Rīgas Stradiņa Universitāte

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ANTIBACTERIAL EFFICIENCY OF ANTIBIOTIC-IMPREGNATED BIOMATERIALS IN AN *IN VITRO* AND *IN VIVO* STUDY

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Speciality – Microbiology

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Doctoral Thesis is available in the RSU library and at RSU webpage: www.rsu.lv

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CONTENTS

ABBREVIATIONS

Abbreviation	In English
BAI	*biomaterial associated infections*
CD	*calcium deficient*
Cipro	*ciprofloxacin*
ELISA	*enzyme-linked immunosorbent assay*
Genta	*gentamicin*
HAp	*hydroxyapatite*
MRSA	*methicillin-resistant Staphylococcus aureus*
MIC	*minimum inhibitory concentration*
PBS	*phosphate-buffered saline*
PCL	*polycaprolactone*
PLGA	*poly(lactic-co-glycolic acid)*
PLLA	*polylactic acid*
SEM	*scanning electron microscope*
TCP	*tricalcium phosphate*
TNF-α	*tumor necrosis factor*
TSA	*trypticase soy agar*
TSB	*trypticase soy broth*
UV	*ultraviolet*

INTRODUCTION

The use of biomaterials is popular in all fields of medicine, and they are used to substitute human organs or parts of human organs, thus improving the life quality of patients and lengthening their life. Biomaterials are used in orthopaedics to substitute joints or parts of bones, in dentistry – as tooth implants, in cardiology – as artificial heart valves or cardio-stimulators. All medical fields use various biomedical implants, such as intravenous catheters, urinary tract catheters, intubation systems and other biomaterials used in medicine, which is a testament to the important role biomaterials play in medicine today (*Huebsch et al*., 2009).

Unfortunately the use of biomaterials is usually also associated with various complications, and one of them is BAI. Despite prophylactic measures taken before and after biomaterial implantation surgeries, the sterility of the surgery and other prophylactic measures taken to decrease BAI risk, the amplitude of infection development varies. Thus, infection risk of orthopaedic implants is 1 to 3 per cent, and death risk is high among patients with this infection (*Darouiche et al*., 2001), which also raises treatment expenses associated with orthopaedic implant replacement and longer hospitalisation time (*Haenle et al*., 2012). BAI is a local infection, which can start after bacterial contamination during the surgery, during the post-operative period, or as a result of haematogenous dissemination from another focal of infection in the human body. Considering the peculiarities of local infections, the most effective BAI prophylaxis is the use of antibiotic-saturated biomaterials in order to decrease bacterial adhesion to biomaterials, and the formation of a biofilm (*van de Belt et al*., 2001).

Local antibiotic substance activity from the biomaterial has a number of benefits if compared to systemic antibiotic use in order to reduce BAI risk. Local antibacterial therapy from the biomaterial allows us to avoid

complications associated with systemically used antibiotic substances. The most common are allergic reactions, toxicity and disbacteriosis, which are only a few of complications, which can stem from the use of antibacterial substances. They can be prevented with aforementioned local therapy (*Campoccia et al*., 2010).

Saturation of biomaterials with antibacterial substances and their alignment with biodegradable polymers ensures antibiotic management, a controlled and prolonged release, which prolongs the antibacterial properties of the biomaterial (*Leprêtre et al*., 2009).

Apart from BAI risk, biomaterials have to be biocompatible with surrounding tissues, because irrespectively of biomaterial synthesis, type and use, it is still a foreign body to the organism (*Franz et al.*, 2011).

The aim of the thesis

Determine the efficiency of a hydroxyapatite saturated with antibiotic substances and covered with a biodegradable polymer against most frequent post-operation infection agents *P. aeruginosa* and *S. epidermidis in vitro* and *in vivo* study

Hypothesis of the thesis

1) PLLA and PCL has different impact on antibacterial efficacy against *S. epidermidis* and *P. aeruginosa*.
2) The expression of inflammatory cytokines and antibacterial peptide in tissue around biomaterials is more intensive, if are implanted biomaterials without antibiotics.

Tasks

1) To evaluate antibacterial efficacy of the different composite materials against *P. aeruginosa* and *S. epidermidis in vitro*.

2) To study how the biodegradable polymer affect on biomaterials antibacterial effectiveness *in vitro*.
3) Evaluate the impact of composite material porosity on antibacterial time.
4) Compare PCL and PLLA efficiency on the antibacterial period of the biomaterial.
5) Determine the intensity of inflammatory cytokines – IL-10, TNF-α and antibacterial peptide - β-defensin–2 expression *in vivo*.

Novelty of the thesis

Studied biomaterials were all originally synthesised in Latvia according to the latest and previously unused methods. This was the first time when the antibacterial characteristics of biomaterials were studied at such detail *in vitro*, and their biocompatibility *in vivo*, determining inflammatory cytokines and antibacterial peptides.

Materials and technologies

The Department of Biology and Microbiology at Rīga Stradiņš University ensured the *in vitro* study, as well as ELIS kits for the *in vivo* part of the study. The *in vivo* part of the study was carried out in the Laboratory of Experimental Animals of Rīga Stradiņš University. Studied biomaterials were originally synthesised in the Riga Biomaterial Innovation and Development Centre of Rūdolfs Cimdiņš at Riga Technical University.

Ethical aspects of the thesis

In order to carry out *in vivo* studies, we received Authorisation licence from the Food and Veterinary Service (*Pārtikas and veterinārais dienests*).

Personal investment

The author carried out all *in vitro* and *in vivo* studies by himself, independently carrying out surgical operations on the animals used in the experiment, extracting samples for examination, and using molecular diagnostic methods.

Structure of the thesis

The thesis is 106 pages long and it consists of the following parts: introduction, literature review, materials and methods, results, discussion, conclusions, bibliography and appendixes. There are 9 tables and 69 figures. Bibliography includes 210 sources.

1. MATERIALS AND METHODS

1.1 Biomaterial samples

1.1.1 HAp/PLLA+cipro un HAp/PLLA+genta

Hydroxyapatite powder was prepared using the wet – chemical deposition method from calcium oxide (*Sigma-Aldrich, UK*) ≥97% and orthophosphoric acid (*Sigma-Aldrich, UK*) ≥ 85% solution (*Sokolova et al.*, 2014). The acquired synthesised powders were pressed into pellets (d=10, H=3 mm). All samples were processed in 1100°C for 1 hour. Prior to saturation with antibiotic substances in HAp samples, gentamicin (concentration of 40mg/ml) or ciprofloxacin (concentration of 100 mg/10 ml) were dissolved in deionised water. HAp samples were saturated with water medicine solution at room temperature at atmospheric pressure, and then dried at 37°C. Parts of the prepared samples were used to coat them with PLLA. PLLA coating of HAp samples was prepared from 10 wt% poly (L-lactic acid) (Nature Works LLC, Mw = 110 kDa) solution with dichloromethane (DCM) (*Sigma-Aldrich, UK*). PLLA was dissolved in DCM, stirring it for 2 hours at room temperature. Polymer solution was infiltrated into HAp bioceramic samples using the vacuum impregnation method at 500 mbar pressure for 15 minutes. Coated samples were dried at room temperature for 24 hours. Open porousness and total porousness was determined with the Archimedes method, which was based on – force used equals the mass of the fluid moved (*Locs et al.*, 2013). Open porousness level was 34 per cent, whereas total porousness was 36 per cent. Upon cross-sectional examination of coated samples with SEM, we can see that HAp/PLLA samples, which were coated with 10 wt% of PLLA have a porous microstructure, with the diapason of pore size being from 200 nm to 500

nm (see Fig. 2.1 on the left side). We acquired samples with PLLA coating, which were 2-5 μm thick (see Fig. 2.1 on the right side). In order to determine, whether PLLA has any antibacterial characteristics, we prepared HAp/PLLA samples without any antibiotic substances.

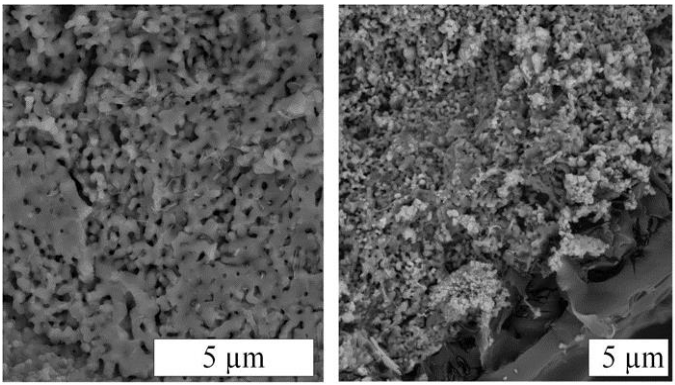


Fig. 2.1 **HAp/PLLA cross-section** (SEM)

According to the same method we prepared ↓HAp/PLLA+cipro, ↓HAp+cipro and ↓HAp/PLLA; their open and total porousness was lower, and made only 15 per cent open porousness, and 19 per cent of total porousness. Samples were synthesised in order to determine how the level porousness affects antibacterial length.

