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INFLUENCE OF ANTIBIOTIC-IMPREGNATED BIOMATERIALS ON INFLAMMATORY CYTOKINES

Ingus Skadiņš[1#], Juta Kroiča[1], Ilze Salma[2], Aigars Reinis[1], Marina Sokolova[3], and Dagnija Rostoka[1]

[1] Department of Biology and Microbiology, Rīga Stradiņš University, 16 Dzirciema Str., Rīga, LV-1007, LATVIA

[2] Department of Oral and Maxillofacial Surgery, Rīga Stradiņš University, 20 Dzirciema Str., Rīga, LV-1007, LATVIA

[3] Faculty of Material Science and Applied Chemistry, Rīga Technical University, 3 Paula Valdena Str., Rīga, LV-1048, LATVIA

[#] Corresponding author, ingus.skadins@rsu.lv

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Local antibiotic therapy has several advantages over systemic antibiotic treatment. Using antibiotics in local biomaterial systems can reduce the number of microorganisms that can adhere to implanted biomaterials. In this in vitro *study, antibacterial properties of hydroxyapatite biomaterials impregnated with antibiotics and biodegradable polymers were examined. The antibacterial efficiency of hydroxyapatite biomaterials impregnated with antibiotics and biodegradable polymers against* Staphylococcus epidermidis *and* Pseudomonas aeruginosa *was studied by evaluating the expression of inflammatory cytokines (Interleukin-10 (IL-10),* β*-defensin-2 and tumour necrosis factor alpha (TNF-*α*)) in tissue surrounding implanted biomaterials* in vivo*. The results of this study demonstrated that hydroxyapatite biomaterials impregnated with antibiotics and biodegradable polymers had a prolonged antibacterial effect in comparison to biomaterials without biodegradable polymers. Surrounding tissue displayed higher levels of inflammatory cytokines when implanted biomaterials had not been previously impregnated with antibiotics.*

Key words: *impregnated biomaterials,* S. epidermidis*,* P. aeruginosa*, interleukin-10,* β*-defensin-2, TNF-*α*.*

INTRODUCTION

Implants can be the culprit of biomaterial-associated infections depending on the location and implantation of the biomaterial in the body. These infections are known to be difficult to treat and implants are subsequently removed in order to avoid life-threatening complications. Biomaterial-associated infections are often linked to additional surgeries, which may lead to increased patient discomfort and expenses (Gristina, 1987).

There are several ways how bacteria can infect biomaterials. Biomaterials can be infected by patients' microbiota of the skin and mucous membranes or that of the surgeon, as well as bacteria present in the air microbiota of operating theatres, where levels of bacteria are up to 20–60 CFU·m^{-3}. Another possible route of infection is via the bloodstream from sites of infection in other parts of the body following surgery (Hughes and Anderson, 1999; McCann *et al*., 2008; Hodgson *et al*., 2014).

Due to the fact that implantation of biomaterials inherently entails trauma to tissue, bacterial contamination of the implant can cause significant production of inflammatory cytokines, or the opposite, its suppression. Inflammatory cytokines (TNF-α, IL-6 and procalcitonin) are promising laboratory markers for evaluating the intensity of inflammation (Bottner *et al*., 2007).

Some authors recommend the use of inflammatory cytokines TNF-α, IL-1, IL-8, to assess the dynamics of the development of inflammation following implantation. If the level of inflammatory cytokines does not decrease within seven days following surgery, this could indicate infection of the implant (Shindo *et al*., 2003). *Staphylococcus epidermidis* (which is part of the skin microbiota) can cause

biomaterial-associated infections. An important characteristic of *S. epidermidis* is its ability to induce beta-defensin production. Increased β-defensin-2 levels generally show activity against Gram-negative bacteria, and β-defensin-3 shows activity against both Gram-positive (*Staphylococcus aureus*) and against Gram-negative bacteria (*Pseudomonas aeruginosa*) (Sawamura *et al*., 2005).

IL-10 is an anti-inflammatory cytokine, which reduces the expression of other cytokines, such as TNF-alpha, IL-6 and IL-1. IL-10 promotes the secretion of other anti-inflammatory cytokines, and reduces the quantity of inflammatory cytokine receptors (Zhang and Jianxiong, 2007). IL-10 inhibits activation of T cells, monocytes and macrophages. Its main function is the reduction and termination of the inflammatory reaction (Moore *et al*., 2001). IL-10 is secreted by Th_2 cells and T regulatory cells, which activate B cell growth and inhibit Th_1 responses (Rousset *et al*., 1992).

