) as the matrix for the degradable implant due to its chemical properties providing control of its 3D mechanical structure (resorption lasts 1.5-4 years) [26, 27]. The HA-filled PCL maintains mechanical strength and demonstrates an osteoinduction effect on the bone regeneration [28, 29]. We hypothesized that the impregnation of an PCL implant with an antibiotic would have an antibacterial effect, what is especially required in early postoperative period.

The antibacterial activity of ceramic nanoparticles (ZnO, TiO_2) applied as a coating on metal implants was demonstrated in the study of Colon et al. [30]. This coating significantly decreased adhesion of *Staphylococcus epidermidis* on the implant surface. In contrast to that, an increased adhesion of osteoblasts was stated. This finding allowed drawing a conclusion that the large surface areas of the nanophase compared to the microphase and high surface energy present in the surface layers of the nanoparticles could lead to increased dissolution rate of the ceramic surface, which disrupts the functions of bacteria. That study suggests that the technology of bioactive coating of an implant with hydroxyapatite allows its additional saturation with a desirable broad-spectrum antibiotic or in accordance with its antimicrobial activity against infected patient tissues. Our findings are consistent with this hypothesis demonstrating the bactericidal effect of nanostructured hydroxyapatite coating of PCL against *Escherichia coli*.

We propose to fill the matrix of bioresorbable implants with antibiotics. Combined with HA-nanostructured coating, degradable implants will ensure stability of osteosynthesis (related to controllable resorption), stimulation of host bone regeneration and prevention of septic complications. Our experimental study demonstrated antibacterial activity of the implant bioactive surface against the most common gram-negative and gram-positive bacteria causing septic complications in surgical departments: *P. aeruginosa*, *S. aureus*, *E. coli*. Most of the impregnated antibiotic released from porous PCL products into the hydrolysate within the first two days of incubation. We established that nanostructured hydroxyapatite on the surface of a biodegradable implant in its pure composition has a pronounced bactericidal activity only against *Escherichia coli*.

CONCLUSION

Porous polycaprolactone implants filled with hydroxyapatite and antibiotics are targeted to stimulate bone regeneration and simultaneously ensure antimicrobial activity. Nanostructured hydroxyapatite on the implant surface does not decrease bactericidal activity.

Conflict of interest *All authors declare no conflict of interest.*

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