1.1.2 CDHAp/PCL+cipro, CDHAp/PCL+genta, CDHAp/PLLA+cipro un CDHAp/PLLA+genta

Calcium deficit hydroxyapatite powder was made using the wet – chemical sedimentation method. The following reagents were used: calcium oxide (*Sigma-Aldrich, UK*) from marble, ≥97% and orthophosphoric acid (*Sigma-Aldrich, UK*) ≥85% and deionised water. A few factors were evaluated

regarding composite formation process, e.g., pH, suspension temperature and additional acid (*Sokolova et al*., 2014).

PCL and CDHAp composites with biopolymer content of 20 wt% and PLLA and CDHAp composites with biopolymer content of 30 wt% were synthesised using the new wet/solid suspension technique.

Gentamicin in concentration of 40 mg/ml, and ciprofloxacin in concentration of 100 mg/10 ml were dissolved in deionised water. Acquired solutions were mixed with a little CDHAp/PCL and CDHAp/PLLA powder. Acquired powder mixtures were dried at room temperature for 24 hours. Dried composites were pressed into pellets (d = 12.5 mm,h = 2.2 mm). CDHAp/PCL and CDHAp/PLLA pellet porousness was determined by the geometric method.

In order to evaluate, whether PLLA and PCL have any antibacterial characteristics, we prepared CDHAp/PCL and CDHAp/PLLA samples without antibiotics.

1.2 Determination of antibacterial characteristics *in vitro*

1.2.1 Determination of antibacterial characteristics in a bacterial suspension

Bacterial suspensions were prepared in sterile circumstances from 1 ml TSB (*Oxoid, UK*) and 1 ml bacteria with an optic density of 0.5 according to the McFarland standard. Prior to placing biomaterial samples into bacterial suspensions, all biomaterials were placed, each separately, in 1ml rabbit plasma (BBL, USA) and incubated in thermostat (*Memmert, Germany*) at 37°C and 100% relative humidity for two hours. After two hours, the biomaterial samples were transferred with the help of sterile pincers into test tubes with bacterial suspension, and they were incubated for 24 hours in 37°C.

After 24-hour incubation, 0.1 ml of bacterial suspension was inoculated on TSA (*Oxoid, UK*) in order to examine the antibacterial characteristics and effectiveness of studied biomaterials. A new bacterial suspension was prepared

at the same time, and with the help of sterile pincers biomaterials of the studied group were transferred into a new TSB and bacterial culture suspension for the next 24 hours. These actions were repeated every 24 hours until no trace of antibacterial characteristics were found in the studied biomaterial groups for two days in a row, and TSA colony number was equal to the colony number in the control group on TSA.

1.2.2 Determination of antibacterial characteristics with the disk diffusion method

Kirby-Bauer disk diffusion method is a standardised method used in microbiology laboratories in order to determine bacterial susceptibility against antibiotic substances (*Bauer et al.*, 1966). We prepared bacterial suspensions according to EUCAST (*EUCAST*, 2015) standards in optic density of 0.5 according to McFarland standard with McFarland optic densitometer (*Biosan, Latvia*). Bacterial suspension was inoculated onto a sterile TSA plate (*Oxoid, UK*) with a sterile cotton swab. After bacterial inoculation, biomaterial disks were placed onto TSA with sterile pincers, and TSA agar was incubated in the thermostat for 24 hours in 37°C degrees. After 24 hours, antibacterial properties of biomaterial samples were analysed by measuring the sterile area (diameter) around disc biomaterials. After the measurements, a new bacterial suspension was prepared, and it was inoculated onto a new sterile TSA agar, and biomaterial discs were transferred from the old to the new TSA culture agar, and incubated for another 24 hours in 37°C. These actions were repeated every 24 hours until no trace of antibacterial characteristics or sterile area around biomaterial samples was found in the studied biomaterial groups for two days in a row.

1.3 Bacterial cultures and they susceptibility against gentamicin and ciprofloxacin

All antibacterial characteristics of the biomaterial were studied, using bacterial reference cultures *S. epidermidis* (ATCC 12228) and *P. aeruginosa* (ATCC 27853).

We used antibiotic strip manufacturer recommendations for the examination procedure to determine bacterial sensitivity against antibacterial substances. We inoculated bacteria on the Mueller Hinton (*Oxoid, UK*) agar with a sterile cotton swab in a suspension prepared according to the 0.5 McFarland standard. It was then placed on gentamicin or ciprofloxacin antibiotic strips (*Liofilchem, Italy*). Agar plates were incubated at 37°C. The results were evaluated after 20 hours of incubation.

1.4 Determination of inflammation intensity *in vivo*

1.4.1 Animals used in the experiment

Three month old male rabbits were used in the experiment in order to determine the intensity of inflammatory cytokines in tissues around the implanted biomaterials. All rabbits used weighed 3 kg. At the start of the experiment they were all healthy, all ethical aspects were considered, and we received authorisation from the Food and Veterinary Service

1.4.2 Procedure of the operation

Prior to the operation, we prepared bacterial suspension of *P. aeruginosa* and *S. epidermidis* with optical density of 0.5 according to the McFarland standard. We shaved the neck of the rabbit and treated the area with iodine solution. Shaved area was treated with antiseptics, applying also local anaesthesia of 2% lidocaine hydrochloride solution onto the shaved area. A 2.5 cm incision was made in a sterile environment with a sterile scalpel.

With the help of the scalpel, a deep enough subcutaneous pocket was made where the biomaterial pellet could be implanted. After the biomaterial was placed in the subcutaneous pocket, the wound was infected with 0.1 ml of bacterial suspension.

After the wound was infected with bacterial suspension, it was sewn back together with sterile 3-4 sutures, closing the wound tightly. The post-operative period went by without any complications – none of the rabbits fell ill or died. Biomaterial samples were removed four weeks later. The following biomaterials were implanted – HAp/PLLA+cipro, HAp+cipro, HAp/PLLA, HAp/PLLA+genta and HAp+genta, which were also infected with *P. aeruginosa* or *S. epidermidis* (Table 1.1).

Table 1.1

Sample groups used in the *in vivo* study

Sample group	HAp	PLLA	cipro	genta	*P. aeruginosa*	*S. epidermidis*
Group A	X	X	X		X	
Group B	X	X	X			X
Group C	X	X		X	X	
Group D	X	X		X		X
Group E	X		X		X	
Group F	X		X			X
Group G	X			X	X	
Group H	X			X		X
Group I	X	X			X	
Group J	X	X				X

1.4.3 Studied material

In order to determine IL-10, TNF-α and beta-defensin-2 level in surrounding tissue around the implanted biomaterial, we used standardised ELISA method with ELISA kits (*USCN life science* and *MyBioSource, USA*). After a four-week biomaterial implantation, the rabbits were euthanized. At the location of the implanted biomaterial, the skin of the rabbit was cut with a

sterile scalpel. Three equal-size tissue samples of surrounding tissues were collected, and three tissue samples were collected at a distance (1.5 cm) from the implanted biomaterial. Tissues were washed with cold PBS (0.01 mol/L, pH 7.0 to 7.2) before the homogenization, in order to wash off blood residue from the tissues. The tissues were cut into fine pieces and they were homogenized. The resulting tissue suspension was placed in an ultrasonic bath, in order to tear cell membranes. Next, the suspension was spun for 5 minutes at 5000 × g, and the supernatant was collected. After the standardized ELISA procedures 96 cavity microplate was analysed by an ELISA reader spectrophotometrically at a wavelength of 450 nm ± 10 nm (TECAN, Switzerland).

1.5 Statistical methods

We selected the non-parametric statistic for the examination of results obtained. A Mann-Whitney test (*Mann et al.*, 1947) was used in order to assess whether there is a statistically significant difference between the study group of antimicrobial duration of biomaterials. *P*-value less than or equal to 0.05 was assumed as statistically significant. The results were entered into Microsoft Excel 2014, and statistical analysis was performed with SPSS 22.0.

2. RESULTS

2.1 Minimally inhibited concentration of antibiotic substances

Using a standardized E-test method, we determined that *P. aeruginosa* and *S. epidermidis* cultures were sensitive to antibiotics used in the study. Ciprofloxacin MIC against *S. epidermidis* was 0.094 μg/ml, and against *P. aeruginosa* it was 0.125 μg/ml. Conversely, gentamicin MIC against *S. epidermidis* was 0.19 μg/mL, and against *P. aeruginosa* it was 1.0 μg/mL.

2.2 HAp/PLLA+genta and HAp/PLLA+cipro antibacterial effectiveness *in vitro*

Using the the antibacterial performance determination method, bacterial suspension showed that the maximum duration of antibacterial activity against *S. epidermidis* in HAp/PLLA+genta biomaterials was 264 h and minimum antibacterial duration was 216 h, hence, the average duration of antibacterial activity against *S. epidermidis* was 249.6 ± 16.78 h (Fig. 2.1). Antibacterial properties were not observed in HAp/PLLA against any of the bacterial cultures.