The principal aim of this study was to compare the antibacterial efficiency of hydroxyapatite (HAp) saturated with ciprofloxacin (cipro) and polyL-lactic acid (PLLA) polymer with a controlled release of antibiotics with HAp saturated with cipro without PLLA. Another aim was to evaluate the expression of inflammatory cytokines (IL-10, β-defensin-2 and TNF-alpha) in tissue surrounding implanted biomaterials *in vivo*.

MATERIALS AND METHODS

Procedure of HAp/PLLA+cipro sample synthesis. HAp powder was prepared using the wet chemical precipitation synthesis method from calcium oxide (CaO, Fluka, ≥ 97%) and orthophosphoric acid solution (Sigma-Aldrich, ≥ 85%) (Sokolova *et al*., 2014). The as-synthesised powders obtained were uniaxial, pressed into pellets (d = 10, h = 3 mm). All samples were sintered at 1100 °C for one hour.

In order to load drugs in the HAp scaffolds, cipro was dissolved in deionised water at a concentration of 10 $mg \cdot ml^{-1}$. The scaffolds were impregnated with 2 ml aqueous drug solution at room temperature, followed by drying at 37 °C. Some of the prepared samples were used for coating with PLLA.

Solution of 10 wt% PLLA (Nature Works LLC, Mw = 110 kDa) in dichloromethane (DCM) (Sigma-Aldrich, UK) was prepared to coat the HAp scaffolds with polymer. PLLA was dissolved in DCM by stirring it for 2 hours at room temperature. Polymer solution was infiltrated in HAp bioceramic scaffolds using the vacuum impregnation technique at 500 mbar pressure for 15 minutes. Coated scaffolds were dried at room temperature for 24 hours.

The open (P_0) porosity and total (P_t) porosity of scaffolds were determined by the Archimedes method based on the principle that buoyant force is equal to the weight of the fluid displaced (Locs *et al*., 2013). Observations of the cross-section microstructure of coated scaffolds by SEM revealed that HAp/PLLA scaffolds coated with 10 wt% of PLLA exhibited a porous microstructure with pore size estimated to be in the range 200 nm to 500 nm (Fig. 1).

Antibacterial efficiency of HAp/PLLA+Cipro samples by disk diffusion method. Antibacterial efficiency was tested via the disk diffusion test (also called Kirby–Bauer method), which is the standard laboratory method for testing antibacterial susceptibility.

HAp/PLLA+cipro, HAp+cipro and HAp/PLLA disks were first eluted in rabbit blood plasma at 37 °C with 5% CO_2 atmosphere and 100% relative humidity for two hours. Then bacterial suspension with optical density 0.5 was prepared according to the McFarland standard. Trypticase soy agar (TSA) (Oxoid, UK) plates were inoculated with 100 µl of the bacterial suspension. The agar plates were inoculated using the streak plate method with a swab containing the inoculum. The plates were rotated and the inoculation procedure was repeated until the distribution of the inoculum was even. Biomaterial discs were placed on the plates and incubated for 24 hours at 37 °C. Antibacterial properties were detected by measuring a sterile zone (diameter) around the biomaterial discs. Biomaterial discs were transferred on fresh TSA plates with bacterial inoculum, and were incubated for an additional 24 hours. Repeated measurements

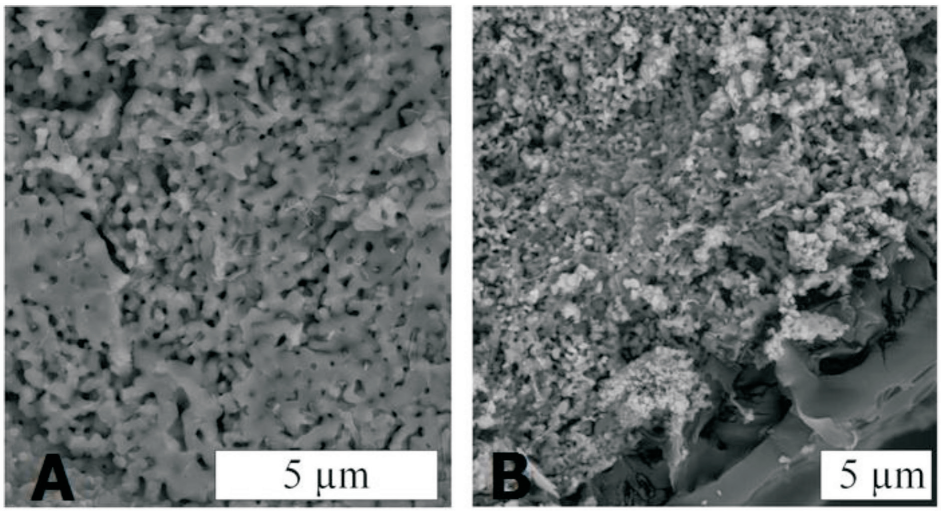

Fig. 1. SEM micrographs of HAp/PLLA coated scaffolds cross-section. A, pore diameter in the range 200 nm to 500 nm; B, PLLA coating of 2–5 µm in thickness.

