An In Vitro and In Vivo Study on the Intensity of Adhesion and Colonization by *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* on Originally Synthesized Biomaterials With Different Chemical Composition and Modified Surfaces and Their Effect on Expression of TNF-α, β-Defensin 2 and IL-10 in Tissues

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Key words: *biomaterials; Staphylococcus epidermidis; Pseudomonas aeruginosa; interleukin 10; tumor necrosis factor α; β-defensin 2.*

Summary. *The aim of this study was to determine adhesion and colonization of bacteria on the surface of originally synthesized glass-ceramic biomaterials and their effect on inflammation reactions in tissues surrounding the implant.*

Materials and Methods. Biomaterial discs were contaminated with bacterial suspensions of 10, 10^2, and 10^3 colony forming units (CFU)/mL (P. aeruginosa ATCC 27853 and S. epidermidis ATCC 12228), and after 2 hours of cultivation, the intensity of bacterial adhesion was determined. For in vivo tests, the samples were contaminated with 10^2 and 10^3 CFU/mL cultivated at 37°C for 2 h to ensure bacterial adhesion. Contaminated biomaterial samples were implanted in the interscapular area of chinchilla rabbits for 2 and 4 weeks. The biomaterials were removed, and using plate count and sonification methods, bacterial colonization on the surface of biomaterials was determined. Moreover, the expression of TNF-α, β-defensin 2, and IL-10 in the surrounding tissues was assessed by using immunohistochemistry methods.

Results. P. aeruginosa more intensively colonized biomaterials in the in vivo study as compared with S. epidermidis. Il-10 is a regulatory cytokine, which reduces the intensity of inflammatory cell activity, thus reducing nonspecific resistance of the organism.

Conclusions. The expression of TNF-α and IL-10 was not affected by short (2 and 4 weeks) biomaterial implantation. Pronounced cytokine expression in tissues around implanted biomaterials contaminated with P. aeruginosa was observed.

Introduction

During the last few years, biomaterials originally synthesized in Riga Technical University have been extensively utilized in clinical practice (maxillofacial and reconstructive surgery, etc.). There are studies that aimed to determine the morphologic and chemical properties of utilized biomaterials as well as to evaluate the adhesion and colonization of various microorganisms on different biomaterials.

Reactogenicity is a characteristic of biomaterials used in implants – they can cause counteraction of various intensity of macroorganism (1). The biomaterial also possesses the characteristic to attract microorganisms.

There are two principal paths for the bacterial contamination of biomaterials: direct implant contamination during surgery, which is the most common contamination form, and the contamination of implants due to hematogenous or lymphogenous bacterial dissemination. The source of bacterial contamination is the microbiota of either skin or mucous membrane (2–4).

Hence, the ability of microorganisms to infect and colonize implanted biomaterials or other appliances is still a common problem and serves as a risk factor in the development of infections at hospitals without regard to the use of aseptic and antiseptic methods (4). Therefore, up-to-date studies on the risk of bacterial contamination of biomaterials and its effect on inflammation and immune reactions in implant-surrounding tissues are still common (5).

Therefore, the aim of this study was to determine the adhesion and intensity of colonization by *Staphylococcus epidermidis* (*S. epidermidis*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) on an originally synthesized surface of glass-ceramic biomaterials

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and to evaluate the effect of bacterial colonization of biomaterials on the expression of inflammatory cytokines in surrounding tissues of implanted biomaterials.

Material and Methods

Production and Description of the Glass-Ceramic. In this study, niobium oxide was added to the basis of calcium phosphate to increase mechanical durability as well as natrium oxide to improve bioactivity and solubility. The glass-ceramic was produced during a two-step process: first, glass powder was produced, which later was pressed into a form, agglutinated, and crystallized with thermal treatment. The temperatures for crystallization were established with a differential thermal analyzer BAHR-DTA 703. A scanning electron microscope Tescan (Mira/LMU, High Resolution Schottky FE SEM) and x-ray diffractometer PANalytical X'Pert Pro were used to analyze the crystalline phases and microstructure.

Microbiological Testing. For microbiological analysis, 3 originally synthesized biomaterials with different degrees of crystallization and surface modification that were created by the aforementioned method were used: A, the raw material and product were crystalline (maximum burning temperature of 1005°C for 30 min); B, the raw material was amorphous and the product was crystalline (760°C for 60 min); B+, acid-dipped B (30 h, H_2O_2, HF, HNO_3), to reduce the easy-soluble amorphous phase on the surface.