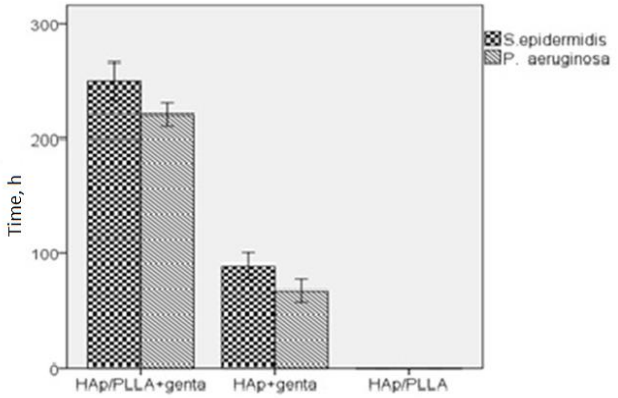


Fig. 2.1 **Average antibacterial duration for various composite materials with gentamicin**

Antibacterial duration of biomaterials without the PLLA polymer (HAp+genta) was much lower, because the maximum antibacterial time reached 96 hours against *S. epidermidis*, and minimum duration reached 72 hours; the average duration of antimicrobial activity was 88.8 ± 11.59 hours. The Mann-Whitney test shows that biomaterials with PLLA and gentamicin have a statistically significantly longer duration of antibacterial activity against *S. epidermidis* than biomaterials without PLLA ($p<0.001$).

The ability of HAp/PLLA+genta and HAp+genta to inhibit *S. epidermidis* growth *in vitro* as a whole is different, but equal to the first three experiment days when almost all *S. epidermidis* bacteria were inhibited. Over the course of the following days of the experiment HAp/PLLA+genta retained high *S. epidermidis* growth inhibition ability, and gradually lost them, while *S. epidermidis* growth inhibition abilities were lost rapidly on HAp+genta.

The Mann-Whitney test shows that there is a statistically significant difference between the HAp/PLLA+genta and HAp+genta biomaterial groups ($p<0.001$) against *P. aeruginosa*, and the maximum and average antibacterial length of this group was less than that against *S. epidermidis*. Maximum HAp/PLLA+genta antibacterial duration against *P. aeruginosa* was 240 h, and the minimum duration was 216 h, which was equal to *S. epidermidis*. Maximum duration of HAp+genta antibacterial activity was 72 hours, and the minimum antibacterial duration was 48 hours against *P. aeruginosa*. Accordingly, the average HAp/PLLA+genta antibacterial time against *P. aeruginosa* was 220.8 ± 10.11 hours and on HAp+genta it was 67.2 ± 10.11 hours.

The ability of HAp+genta and HAp/PLLA+genta to inhibit *P. aeruginosa* growth dynamic in the first 48 hours did not differ statistically significantly ($p>0.05$), however, starting from the third day of the experiment we saw statistically significant differences ($p<0.001$) between the HAp/PLLA+genta and HAp+genta biomaterials against *P. aeruginosa*.

Same as in the *S. epidermidis* case, HAp/PLLA+genta biomaterials against *P. aeruginosa* showed gradual loss of antimicrobial properties.

Similar trends were demonstrated by HAp/PLLA+cipro and HAp+cipro against both bacterial cultures used in the study. Compared to HAp/PLLA+genta, HAp/PLLA+cipro had a maximum duration of antibacterial activity against *S. epidermidis* of 288 hours, and the minimum time was 264 hours. Conversely, maximum and minimum antibacterial activity on HAp+cipro against *S. epidermidis* was similar to that of HAp+genta, and amounted to 96 and 72 hours. Thus the average antibacterial duration of PLLA biomaterials was longer than sans-PLLA biomaterials (Fig. 2.2).

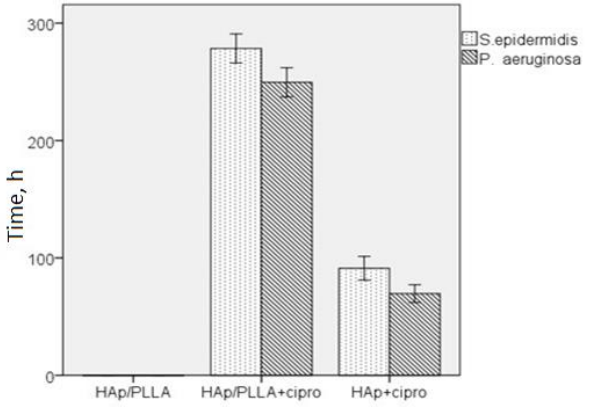


Fig. 2.2 **The average time for a variety of antibacterial composite materials with ciprofloxacin**

Average antibacterial duration of HAp/PLLA+cipro against *P. aeruginosa* was shorter than that against *S. epidermidis*. No statistically significant differences ($p<0.001$) were observed between the HAp/PLLA+cipro and HAp+cipro against *P. aeruginosa*.

HAp/PLLA+cipro and HAp+cipro completely inhibited the growth of *S. epidermidis*, which did not statistically differ ($p>0.05$) in the first three days of the experiment, but starting from the fourth day of the experiment – within

96 hours a statistically significant ($p<0.005$) difference was observed. We observed statistically significant differences in the growth dynamic of *P. areruginosa* growth inhibition already in the 72 hour of the experiment.

The Mann-Whitney test showed that HAp/PLLA+genta, HAp+genta, HAp/PLLA+cipro and HAp+cipro biomaterials did not alter statistically significantly the bacterial inhibition percentage during the first 24h to 48h period on any of the bacterial cultures ($p>0.05$).

In determining the antimicrobial properties of HAp/PLLA+cipro and HAp+cipro with the disc diffusion method against both bacterial cultures, it is evident that the method does not affect the examination of the antibacterial time against any of the bacterial cultures used in the study on biomaterial samples. Nor are there any differences in *S. epidermidis* growth inhibition, because HAp/PLLA+cipro maintained its antibacterial properties for long periods, losing them gradually, however HAp+cipro lost its antibacterial properties rapidly, and within 5 days it equalled to zero. The disk diffusion method showed that HAp+cipro biomaterials emit higher levels of antibiotic concentration than HAp/PLLA+cipro, because we observed a larger sterile area diameter on the first day of the experiment, which shows greater antibiotic substance defunding from the sample of the biomaterial. HAp+cipro had also larger antibiotic substance quantities against *P. aeruginosa*, since the sterile area diameter was greater than that on HAp/PLLA+cipro.

However, the overall antibacterial time against *P. aeruginosa* on HAp/PLLA+cipro was longer than that on HAp+cipro, and antibacterial properties were lost progressively throughout the *in vitro* study.

Biomaterials with lower porosity – ↓HAp/PLLA+cipro had a statistically different antibacterial time ($p<0.001$) compared to biomaterials with higher porosity – HAp/PLLA+cipro. Statistically significant differences ($p<0.001$) were also observed on the different levels of porosity in biomaterials without PLLA with antibiotics (Fig. 2.3).

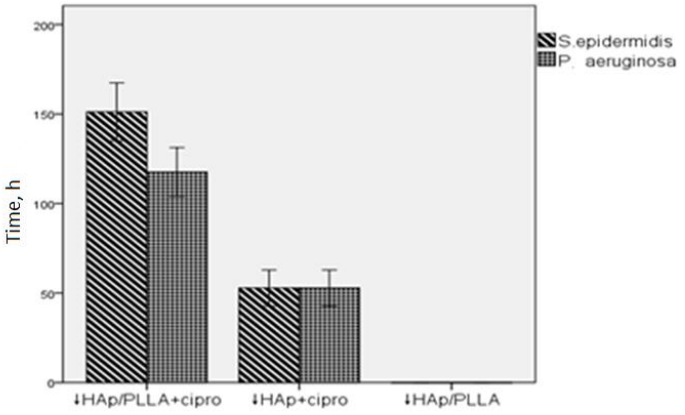


Fig 2.3 **Average antibacterial length in composite materials with a decreased porosity and ciprofloxacin**

S. epidermidis growth inhibition ability on ↓HAp+cipro was shorter than that on HAp+cipro biomaterials with higher porosity, which shows that the level of porosity depends on the ability of biomaterials to inhibit the growth of bacteria. The ability of PLLA biomaterials to inhibit the growth of *S. epidermidis* also depends on the porosity level, taking into account that the HAp/PLLA+cipro antibacterial duration is longer than that of ↓HAp/PLLA+cipro. The impact of the porosity level on antimicrobial time was also proved by the study of *P. aeruginosa* bacterial culture, showing that biomaterials with a lower level of porosity altered the dynamics of *P. aeruginosa* growth inhibition compared to biomaterials with higher porosity.

Within the first 24 to 48 hours in the group of low porosity biomaterials we observed statistically significant percentage changes in inhibited bacterial dynamic on ↓HAp+cipro against *S. epidermidis* cultures (Mann-Whitney test, $p<0.05$), as well as statistically significant decrease against *P. aeruginosa* cultures ($p<0.05$).