and fresh TSA late inocula were made every 24 hours until no antibacterial properties were detected on any of the biomaterial samples for two consecutive days. Antibacterial efficiency was also tested with this method for HAp+cipro and HAp/PLLA samples.

Animal model. Chinchilla rabbits (n = 9) were used in this study for the determination of levels of IL-10, beta-defensin-2 and TNF-alpha in tissues around implanted biomaterial samples. All the male rabbits weighed 3 kg and were 3 months old. The *in vivo* study was approved by the Food and Veterinary Service of Latvia and the Ministry of Agriculture of the Republic of Latvia.

Prior to the surgery, we prepared bacterial suspensions of *P. aeruginosa* and *S. epidermidis* with an optical density of 0.5 according to the McFarland standard. The fur on the back under the scapulae was shaved and the area was sterilised with iodine solution. A 2.5 cm incision of the skin with a sterile scalpel and a sufficiently large subcutaneous pocket (where biomaterial disc could be implanted) was made under local anaesthesia with 2% lidocaine solution. After biomaterial implantation in the subcutaneous pocket, the wound was infected with 0.1 ml of the bacterial suspension; the wound was closed with a separate suture. HAp/PLLA+cipro, HAp+cipro, HAp/PLLA were contaminated with *S. epidermidis* or *P. aeruginosa*. Four weeks later, using standard ELISA kits (USCN life science and MyBioSource, USA) according to the manufacturer's instructions, the levels of IL-10, TNF-alpha and beta-defensin-2 were determined in the surrounding tissue (internal and external zone) around the biomaterial and in tissue within a distance of 1.5 cm from the biomaterial. The tissue samples were homogenised with PBS (pH 7.2–7.5) and centrifuged for 5 minutes at 5000× g. The supernatant was removed immediately.

Bacterial cultures. Antibacterial efficiency of all biomaterials was tested using *S. epidermidis* (ATCC 12228) and *P. aeruginosa* (ATCC 27853) bacteria reference cultures, which were acquired from the American type culture collection.

Statistical analysis. The results were analysed by non-parametric statistics. The Mann–Whitney test was used to test for statistically significant differences between duration of antibacterial effect of different biomaterial samples. Statistically significance was assumed if the p value was less than or equal to 0.05. Statistical analysis was performed with SPSS 22.0.

RESULTS

Antibacterial efficiency *in vitro*. The antibacterial efficiency test showed that the period of antibacterial effect of HAp/PLLA+cipro against *S. epidermidis* was significantly longer ($p < 0.001$) compared to that of HAp+cipro. The period of the antibacterial effect against *P. aeruginosa* for HAp/PLLA+cipro compared to HAp+cipro was significantly longer ($p < 0.001$). The mean antibacterial time of HAp/PLLA+cipro samples against *S. epidermidis* was 278.4 hours, whereas for HAp+Cipro it was 91.2 hours (Fig. 2). Antibacterial time against *P. aeruginosa* for HAp/PLLA+cipro and HAp+cipro was shorter compared to the antibacterial time against *S. epidermidis*, respectively 249.6 hours for HAp/PLLA+cipro and 69.6 hours for HAp+cipro.

Within the first three days of the study, HAp+cipro compared to HAp/PLLA+cipro showed a larger sterile zone against *S. epidermidis* (Fig. 3), which means that HAp+cipro released more antibiotics than HAp/PLLA+cipro. Within the first two days of the study, HAp+cipro showed an even larger sterile zone against *P.aeruginosa* (Fig. 4). HAp/PLLA+cipro gradually released antibiotics against both bacterial cultures, and gradually lost antibacterial properties against *S. epidermidis* and *P. aeruginosa*.

No antibacterial properties were observed for HAp/PLLA samples against both bacterial cultures.

Expression intensity of IL-10 in the animal model. The highest expression of IL-10 was observed in tissues around HAp/PLLA. There were no statistically significant ($p > 0.05$) differences of intensity of IL-10 in direct contact tissues (internal and external zone) and distance zone tissues compared to the control group, when HAp/PLLA+cipro or HAp+cipro were implanted and contaminated with *S. epidermidis* (Fig. 5) or *P. aeruginosa* (Fig. 6).