Reference cultures of *P. aeruginosa* ATCC 27853 and *S. epidermidis* ATCC 12228 were used in the study. Suspensions of bacteria were made from the microbiological cultures in 1 mL of trypticase soy broth (Oxoid, UK) with the concentration of 10, 10^2, and 10^3 colony forming units (CFU)/mL. The samples were cultivated at 37°C for 2 hours to determine the intensity of the adhesion. The sonication-plate count method was used to determine the intensity of adhesion (6, 7); the unattached microorganisms were washed away after the incubation. To separate the bacteria attached to the surface of biomaterials, the discs were treated in an ultrasonic bath for 1 minute at the frequency of 45 kHz and for 1 minute in a Vortex centrifuge at maximum rpm. The several subcultures were made on a trypticase soy agar (TSA) growth medium (Oxoid, UK) and cultivated at 37°C for 24 hours to determine the total count of microorganisms, and CFUs were calculated on 1-mm^2 surface of biomaterial disc. The samples for scanning electron microscope (SEM) analysis were fixed in a mixture of ether-ethanol (1:1). The data were analyzed using the Microsoft Office Excel program.

The intensity of colonization was determined in an in vivo study that was approved by the Council of the Ethics of Animal Protection of the Ministry of Agriculture according to the legislation of the Republic of Latvia.

Suspensions of bacteria were made from the microbiological cultures in 1 mL of trypticase soy broth (Oxoid, UK) at the concentrations of 10^2 and 10^3 CFU/mL. The samples were cultivated at 37°C for 2 hours, the unattached microorganisms were washed, and then biomaterial samples were implanted to the experimental animals. Chinchilla rabbits were used for the experiments, a 2-cm cut was made on the back below the scapulae under combined general and local anesthesia. The subcutaneous tissue was detached, and then the biomaterial (with or without bacteria) was implanted in the subcutaneous tissues. Hemostasis was made, and the wound was closed with separate stitches. After the 2- and 4-week exposure, the rabbits were euthanized, the samples of biomaterial were removed and treated in a sonicator for 1 minute as well as vortexed for 1 minute to deattach the bacteria colonizing the sample. The sample was planted on a TSA growth medium and cultivated at 37°C for 24–48 hours. The colonies grown were counted and recalculated in count of bacteria on a 1-mm^2 surface of biomaterial samples (6, 7). The data were analyzed using the Microsoft Office Excel program. The expression of interleukin 10 (IL-10), β-defensin 2, and tumor necrosis factor α (TNF-α) in the tissues surrounding the biomaterial sample was determined using immunohistochemistry methods.

Results

Using the plating method, it was revealed that the adhesion of both microorganisms used in the study was more intense on the surface of the biomaterial A, which was expressly crystalline. A low ability to attract both bacteria at a concentration of 10^3 CFU/mL was shown by biomaterials B (0.005 CFU/mm^2 for both microorganisms) and B+ (0.005 CFU/mm^2 for *S. epidermidis* and 0.016 CFU/mm^2 for *P. aeruginosa*) (Table 1).

Analysis of the microbiological subcultures of the in vivo samples showed that the colonization of the biomaterial in vivo by *S. epidermidis* was minimal: in both cases, in the biomaterial sample subcultures contaminated with 10^2 and 10^3 CFU/mL, a small number of staphylococci were present (Table 2). A difference was observed in the subcultures from samples that were contaminated with *P. aeruginosa* (Table 3): the lowest colonization of the implanted biomaterial was observed in the sample B contaminated with 10^2 CFU/mL, but the greatest was observed in the sample B+.