During the first 24 hours, regardless of the presence of PLLA in low porosity biomaterials we observed statistically significant changes ($p<0.05$), but changes in the *in vitro* study began to occur after a 48h reference period and the Mann-Whitney test indicated that the decrease of percentage changes of inhibited bacteria in low porosity biomaterials without PLLA within 48 h is statistically significantly higher than in low porosity biomaterials with PLLA ($p<0.05$) regardless of the bacterial cultures.

2.3 CDHAp/PLLA+genta and CDHAp/PLLA+cipro antibacterial effectiveness *in vitro*

Using antibacterial performance test we found out that the maximum antibacterial time on CDHAp/PLLA+genta against *S. epidermidis* is 264 hours, and the minimum antibacterial time reaches 240 hours, presenting the average antibacterial time against *S. epidermidis* as 249.6 ± 16.78h (Fig. 2.4). Antibacterial properties were not observed on CDHAp/PLLA against *S. epidermidis* and *P. aeruginosa*.

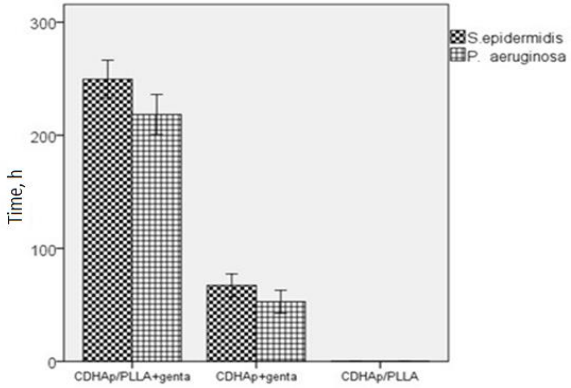


Fig. 2.4 **Average antibacterial time on composite materials with gentamicin and PLLA**

The Mann-Whitney test showed that antibacterial time on CDHAp/PLLA+genta and CDHAp+genta differ statistically significantly

($p<0.001$) against *S. epidermidis*. CDHAp/PLLA+genta completely inhibited the growth of *S. epidermidis* within the first 5 days, and then gradually lost this ability, while CDHAp+genta completely inhibited the growth of *S. epidermidis* in the first study days, and then progressed to a rapid loss of antimicrobial properties.

The maximum antibacterial time of CDHAp+genta against *S. epidermidis* was 72 hours, and the minimum antibacterial time was 48 hours, representing an average antibacterial time of 67.2 ± 10.11 hours. The ability of CDHAp/PLLA+genta and CHAp+genta to inhibit the growth of *P. aeruginosa* was similar to that against *S. epidermidis*, except that *P. aeruginosa* in its general growth inhibition dynamic shows rapid loss of antimicrobial properties as a whole on CDHAp/PLLA+genta, and the maximum and minimum antibacterial time varies, and reaches 240 and 196 hours respectively.

We observed statistically significant changes within the first 24h to 48h on HAp+genta against *P. aeruginosa* bacterial culture (Mann-Whitney test, $p<0.05$), as well as a statistically significant decrease against *S. epidermidis* culture ($p<0.05$). We did not observe statistically significant changes on any of the other CDHAp/PLLA+genta biomaterials (Mann-Whitney test, $p>0.05$).

Nor did we observe statistically significant changes within the first 24 to 48 hours on CDHAp/PLLA+cipro and CDHAp+cipro ($p>0.05$) against *S. epidermidis* and *P. aeruginosa*. We observed statistically significant changes ($p<0.05$) within the first 24 to 48 hours on CDHAp+cipro against both bacterial groups used in the *in vitro* study, but we did not observe any reliable statistical changes within the same period on CDHAp/PLLA+cipro against any of the strains. We observed gradual loss of *S. epidermidis* growth inhibition on CDHAp/PLLA+cipro; compared to biomaterials of the same group without PLLA (CDHAp+cipro), the antibacterial time is statistically significantly longer ($p<0.001$) against *S. epidermidis*.

If we compare CDHAp/PLLA+cipro with CDHAp/PLLA+genta against *S. epidermidis*, we can see that antibacterial period on CDHAp/PLLA+cipro was shorter and reached 213.6 ± 13.62 hours (Fig. 2.5). Maximum antibacterial time shown by CDHAp/PLLA+cipro against *S. epidermidis* was 240 hours and minimum antibacterial time was 192 hours. CDHAp+cipro showed a longer antibacterial time against *S. epidermidis* than CDHAp+genta, but we did not observe statistically significant changes ($p>0.05$).

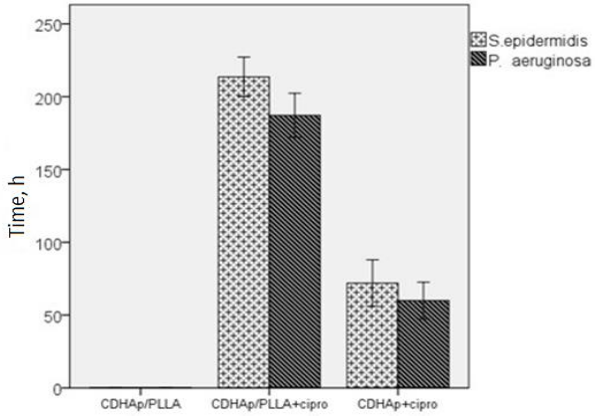


Fig. 2.5 **Average antibacterial length in composite materials with and PLLA**

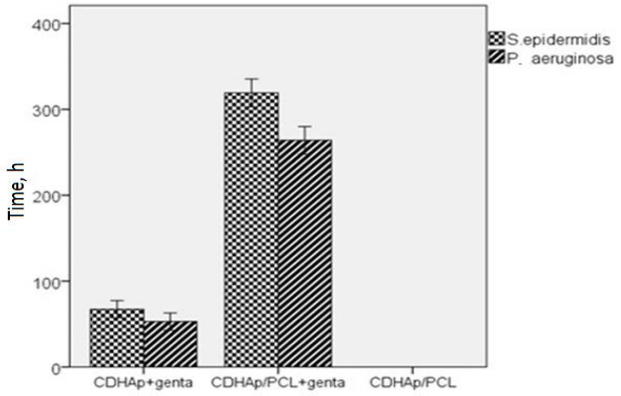
Inhibition ability of CDHAp/PLLA+cipro and CDHAp+cipro against *P. aeruginosa* remained in accordance with the biomaterial group, because in the case of CDHAp/PLLA+cipro we observed a prolonged ability to inhibit the growth of *P. aeruginosa*, and it lost this capacity gradually; in the case of CDHAp+cipro, we observed distinct antibacterial ability during the first few days of the study, which was followed by rapid antimicrobial ability decrease along with rapid distribution of antibiotic substances.

We observed statistically significant inhibited bacteria percentage changes (Mann-Whitney test, $p<0.05$) on CDHAp+cipro against *P. aeruginosa*

in the 48 to 72 hour period. Maximum antibacterial time against *P. aeruginosa* on CDHAp+cipro was 72 hours, and the minimum antibacterial time was 48 hours. Maximum antibacterial time against *P. aeruginosa* on CDHAp/PLLA+cipro was considerably longer than that on CDHAp+cipro, and reached 192 hours, but it was less effective against *S. epidermidis*. The Mann-Whitney test showed a statistically significant difference ($p<0.001$) between CDHAp/PLLA+cipro and CDHAp+cipro antibacterial times against *P. aeruginosa*. We can see that across a range of composite materials and antibacterial properties there is a statistically significant (Mann-Whitney test, $p<0.001$) difference between HAp/PLLA+cipro and CDHAp/PLLA+cipro against both bacterial cultures; at the same time, the same gentamicin composite material did not show a statistically significant (Mann-Whitney test, $p>0.05$) difference against any of the bacterial cultures.

2.4 CDHAp/PCL+genta and CDHAp/PCL+cipro antibacterial effectiveness *in vitro*

During the study we examined composite materials with other biodegradable polymers PCL, which were connected by antibiotic substances. The Mann-Whitney test showed that CDHAp/PCL+genta biomaterials have statistically significant ($p<0.001$) different antibacterial times against *S. epidermidis* than CDHAp/PLLA+genta. Antibacterial time against *S. epidermidis* on CDHAp/PCL+genta differs statistically significantly ($p<0.001$) compared to CDHAp+genta. Study of CDHAp/PCL+genta showed the longest antibacterial time (Fig. 2.6) against *S. epidermidis* reaching antibacterial length of 319.2 ± 16.19 hours.



Fid. 2.6 **Average antibacterial time on composite materials with gentamicin and PCL**

The long antibacterial time on CDHP/PCL+genta was related to the maximum length of time against antimicrobial *S. epidermidis* of some samples, which was 336 hours, and the minimum antibacterial time was 288 hours. Maximum antibacterial time on CDHAp/PCL+genta against *P. aeruginosa* reached 288 hours, and minimum antibacterial time was 240 hours.