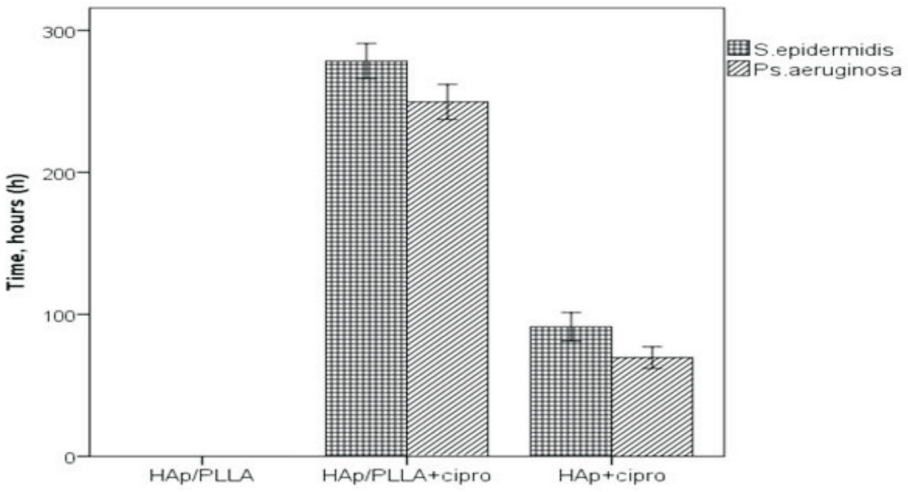

Fig. 2. Antibacterial efficiency testing *in vitro* of Hap/PLLA+cipro (n = 5), Hap+cipro (n = 5) and Hap/PLLA (n = 5) against *S. epidermidis* and *P. aeruginosa*. Data presented as mean antibacterial time ± SD.

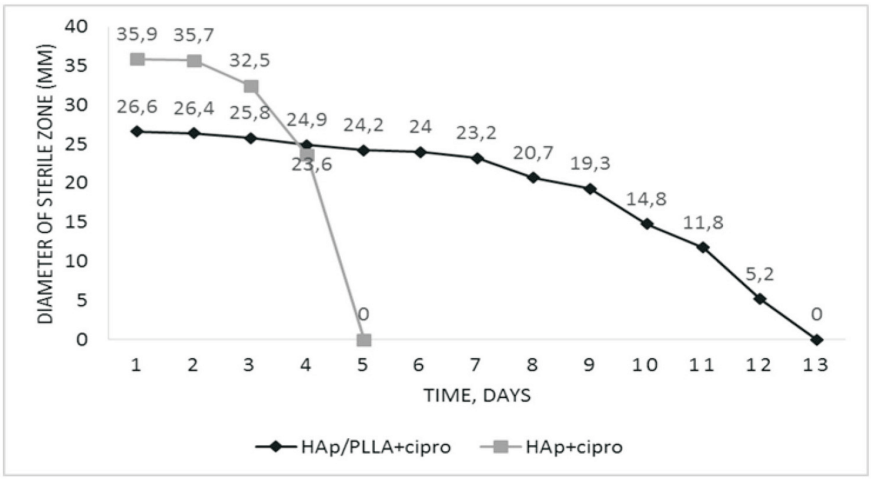

Fig. 3. The dynamics of *S. epidermidis* growth inhibition. Data presented as mean diameter (mm) of the sterile zone around Hap/PLLA+cipro or Hap+cipro disks.

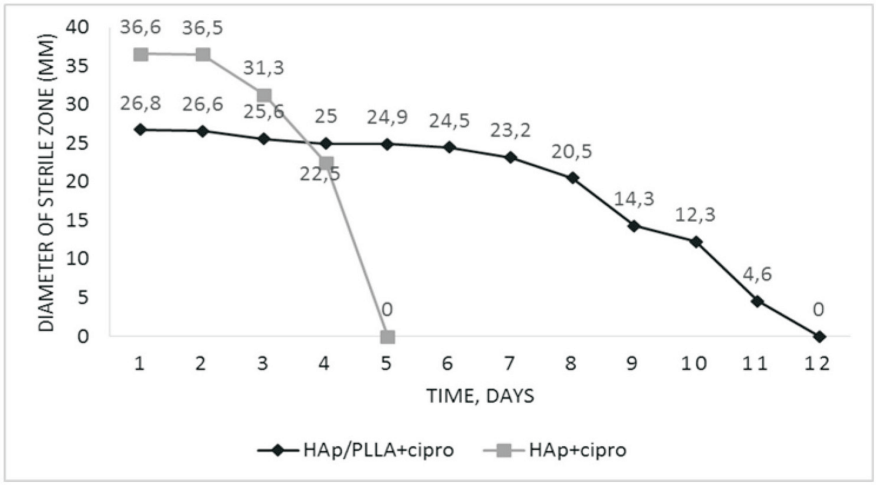

Fig. 4. The dynamics of *P. aeruginosa* growth inhibition. Data presented as mean diameter (mm) of the sterile zone around Hap/PLLA+cipro or Hap+cipro disks.

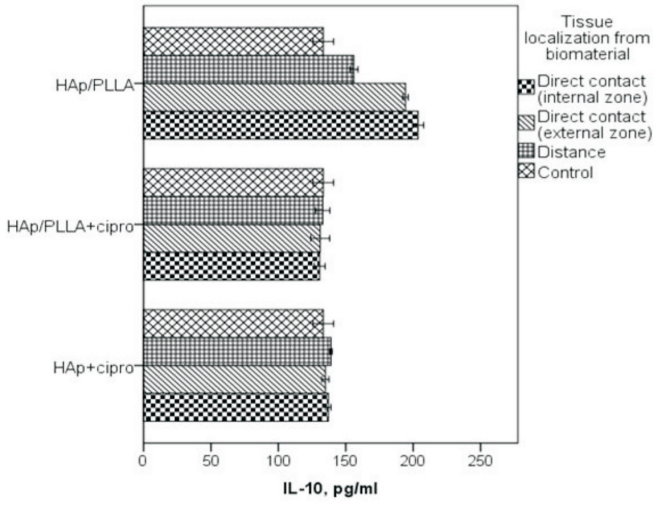

Fig. 5. The levels of IL-10 in wound tissues contaminated with *S. epidermidis* after Hap/PLLA (n = 3), Hap/PLLA+cipro (n = 3) or Hap+cipro (n = 3) implantation. The level of IL-10 was measured 4 weeks following implantation. Data presented as mean ± SD.

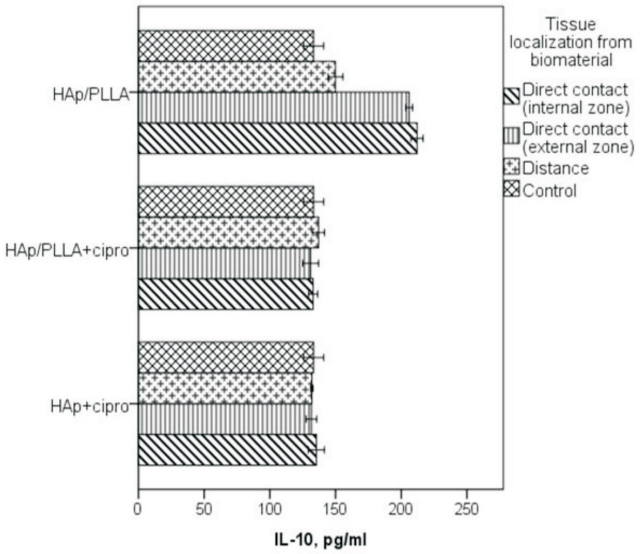

Fig. 6. The levels of IL-10 in wound tissues contaminated with *P.aeruginosa* after Hap/PLLA (n=3), Hap/PLLA+cipro (n=3) or Hap+cipro (n = 3) implantation. The level of IL-10 was measured 4 weeks following implantation. Data presented as mean ± SD.

A significant ($p < 0.001$) increase of IL-10 compared to the control group was expressed in direct contact tissues when HAp/PLLA was implanted and contaminated with *S. epidermidis* or *P. aeruginosa*. The level of IL-10 decreased significantly ($p < 0.001$) from direct contact zone tissues to distance zone tissues, which means that the inflammatory zone became smaller.