A total growth inhibition of *P. aeruginosa* on CDHAp/PCL+genta was observed until the 168 hour mark. Continued study showed an overall trend of composite materials with antibiotic agents and biodegradable polymer coatings losing their antibacterial capabilities gradually.

CDHP/PCL+genta was capable of inhibiting *S. epidermidis* growth totally for a prolonged period (192 h – 216 h), and even later *S. epidermidis* growth was not fully suppressed, as anti-bacterial properties were lost gradually. Biomaterial samples without PCL lost their antibacterial abilities fast, and using the Mann-Whitney test *in vitro* study at 24 to 48 hours showed statistically significant differences in inhibition of the bacterial percentage index ($p<0.05$).

CDHAp/PCL+genta abilities against *P. aeruginosa* showed statistically significant ($p>0.05$) changes in the 24 to 48 hour period, as well as the 48 to 72

hour period, but CDHAp+genta showed statistically significant ($p<0.05$) changes in the 24 to 48 hour period, and the 48 to 72 hour period.

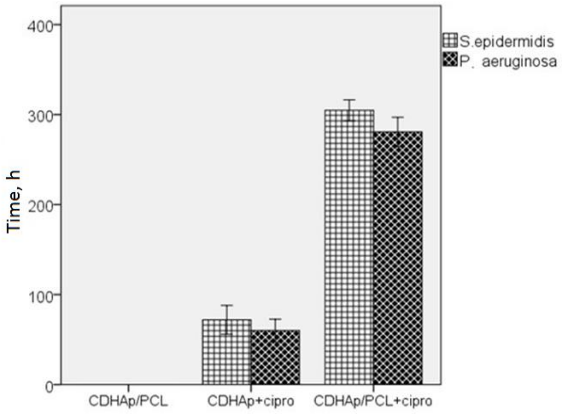


Fig. 2.7 **Average antibacterial time on composite materials with ciprofloxacin and PCL**

The longest antibacterial time against *P. aeruginosa* was demonstrated by CHAP/PCL+cipro, reaching 280.8 ± 16.19 hours. Maximum antibacterial time against *P. aeruginosa* on CDHAp/PCL+cipro was 312 hours, and the minimum antibacterial time against *P. aeruginosa* was 264 hours (Fig. 2.7). Mann-Whitney test showed that there is a statistically significant difference ($p<0.001$) between CDHAp/PCL+cipro and CDHAp+cipro against *P. aeruginosa*.

As the growth inhibition dynamic of *P. aeruginosa* showed, the antibiotic substances from CDHAp/PCL+cipro were released gradually and in sufficiently large quantities to inhibit completely all *P. aeruginosa* bacteria for 168 to 192 hours. After this period, the amount of released antibiotic substances was insufficient to suppress all *P. aeruginosa* bacteria, and after 336 hours it ended with a complete loss of antibiotic properties. Antibiotic property loss of CDHAp+cipro against *P. aeruginosa* from 24 to 48 hours was statistically

significant ($p<0.05$) according to the Mann-Whitney test. Such changes were not observed on CDHAp/PCL+cipro (statistically not different, $p>0.05$).

If we compare the ability of CDHP/PCL+cipro to inhibit the growth of *P. aeruginosa* with *S. epidermidis*, we can see that CDHAp/PCL+cipro inhibited a greater percentage of *S. epidermidis* for a longer period of time than *P. aeruginosa*. Total antibacterial time of CDHAp/PCL+cipro against *S. epidermidis* was 304.8 ± 11.59 hours, with a maximum antibacterial time of 312 hours, and minimum time of 288 hours. We observed statistically significant ($p<0.001$) differences in the antibacterial time between CDHAp/PCL+cipro and CDHAp+cipro against *S. epidermidis*.

2.5 IL-10 expression intensity *in vivo*

Regardless of the examined tissue and implanted biomaterial, there were no statistically significant changes ($p>0.05$, Mann-Whitney test) of IL-10 expression intensity (table 2.1) from tissue around composite materials HAp/PLLA+genta, HAp/PLLA+cipro, Hap+genta and HAp+cipro contaminated with *S. epidermidis* compared to the control group. By contrast, composite materials without antibiotics (ciprofloxacin or gentamicin) contaminated with *S. epidermidis* bacterial suspension showed statistically significant ($p<0.05$ Mann-Whitney test) IL-10 expression increased in all target tissues compared to the control group. Greater IL-10 intensity was observed in tissues that were in direct contact with the implanted HAp/PLLA composite material. A statistically significant ($p>0.05$, Mann-Whitney test) change was observed in IL-10 levels of intensity between tissues that had been in contact with biomaterials and tissues that had been in direct contact in the outer area. Distant tissues from the implanted HAp/PLLA demonstrated statistically significant ($p<0.05$) IL-10 expression reduction compared to the intensity of the IL-10 expression in direct contact tissues. We saw statistically significant

($p<0.05$) differences between IL-10 intensity in distant tissues and the control group.

Contamination of HAp/PLLA+genta, HAp/PLLA+cipro, HAp+genta and HAp+cipro with *P. aeruginosa* bacterial cultures did not show statistically significant differences ($p>0.05$) in IL-10 expression, regardless of the target tissue location from the biomaterial (table 2.2).

The Mann-Whitney test showed statistically significant ($p<0.05$) differences between the IL-10 levels in the control group and other examined tissues in the case of HAp/PLLA implants contaminated with *P. aeruginosa*. Compared to the control group, statistically significant ($p<0.05$) intensity of IL-10 was observed in direct contact tissues – both in direct contact tissues with HAp/PLLA and in the case of direct contact outer edge; however we did not find statistically significant ($p>0.05$) differences between both direct contact groups in the tissues. IL-10 intensity level decreased statistically significantly ($p<0.05$) in the examined distant tissues, but it still showed statistically significant ($p<0.05$) differences with the examined control group (healthy animals without biomaterial implantation).

Table 2.1

IL-10 levels after wound contamination with *S. epidermidis* (pg/ml)

Biomaterial sample	Direct contact inner area	Direct contact outer area	At a distance	Control group
HAp/PLLA+cipro	131 ± 4.04	131 ± 7.00	133 ± 5.29	133 ± 7.63
HAp+cipro	137 ± 2.00	135 ± 2.64	139 ± 1.00	133 ± 7.63
HAp/PLLA+genta	133 ± 5.69	134 ± 6.03	133 ± 7.64	133 ± 7.63
HAp+genta	132 ± 5.69	135 ± 6.56	133 ± 8.19	133 ± 7.63
HAp/PLLA	204 ± 4.04	194 ± 2.04	156 ± 3.00	133 ± 7.63

Table 2.2

IL-10 levels after wound contamination with *P. aeruginosa* (pg/ml)

Biomaterial sample	Direct contact inner area	Direct contact outer area	At a distance	Control group
HAp/PLLA+cipro	133 ± 5.51	132 ± 3.79	133 ± 6.66	133 ± 7.63
HAp+cipro	135 ± 6.03	131 ± 7.37	133 ± 6.11	133 ± 7.63
HAp/PLLA+genta	133 ± 3.46	131 ± 6.00	137 ± 4.58	133 ± 7.63
HAp+genta	135 ± 6.11	132 ± 4.04	132 ± 1.01	133 ± 7.63
HAp/PLLA	212 ± 4.58	206 ± 2.64	150 ± 5.56	133 ± 7.63

2.6 TNF-alpha expression intensity *in vivo*

TNF-alpha intensity levels in target tissues in the case of both *S. epidermidis* and *P. aeruginosa* contamination did not change and we observed a statistically significant ($p>0.05$) difference with TNF-alpha intensity level in the control group, when composite materials with antibiotics were implanted.

Intensified TNF-alpha expression was observed in direct contact with existing tissue with HAp/PLLA contaminated with *S. epidermidis* (table 2.3) or *P. aeruginosa* (table 2.4). TNF-alpha expression in both bacterial contamination cases was statistically significantly ($p<0.001$) more intense than in the control group. When HAp/PLLA is contaminated with *P. aeruginosa*, we see a more intense expression of TNF-alpha in the direct contact tissues. Based on TNF-alpha intensity in distant tissues of HAp/PLLA, we can see that the inflammatory area is reduced, because we can observe a statistically significant ($p<0.05$) difference in TNF-alpha intensity between direct contact tissue and distant tissues in the case of both bacterial cultures. It is possible that the inflammation area is larger, given that the TNF-alpha level of intensity in the

distant tissues was statistically significantly ($p<0.05$) higher than that in the control group of both bacterial cultures.