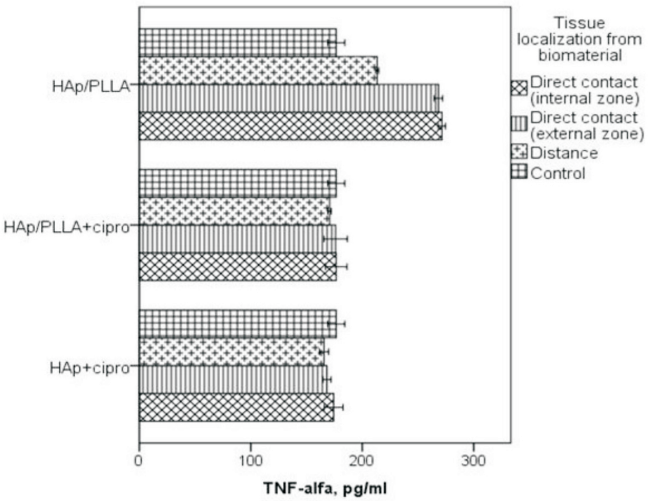

Fig. 7. The levels of TNF-alpha in wound tissues contaminated with *S. epidermidis* after Hap/PLLA (n = 3), Hap/PLLA+cipro (n = 3) or Hap+cipro (n = 3) implantation. The level of TNF-alpha was measured 4 weeks following implantation. Data presented as mean ± SD.

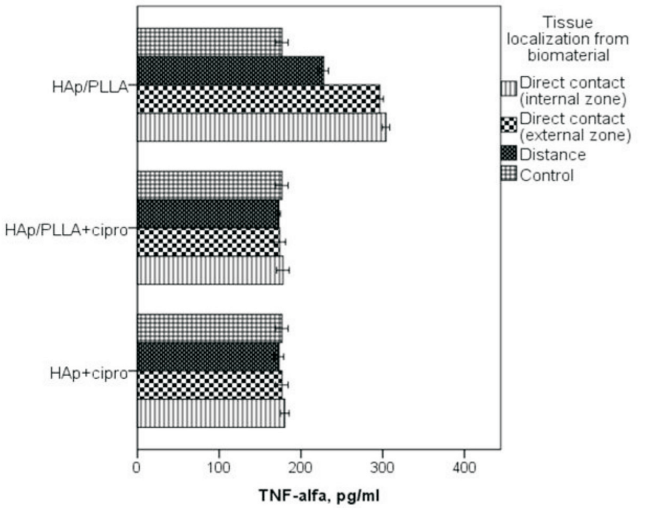

Fig. 8. The levels of TNF-alpha in wound tissues contaminated with *P. aeruginosa* after Hap/PLLA (n = 3), Hap/PLLA+cipro (n = 3) or Hap+cipro (n = 3) implantation. The level of TNF-alpha was measured 4 weeks following implantation. Data presented as mean ± SD.

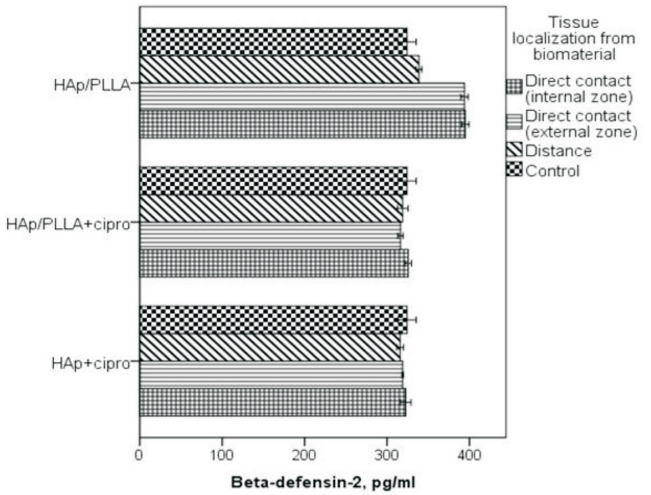

Fig. 9. The levels of beta-defensin-2 in wound tissues contaminated with *S. epidermidis* after Hap/PLLA (n = 3), Hap/PLLA+cipro (n = 3) or Hap+cipro (n = 3) implantation. The level of beta-defensin-2 was measured 4 weeks following implantation. Data presented as mean ± SD.

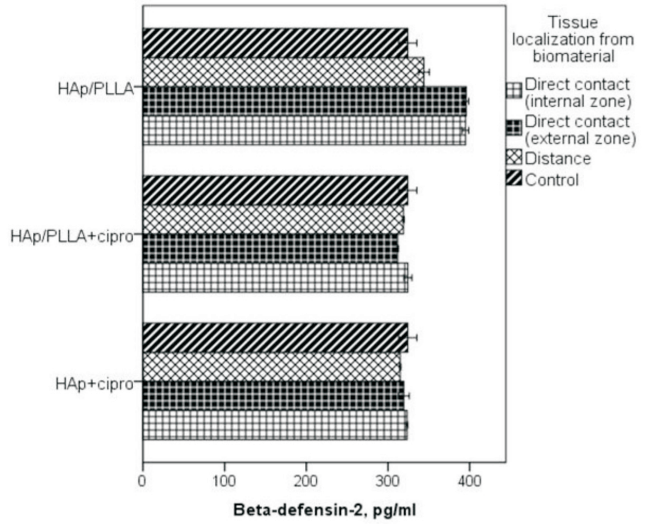

Fig. 10. The levels of β-defensin-2 in wound tissues contaminated with *P. aeruginosa* after Hap/PLLA (n = 3), Hap/PLLA+cipro (n = 3) or Hap+cipro (n = 3) implantation. The level of beta-defensin-2was measured 4 weeks following implantation. Data presented as mean ± SD.