Table 2.3

TNF-alpha levels after wound contamination with *S. epidermidis* (pg/ml)

Biomaterial sample	Direct contact inner area	Direct contact outer area	At a distance	Control group
HAp/PLLA+cipro	177 ± 9.60	176 ± 10.5	171 ± 1.52	177 ± 7.63
HAp+cipro	174 ± 8.38	168 ± 3.51	166 ± 4.04	177 ± 7.63
HAp/PLLA+genta	181 ± 5.03	179 ± 2.08	176 ± 7.09	177 ± 7.63
HAp+genta	185 ± 2.08	182 ± 1.53	173 ± 6.11	177 ± 7.63
HAp/PLLA	271 ± 3.21	268 ± 3.78	213 ± 1.52	177 ± 7.63

Table 2.4

TNF- alpha levels after wound contamination with *P. aeruginosa* (pg/ml)

Biomaterial sample	Direct contact inner area	Direct contact outer area	At a distance	Control group
HAp/PLLA+cipro	178 ± 7.54	174 ± 7.21	173 ± 2.00	177 ± 7.63
HAp+cipro	180 ± 5.13	177 ± 7.63	173 ± 5.77	177 ± 7.63
HAp/PLLA+genta	170 ± 2.65	168 ± 2.52	174 ± 6.43	177 ± 7.63
HAp+genta	174 ± 11.14	170 ± 12.86	170 ± 8.66	177 ± 7.63
HAp/PLLA	271 ± 3.21	268 ± 3.78	213 ± 1.52	177 ± 7.63

2.7 Beta-defensin-2 expression intensity *in vivo*

More intensive beta-defensin-2 expression occurred in tissues that had the most direct contact with HAp/PLLA, contaminated with *S. epidermidis* (table 2.5) compared to the control group where we observed statistically significant changes ($p<0.05$) in beta-defensin-2 levels. We did not observe statistically significant changes ($p>0.05$) between direct contact outer and inner area tissues. Statistically significant ($p<0.05$) changes of beta-defensin-2 intensity level occurred between the direct contact area and distantly located tissues in the case of HAp/PLLA implantation, which shows that beta-defensin-2 intensity reduced. We did not observe beta-defensin-2 level changes in tissues after implantation of composite materials with antibiotics.

By contaminating HAp/PLLA+cipro, HAp+cipro, HAp/PLLA+genta and HAp+genta with *P. aeruginosa* (table 2.6) after 4 weeks implantation we observed beta-defensin-2 level changes compared to the control group. Changes in beta-defensin-2 intensity levels were observed in tissues following HAp/PLLA implantation and contamination with *P. aeruginosa*. Same as in the case with *S. epidermidis* contamination, in *P. aeruginosa* contamination too we observed the most intense beta-defensin-2 expression in direct contact tissues surrounding the HAp/PLLA. Beta-defensin-2 intensity level decreased statistically significantly ($p<0.05$) in the distant tissues compared to direct contact tissues, but there was not a statistically significant ($p>0.05$) difference between beta-defensin-2 level of intensity in distant tissues and the control group.

Table 2.5

Beta-defensin-2 levels after wound contamination with *S. epidermidis* (pg/ml)

Biomaterial sample	Direct contact inner area	Direct contact outer area	At a distance	Control group
HAp/PLLA+cipro	326 ± 4.04	316 ± 3.21	319 ± 6.55	324 ± 11.01
HAp+cipro	323 ± 6.24	319 ± 1.00	316 ± 4.00	324 ± 11.01
HAp/PLLA+genta	325 ± 2.52	317 ± 2.65	321 ± 3.21	324 ± 11.01
HAp+genta	319 ± 3.21	320 ± 2.00	317 ± 4.58	324 ± 11.01
HAp/PLLA	395 ± 4.35	394 ± 4.35	339 ± 3.50	324 ± 11.01

Table 2.6

Beta-defensin-2 levels after wound contamination with *P. aeruginosa* (pg/ml)

Biomaterial sample	Direct contact inner area	Direct contact outer area	At a distance	Control group
HAp/PLLA+cipro	324 ± 4.93	312 ± 1.00	319 ± 1.00	324 ± 11.01
HAp+cipro	323 ± 1.12	319 ± 6.65	315 ± 1.00	324 ± 11.01
HAp/PLLA+genta	323 ± 6.11	317 ± 2.65	317 ± 3.61	324 ± 11.01
HAp+genta	318 ± 1.00	319 ± 3.51	317 ± 1.00	324 ± 11.01
HAp/PLLA	395 ± 4.35	394 ± 4.35	339 ± 3.50	324 ± 11.01

3. DISCUSSION

3.1 Efficiency of biomaterials impregnated with antibiotic substances *in vitro*

Almost all fields of medicine use biomedical implants to provide patients with diagnostic or therapeutic purpose manipulations – intravenous catheters, intubation equipment, bladder catheters, joint replacements and other biomedical equipment. However, the use of these biomaterial implants has also the ability to develop various infections (*Huebsch et al*., 2009).

An additional infection to the existing illness increases the patient's hospitalisation time, the cost of treatment and risk of death. The risk of developing biomaterial associated infections (BAI) is not the same for all patients, as it is determined by several factors related to the same patient, e.g., implant type and extent of surgery. Greater BAI risk exists in the elderly, immunosuppressed patients, cancer patients, and patients with skin lesions, since their immune system's ability against BAI agents is reduced (*Laupland et al*., 2006; *Goldmann et al*., 1993, *Greco et al*., 2015).

BAI risk is determined by the scope of the operation as well as the sterility of the surgical site of the human body part. During orthopaedic operations, wounds are large, which can serve as bacterial entrance gate, followed by the development of the BAI. The source of these bacterial infections are air microflora of the operating theatre, the patient's skin and mucous membrane microflora and staff microflora (*Vinh et al*., 2005).

Dental implants and oral cavity operations take place in the human oral cavity, where we find one of the largest amounts of the normal human microflora. Although these bacteria belong to the normal microflora of humans, at the same time they are opportunistic bacteria. This manipulation enables bacteria to initiate BAI (*Heydenrijk et al*., 2002).

The development risk of individual BAI is not great, but as these infections develop and taking into account the patient's medical condition, a great risk of patient mortality exists due to these infections. Urinary tract infections following bladder catheterization develop in approximately 30% of cases, although patient lethality due to these infections is assessed as very low. By contrast, artificial heart valve infections develop in 1-3 per cent of cases, but patients' mortality risk of this type of infection is valued as high (*Darouiche et al*., 2001, *Wright et al*., 2013)

Antibiotic therapy is prescribed in order to reduce BAI development opportunities in hospitals before and after implantation surgery, which aims to reduce the potential for opportunistic bacteria and the possibility to initiate infections, and reduce biomaterial contamination risks during surgery or during the post-operative period (*von Eiff et al*., 2005).

Systemic antibiotic use has a number of disadvantages compared to use of topical antibiotics. One of the most frequent problems is the development of dysbacteriosis, when the normal microflora of the gastro-intestinal tract, the mouth or other body parts is destroyed. Dysbacteriosis can serve as a factor contributing to other bacteria caused infections, e.g., *Cl.difficile* initiated pseudomembranous colitis (*Mylonakis et al*., 2001, *Aldrete Sdel et al*., 2015).

By using antibiotics systemically, they are released at low concentrations throughout the human body. Small concentrations of antibiotic substances are the cause for the normal microflora of bacteria and infectious agents of bacteria to develop resistance towards antibiotics. Hepatotoxicity, nephrotoxicity, ototoxicity and other complications are the result of systemic antibiotic use. Limited use of systemic antibiotic substances usually takes place in cases where patients experience nausea, vomiting, allergic reactions, rash etc. due to use of antibiotic substances (*Cunha et al*., 2001, *Soothill et al*., 2015).

There are advantages to topical antibiotic substance use, in particular in the case of biomaterial implant operations where antibiotics are isolated from a biomaterial because they are impregnated with antibiotic substances (*Gottenbos et al*., 2002, *Pritchard et al*., 2013).

In this study, the main task was to investigate the antibacterial properties of a hydroxyapatite saturated with antibiotic substances, and biodegradable polymers *in vitro* and their biocompatibility in an *in vivo* study after biomaterial implantation and contamination with bacterial cultures, identifying inflammatory cytokines IL-10, TNF-α and antimicrobial peptides – β-defensin-2 intensity in the surrounding tissues around the implanted biomaterials. These biomaterials have the potential to be used in bone regeneration in patients with BAI risk.

The diversity of hospital acquired infection varies both between Gram-negative bacteria (*E. coli*, *P. aeruginosa*, *K. pneumoniae*) and the Gram-positive bacteria (*S. aureus*, *S. epidermidis*), and the majority among them are to be found not only in the hospital environment, but also on the normal human flora. In rare cases, studies also test the antifungal properties of biomaterials, with *C. albicans* or any other representative of the genus *Candida* selected for this purpose. These agents have the ability to cause BAI. The great diversity of agents is reflected in research, because several bacterial cultures are used in one study in order to test the antibacterial activity spectrum of biomaterials impregnated with antibiotics. One bacterial culture is chosen, if the bacterium is the most common specific disease agent, and then its potential antibacterial activity against this particular agent is studied in prevention, e.g., the case of *S. aureus*, or *MRSA* osteomyelitis. The use of Gram-positive and Gram-negative bacteria in a single study is based on the fact that these bacteria reflect the normal microflora of a human, which causes BAI (*Peel et al*., 2012; *Vinh et al*., 2005; *von Eiff et al*., 2005)

In order to assess the antibacterial properties of these original synthetic biomaterials produced in Latvia, we used *S. epidermidis* and *P. aeruginosa* bacterial cultures. This bacterial culture selection was based on the fact that, *S. epidermidis* is one of the most common BAI, nosocomial and opportunistic infection agents. The notable resistance of *P. aeruginosa* against antibiotic agents was the reason we chose it in the study as BAI and nosocomial infections agent.