Expression intensity of TNF-alpha in the animal model. When composite materials with antibiotics (HAp/PLLA+cipro and HAp+cipro) were implanted and contaminated with *S. epidermidis* (Fig. 7) or *P. aeruginosa* (Fig. 8) the level of TNF-alpha intensity in tissue did not show any significant ($p > 0.05$) differences compared to the control group. The most intensive TNF-alpha expression was observed in direct contact tissues around HAp/PLLA for both bacterial cultures and its expression was significantly more intense ($p < 0.001$) than in the control group.

Expression intensity of β-defensin-2 in the animal model. The most intensive β-defensin-2 expression occurred in tissues that were in close contact with HAp/PLLA and were contaminated with *S. epidermidis* (Fig. 9). β-defensin-2 levels significantly differed ($p < 0.05$) between treatments and the control group. Between the external and internal tissue zones, there were no significant differences ($p > 0.05$).

We observed significant ($p < 0.05$) differences in β-defensin-2 intensity levels between the direct contact zone tissues and the distanced zone tissues of the HAp/PLLA, which means that over time beta-defensin-2 intensity reduced. The level of β-defensin-2 did not change after implantation of composite materials with antibiotics.

In comparison to the control group, no difference in expression of β-defensin-2 wa observed after HAp/PLLA+cipro and HAp+cipro implantation and contamination with *P. aeruginosa* (Fig. 10). The highest expression in

β-defensin-2 was observed after HAp/PLLA implantation. The most intensive β-defensin-2 expression was observed in direct contact zone tissues around HAp/PLLA. The intensity level of β-defensin-2 was significantly ($p < 0.05$) lower in distant tissues compared to the direct contact zone, but there were no significant ($p > 0.05$) differences between the level of β-defensin-2 in the distant tissue zone and the control group.

DISCUSSION

The widespread use of biomaterials is often associated with unwanted effects, e.g. bacterial contamination and biofilm formation. In this study, newly synthesised biomaterials were impregnated with antibiotics in order to avoid these unwanted effects. The *in vitro* study showed that such an approach is promising and bacterial adhesion and colonisation of biomaterial surfaces is significantly inhibited. In this study we modelled infection *in vivo* and evaluated the level of inflammatory cytokines and antibacterial peptides.

Biofilms can form on implanted biomaterials after contamination of biomaterials with *S. epidermidis* and *P. aeruginosa*. Biofilms can protect bacteria from antibiotic substances and immune cells; thereby impeding the treatment process of biomaterial-associated infections (Aybar *et al*., 2012; Drenkard, 2013; Vassena *et al*., 2014). Thus, antibiotics can be used to prevent biofilm formation on biomaterials prior to adhesion and colonisation of bacteria. Higher concentration of antibiotics is achieved if antibiotics are used locally at the site of biomaterial implantation. Combining local, long-term and controlled release of antibiotics from biomaterials may reduce contamination risk, which in turn contributes to faster patient recovery (Hanssen, 2005; Antoci *et al*., 2008).

In this study, HAp/PLLA+cipro showed good antibacterial properties against *S. epidermidis* and *P. aeruginosa* and in comparison to HAp+cipro, as it showed controlled and gradual release of antibiotics from the biomaterial for a longer period of time. Because of differences in the bacterial cell wall structure (*S. epidermidis* and *P. aeruginosa*), the chosen antibiotics must be able to kill both bacterial cultures. The experimental data of this study are similar to other authors' studies, which achieved controlled release of antibiotics from biomaterials for longer periods of time using biodegradable polymer coatings (Li *et al*., 2011; Guillaume *et al*., 2012). For example, antibacterial properties were observed up to 14 days, depending on the type of polymer used in the study. Antibacterial properties lasted for an average of 240 hours, reaching even 264 hours of activity if PLLA were used (Kroica *et al.,* 2016). The antibacterial properties of these biomaterials were studied using the most common causative agents of biomaterial-associated infections. The choices of antibiotics in these studies varied, but there were common features such as the ability to kill various bacterial cultures (Chai *et al*., 2007; Guo *et al*., 2013; Lyndon and Birbilis, 2014).

The level of inflammatory cytokines and β-defensin-2 in surrounding tissue after HAp/PLLA+cipro, HAp+cipro and HAp/PLLA implantation and contamination with bacterial cultures (*S. epidermidis* and *P. aeruginosa*) were determined in an *in vivo* study.

High expression of IL-10 was detected in tissue surrounding the implanted biomaterials, which persisted up to 21 days following implantation (Gretzer *et al*., 2006). There is evidence of a difference between IL-10 production in tissue around the non-toxic (TiO_2 based biomaterials) and toxic (copper based biomaterials) implants. In both cases, the dynamics of IL-10 production are similar (Suska *et al*., 2005). The difference is observed between biomaterial samples with and without bacterial contamination. Expression of IL-10 is lower around tissue samples without bacterial contamination (Duarte *et al*., 2009).

TNF-α is the most accurate cytokine, which can show the intensity and dynamics of inflammation (Gul *et al*., 2010). The presence of TNF-α in synovial fluid may indicate development of an implant-associated infection (Gollwitzer *et al*., 2013).

The release of β-defensin-2 is stimulated by inflammatory cytokines: TNF-α and IL-1, and *P. aeruginosa* (Harder *et al*., 2001; Lai *et al*., 2010; Skadins *et al*., 2017). Beta-defensin-2 is also active against *S. epidermidis*, a representative of the normal microbiota (Lai *et al*., 2010). We also observed elevated levels of inflammatory cytokines and beta-defensin-2 in our study on biomaterial implantation with and without bacterial contamination.

In comparison to the control group, no differences were found in the expression of IL-10, TNF-α and β-defensin-2 after HAp/PLLA+cipro, HAp+cipro and HAp/PLLA implantation and contamination with *S. epidermidis* or *P. aeruginosa*. Antibiotics in biomaterials ensured the killing of bacteria, thus preventing the inflammation process. We observed increased levels of IL-10, TNF-α and β-defensin-2 after HAp/PLLA implantation, which clearly showed an active and strong inflammatory process. The results showed that the inflammatory process was localised and developed not only in the surrounding tissue around the biomaterials, but also spread from the site of biomaterial implantation. The high expression of cytokines was a result of the immune response against bacterial infection after HAp/PLLA implantation; in this case Hap/PLLA did not contain antibiotics that inhibited bacteria (Schutte *et al*., 2009; Sun *et al*., 2010).

CONCLUSION

Biomaterials with ciprofloxacin and PLLA polymer coating exhibit antibacterial effects longer against both bacterial cultures. A biodegradable coating ensures the gradual release of ciprofloxacin from the biomaterial. Without a biodegradable coating, ciprofloxacin is rapidly released from the biomaterial. An increased level of inflammatory

cytokines (IL-10, β-defensin-2, TNF-α) compared to the control group indicates an active inflammatory process after HAp/PLLA implantation and contamination with bacterial cultures. The practical use of biomaterials impregnated with antibiotics may reduce the expression of inflammatory cytokines in tissue surrounding implanted devices.

CONFLICT OF INTERESTS

Authors declare that there is no conflict of interest regarding the publication of this paper.

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BIOMATERIĀLU AR ANTIBIOTISKAJĀM VIELĀM IETEKME UZ IEKAISUMA CITOKĪNIEM

Lokālai antibiotisko vielu terapijai piemīt vairākas priekšrocības, salīdzinot ar sistēmisku ārstēšanu. Šīs priekšrocības var izmantot lokālu biomateriālu lietošanā, lai samazinātu to mikroorganismu skaitu, kas piesaistās pie implanta. Šajā *in vitro* pētījumā mēs pētījām antibakteriālās īpašības hidroksiapatīta biomateriāliem ar antibiotiskajām vielām un bionoārdošu polimēru. Antibakteriālās īpašības tika pētītas pret *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* baktērijām. *In vivo* pētījumā tika noteikts iekaisuma citokīnu līmenis (Interleikīns-10 (IL-10), beta-defensīns-2 and tumora nekrotizējošais factors alfa (TNF-alpha)) apkārtējos audos ap biomateriālu. Šajā pētījumā tika atrasts, ka biomateriāliem ar bionoārdošos polimēru ir ilgākas antibakteriālas īpašības nekā biomateriāliem bez bionoārdoša polimēra. Ja tika implantēts biomateriāls bez antibiotiskajām vielām, *in vivo* pētījumā apkārtējos audos tika atrasts augstāks iekaisuma citokīnu līmenis, salīdzinot ar kontroles grupu.