Both of these bacterial cultures are united by their ability to attach to the surface of artificial biomaterials, followed by biofilm formation process. Considering the complex and sometimes unsuccessful treatment of biofilms on biomaterial surfaces, we chose these bacterial cultures for the study in order to protect them with the help of antibiotics from the irreversible bacterial adhesion and colonization.

We can observe the diversity of antibiotic substances in the distribution systems of local antibiotics in biomaterials. Diverse selection is based on the use of antibiotic substances in clinics, their mechanism of action and efficacy against agents and bacterial resistance factors. Ciprofloxacin is used in research, because it penetrates the bone well (*Ahola et al*., 2013). In some studies authors prefer the use of vancomycin, because it shows excellent antibacterial properties against Gram-negative and Gram-positive bacteria as well as resistant bacteria (*Liana et al*., 2013). Studies of local and controlled antibiotic substance release use gentamicin (*Loca et al*., 2011), ceftriaxone (*Kundu et al*., 2010), cefalexin (*Li et al*., 2011), amoxicillin (*Xu et al*., 2008), doxycycline (*Feng et al*., 2010) and others.

In the study we impregnated hydroxyapatite with ciprofloxacin or gentamicin as *S .epidermidis* and *P. aeruginosa* have varying cell wall structures, so the mechanism of antibiotic substances should focus on other bacterial structures. According to their mechanism of action, ciprofloxacin and

gentamicin are different antibiotic substances, but their spectrum includes both *S. epidermidis* and *P. aeruginosa*.

When we analysed the literature, we did not find an identical study of this type of antibacterial properties in composite biomaterials. There are studies that reflect the release of antibiotic substances or antibacterial properties in similar biomaterials, but different antibiotic substances, bacteria or polymer choices. However, what unites this study to a number of other studies is the comparison of antibacterial properties among various biomaterials with biodegradable polymers and without polymers. There are some studies on IL-10, TNF-α and β-defensin-2 expression in the surrounding tissue after biomaterial implantation.

In this study we established that HAp/PLLA+cipro antibacterial properties against *S. epidermidis* remain for 278.4 ± 12.39 hours, but in CDHAp/PLLA+cipro for 213.6 ± 13.26 hours, and in CDHAp/PCL+cipro for 304.8 ± 11.59 hours. The antibacterial length against *S. epidermidis* in biomaterials without polymers, but with antibiotic agents is significantly shorter than in polymers, and respectively stands at 91.2 ± 10.11 hours in HAp+cipro and 72 ± 16 hours in CDHAp+cipro.

Hydroxyapatites connected to a cyclodextrin polymer and antibacterial properties of ciprofloxacin against *S. aureus* were observed up to 240 hours, whereas antibacterial time against *S. aureus* in polymer samples was only 144 hours. The choice for *S. aureus* in this study was due to the fact that the bacterium may be resistant to antibiotics, but its cell wall structure resembles many Gram-positive BAI agents (*Leprêtre et al*., 2009). In another study, we observed *S. aureus* in a sterile area around hydroxyapatite with PLLA and vancomycin for 432 h (*Lian et al*., 2013).

In our study we used HAp/PLLA+cipro antibacterial properties against *P. aeruginosa* and found that it lasts for 249.6 ± 13.39 hours, on CDHAp/PLLA+cipro for 197 ± 15.17 hours and on CDHAp/PCL+cipro for

280.8 ± 16.19 hours. Just as in the case of *S. epidermidis*, biomaterials without polymers against *P. aeruginosa* show a shorter antibacterial time period. Antibacterial time on HAp+cipro against *P. aeruginosa* was 69.6 ± 7.58 hours and on CDHAp+cipro it was 60 ± 12.64 hours.

The antibiotic activity of composite material PCL/β-TCP+cipro against *P. aeruginosa* was present immediately after two-hour incubation. Other authors also stress that antibiotic substances release quickly, 2 hours after the start of incubation, although in this case it was used for other antibiotics – tetracycline, which was recovered from the HAp/PCL composite. The total distribution time of antibiotic substance of the composite material is affected by the amount of antibiotic substances used in composite materials (*Kim et al*., 2004, *Ahola et al*., 2013). Quick antibiotic substance release from biomaterials is an important prevention against biofilm formation on its surface. Bacterial adhesion to biomaterials takes place within the first few hours of the biofilm formation process, followed by proliferation of bacteria and biofilm maturation. If antibiotics are released quickly from the biomaterial, bacterial adhesion is inhibited by killing bacteria. Bacterial inability to adhere onto biomaterials precludes further biofilm formation stages.

Vancomycin is used in addition to ciprofloxacin in orthopaedic infections. This antibiotic substance is used in treatment where the agent is multiresistant against antibiotics. This justifies the use of vancomycin in biomaterials with cyclodextrin polymer for a long-term antibiotic substance release. The composite antimicrobial properties against *S. aureus* lasts for about 144 hours, the antimicrobial period of samples without cyclodextrin polymer is 96h (*Leprêtre et al*., 2009).

Other authors too have shown in their studies the impact of biodegradable polymers on antibiotic substance release speed and duration from the biomaterial. For example, hydroxyapatite with PCL and vancomycin

compared to hydroxyapatite vancomycin without PCL, emit vancomycin longer and more gradually (*Kim et al*., 2005).

PLLA/β-TCP with vancomycin are recommended against MRSA because they show better antibacterial properties *in vitro* against MRSA compared to other biomaterial samples (*Kankilic et al*., 2011) and the good bone regeneration capacity after MRSA contamination *in vivo* (*Kankilic et al*., 2014).

Bone cement biomaterials of modified calcium phosphate with PLLA and vancomycin are able to release antibiotic substances up to 43 days (*Loca et al*., 2015).

In determining the impact of hydroxyapatite porosity level on antimicrobial time in this study, we found that a lower porosity composite material, e.g., the antibacterial time of ↓HAp/PLLA+cipro against *S. epidermidis* was 151.2 ± 16.19 hours and against *P. aeruginosa* for 117.6 ± 13.62 hours. In lower porosity composite materials without a biodegradable polymer – the antibacterial time of ↓+HAp+cipro against *S. epidermidis* and *P. aeruginosa* was only 52.8 ± 10.11 hours.

Other authors' data support effect of composite material porosity on antimicrobial time. Irrespectively of antibiotics used in the study (ciprofloxacin, gentamicin, vancomycin), microporous hydroxyapatites were impregnated with more antibiotic substances than denser hydroxyapatites. Antibacterial time against *E. coli*, *S. aureus* and *P. aeruginosa* bacterial cultures in all three cases was longer when microporous hydroxyapatites were used rather than denser ones. Saturated antibiotic concentration depends on the pore size and the percentage grade. By increasing porosity, the potential of antibiotic substance saturation also increases, but by reducing porosity, the potential of antibiotic substance saturation decreases (*Chai et al*., 2007; *Schnieder et al*., 2011). It is important to find the optimal balance between porosity and antibiotic substance quantities so that the biomaterial would ensure sufficiently long-term anti-

bacterial properties, but at the same time, by increasing porosity level hydroxyapatite would reduce the mechanical resistance capabilities.

In our study, the antibacterial time of composite materials with gentamicin showed the same trend as composite materials impregnated with ciprofloxacin. Regardless of the antibiotic substance used, polymer composites had longer antibacterial properties than without polymer. Antibacterial time of HAp/PLLA+genta against *S. epidermidis* was 249.6 ± 16.78 hours; but against *P. aeruginosa* it was 220.8 ± 10.11 hours. Conversely, antibacterial time of CDHAp/PCL+genta against *S. epidermidis* was 319.2 ± 16.19 hours and against *P. aeruginosa* it was 264 ± 16 hours. Antibacterial time of CDHAp/PLLA+genta against *S. epidermidis* was 249.6 ± 16.78 hours and against *P. aeruginosa* it was 218.4 ± 17.7 hours. Composite materials with no polymer – antibacterial time of HAp+genta, CDHAp+genta against *S. epidermidis* was reduced to 88.8 ± 11.59 hours and 67.2 ± 10.11, and against *P. aeruginosa* to 67.2 ± 10.11 hours and 52.8 ± 10.11 hours respectively.

Studies on gentamicin as an antibiotic substance against *S. aureus* initiated illnesses show that hydroxyapatite pellets with gentamicin and keratin show a stabilizing and slower gentamicin release, and its antibacterial properties are observed for 121 days against *S. aureus* (*Belcarz et al*., 2009).

Throughout the study, all PCL composites, regardless of the antibiotic substances or the bacterial cultures, show longer antibacterial properties than composite materials with PLLA. This is explained by the varying biodegradation speeds of PLLA and PCL as PCL degrades more slowly than PLLA (*Tokiwa et al*., 2009). Perhaps a greater concentration of antibiotic substances was released from the composite materials with PLLA during the first days of the study when bacterial growth was inhibited completely. Released amount of antibiotic substances from PCL composite material was not as large as in PLLA composite materials, but sufficient to completely suppress the growth of bacteria in the first days of the study.

Without the PLA and PCL, other authors in their studies have used other biodegradable polymers in order to achieve a local and steady release of antibiotic substances. The great diversity of polymers and their diverse properties, allows authors to choose the most suitable polymer according to the application or function of the biomaterial. For example, PLGA is a biodegradable polymer used in composite materials to release antibiotics – gentamicin (*Schnieder et al*., 2006), since PLGA with gentamicin shows longer antimicrobial properties against *S. aureus* for up to 432 h (*McLaren et al*. 2014).

Thanks to the effectiveness of ciprofloxacin against Gram-positive and Gram-negative bacteria, it is used in many studies to provide biomaterials with antibacterial properties, and reduce the risk of BAI.

In vitro studies have shown that ciprofloxacin is able to function well when it is adsorbed on biomaterials. Studies have found that ciprofloxacin coated PLLA nanofibers, show antimicrobial activity against *S. aureus* after 24 hour incubation at 37°C. Survey data show that in order to achieve similar efficacy against *S. aureus* pure ciprofloxacin concentration should be 8 times higher than when it is adsorbed on PLLA nanofibers. Better antibacterial properties were achieved with ciprofloxacin coated PLLA nanofibers against *E. coli* (*Parwe et al*., 2014).

Cochlear implant with ciprofloxacin retains its antimicrobial properties for 5 weeks against *S. pneumoniae in vitro* conditions at 23°C and 37°C. On the other hand, *in vivo* studies showed that cochlear implant with ciprofloxacin protects rats from *S. pneumoniae* meningitis, which may be obtained in a haematogenous way. By contaminating these implants with bacteria directly in the *otitis media*, it did not protect from the development of meningitis, but it was able to prolong the development period of meningitis (*Wei et al*., 2006).

Polymerised nano-spheric contact lenses with ciprofloxacin are able to inhibit the growth of *S. aureus* and *P. aeruginosa* up to 96 hours (*Garhwal et al*., 2012).

The use of cyclodextrins prolongs antibacterial time of ciprofloxacin against *S. aureus*, *S. epidermidis* and *E. coli* (*Laurent et al*., 2011).

Excellent antibacterial properties of gentamicin against Gram-negative bacteria is the reason why this antibiotic substance is widely used in biomaterials.

By using gentamicin in the creation of antibacterial biomaterials, it is important to determine its thermostability. Before bone reconstruction, the biomaterial coated with gentamicin and used in bone reconstruction can be stored frozen. According to research data, gentamicin release from prostheses was relatively similar in temperatures of –20°C and –80°C (*Corac-Huber et al*., 2013). By exposing gentamicin solution to 50°C for 30 minutes, 77.91% of gentamicin was degraded (*Naveed et al*., 2014).

Gentamicin is widely used in the creation of antimicrobial biomaterials, because it shows a broad spectrum of activity and high thermostability. Synthetic hernia reticule coated with gentamicin *in vitro* studies show high antimicrobial properties against *S. aureus*, thus protecting patients from potential postoperative infections (*Wiegering et al*., 2014). Studies have also shown that after vascular implant insertion, about 20% of patients develop an operation related infection, but a collagen vascular implant coated with gentamicin, ensures a complete antibacterial activity after the surgery. Patients who used the implant with gentamicin observed shorter hospitalisation time (*Costa Almeida et al*., 2014).

Gentamicin presented a pronounced antibacterial effectiveness in the development of orthopaedic biomaterials. Bone cement covered in gentamicin has distinctly prolonged antimicrobial properties against *S. aureus*, MRSA, coagulase-negative staphylococci, *E. coli*, *P. aeruginosa* and *Klebsiella spp*.,

than cement, coated with other antibiotics such as vancomycin, piperacillin, or imipenem (*Chang et al*., 2013). Treatment of heel bone chronic osteomyelitis with gentamicin impregnated calcium phosphate cement is successful in diabetic patients (*Iwakura et al*. 2014). Patients who used gentamicin polymethylmethacrylate in bone cement regeneration showed release of gentamicin in patients' urine on average of 43 to 71 days (Webb et al., 2013).

3.2 Inflammatory cytokine expression *in vivo*

The *in vivo* part of the study tried to determine inflammatory cytokine (TNF-α and IL-10) and antimicrobial peptide (β-defensin-2) expression in the surrounding tissue after 4 weeks of HAp/PLLA+cipro, HAp/PLLA/+genta, HAp+cipro, HAp+genta and HAp/PLLA subcutaneous implantation and implant contamination with *S. epidermidis* or *P. aeruginosa*.

Inflammatory cytokine production is the body's response to an implanted biomaterial or biomaterial bacterial contamination. Altered inflammatory cytokine findings can serve as diagnostic criteria in biomaterial infections (*Franz et al*., 2011).

In the case of HAp/PLLA+cipro, HAp/PLLA/+genta, HAp+cipro and HAp+genta implantation with subsequent bacterial contamination we observed TNF-α, IL-10 and β-defensin-2 expression changes in the surrounding tissues around biomaterials. Irrespectively of what bacterial culture was used. Despite the presence or absence of PLLA, we did not find any changes in the control group. Normal amount of inflammatory cytokines and antimicrobial peptides point to the lack of development in the inflammatory process in the composite material implantation and contamination associated with antibiotic substance release from the biomaterial and bacteria destruction. The amount of bacteria used *in vivo* study was small enough for composite materials with and without polymers to be able to destroy the bacteria completely, as was demonstrated by the *in vitro* study.

This differs from the case of HAp/PLLA implantation with bacterial contamination. After 4 weeks of implantation, we observed an increase in surrounding tissues of TNF-α, IL-10 and β-defensin-2 expression, which clearly point to the inflammatory process. Given that this composite material was not impregnated with the antibiotics, the quantity of used bacteria was sufficient for the development of the inflammatory process.

According to data, in the case of implant infections in tissues around the implant we observed an increase in IL-10 and TNF-α levels compared to implants without infections (*Duarte et al*., 2009). These results are corroborated by other authors' studies on tissues around dental implants, comparing them to healthy tissues around dental implants (*Ata-Ali et al*., 2015).

Staphylocuccus spp. initiated joint implant infections in the joint capsule fluid reveal elevated TNF-α and β-defensin-2 levels. This study revealed an increased presence of other antimicrobial peptide, i.e., β-defensin-3 in the joint fluid (*Gollwitzer et al*., 2013).

We observed an increased TNF-α, β-defensin-2 and IL-10 production in the tissues around biomaterials after subcutaneous implantation of composite materials and contamination with *P. aeruginosa* or *S. epidermidis* (*Rhine et al*., 2011). Biomaterials that were used in the study were free of antibiotics, and less contaminated with smaller amount of bacteria than in our study, but even this amount of bacterial concentration was sufficient to stimulate the inflammatory cytokine and antimicrobial peptide production in tissues around the biomaterial.

We observed unchanged TNF-α levels compared to the control group where the local vascular implant with vancomycin was contaminated with MRSA. The same implant without vancomycin, but contaminated with bacteria culture causes an increased TNF-α production (*Gul et al*., 2011).

4. CONCLUSIONS

1. Biomaterials with biodegradable polymers and antibiotics have longer antibacterial properties than biomaterials with antibiotics and without biodegradable polymers against *P. aeruginosa* and *S. epidermidis*.
2. The antibacterial time of biomaterials with antibiotic agents and polymers, as well as biomaterials with antibiotics but without the polymer, is longer against *S. epidermidis* than the same biomaterials against *P. aeruginosa*.
3. Antibacterial properties of biomaterials with PCL polymers and antibiotic substances last longer than the same kind of biomaterials with PLLA, regardless of the antibiotic substance or bacterial culture.
4. Antibacterial time in biomaterials with lower porosity levels and antibiotic substances and polymers against *S. epidermidis* and *P. aeruginosa* is shorter than that in biomaterials with a higher porosity levels and antibiotic agents and polymers.
5. Inflammatory cytokines and antimicrobial peptide expression in tissues around the biomaterial does not change after implantation of the biomaterial and contamination with *S. epidermidis* and *P. aeruginosa*, regardless of antibiotic substances or bacterial cultures.
6. We observed an increased intensity of inflammatory cytokines and antimicrobial peptides in tissues around biomaterials, after implantation of biomaterials without antibiotic substances and contamination with *S. epidermidis* or *P. aeruginosa*.

5. PUBLICATION LIST RELATED TO THE STUDY

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