Expression of Cytokines in the Tissue Around the Control Samples. A moderate expression of TNF-α and β-defensin 2 in macrophages and fibroblasts was

Table 1. Intensity of Bacterial Adhesion after 2 hours (CFU/mm^2)

Sample	10 CFU/mL	10^2 CFU/mL	10^3 CFU/mL
S. epidermidis, sample A	Adhesion not observed	0.005±0.001	0.05±0.006
P. aeruginosa, sample A	Adhesion not observed	0.01±0.002	0.048±0.006
S. epidermidis, sample B	Adhesion not observed	0.003±0.0005	0.005±0.001
P. aeruginosa, sample B	Adhesion not observed	0.003±0.0004	0.005±0.001
S. epidermidis, sample B+	Adhesion not observed	0.003±0.0004	0.005±0.001
P. aeruginosa, sample B+	Adhesion not observed	0.008±0.0008	0.016±0.001

Table 2. Intensity of Colonization by *S. epidermidis* on Biomaterial Surface After 2- and 4-Week Exposure (CFU/mm^2)

Sample	2 Weeks	4 Weeks
Sample A, contaminated with 10^2 CFU/mL	No growth	No growth
Sample A, contaminated with 10^3 CFU/mL	No growth	0.005±0.001
Sample B, contaminated with 10^2 CFU/mL	No growth	0.005±0.001
Sample B, contaminated with 10^3 CFU/mL	No growth	0.005±0.001
Sample B+, contaminated with 10^2 CFU/mL	0.01±0.002	0.005±0.001
Sample B+, contaminated with 10^3 CFU/mL	0.01±0.002	0.005±0.001

Table 3. Intensity of Colonization by *P. aeruginosa* on Biomaterial Surface After 2- and 4-Week Exposure (CFU/mm^2)

Sample	2 Weeks	4 Weeks
Sample A, contaminated with 10^2 CFU/mL	1.17±0.02	2.23±0.22
Sample A, contaminated with 10^3 CFU/mL	1.65±0.13	2.34±0.08
Sample B, contaminated with 10^2 CFU/mL	0.21±0.01	0.9±0.08
Sample B, contaminated with 10^3 CFU/mL	1.8±0.14	3.13±0.09
Sample B+, contaminated with 10^2 CFU/mL	8.01±0.17	0.42±0.02
Sample B+, contaminated with 10^3 CFU/mL	8.7±0.19	2.18±0.23

observed in tissues surrounding the biomaterial in the control sample A (Figs. 1–3), which was taken after 2-weeks exposure; it was slightly decreased in the samples after 4 weeks of exposition. There was almost no difference in the expression of TNF-α in the tissue samples surrounding the biomaterial samples B and B+ – a moderate expression of TNF-α in macrophages and fibroblasts was documented. In addition, a very intensive expression of TNF-α in fibroblasts in the capsule of connective tissue surrounding the biomaterial itself was observed in the case of biomaterial B+. The expression of the aforementioned cytokine was slightly decreased in the tissue samples after 4 weeks.

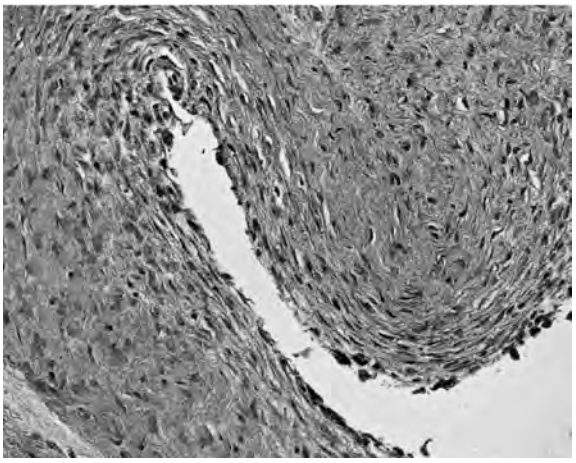

Fig. 2. Sample A, sterile, IL-10, 2-week exposure (×250)

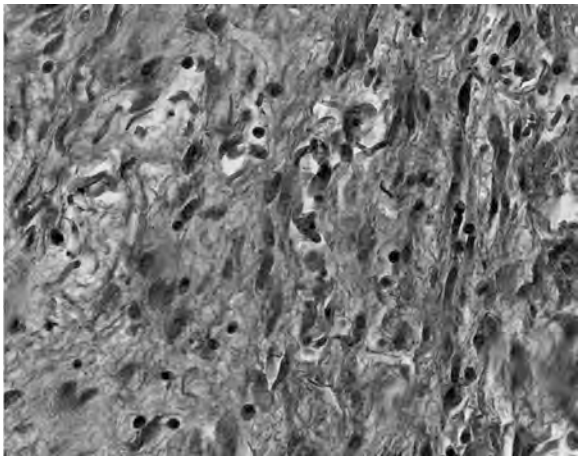

Fig. 1. Sample A, sterile, TNF-α, 2-week exposure (×400)

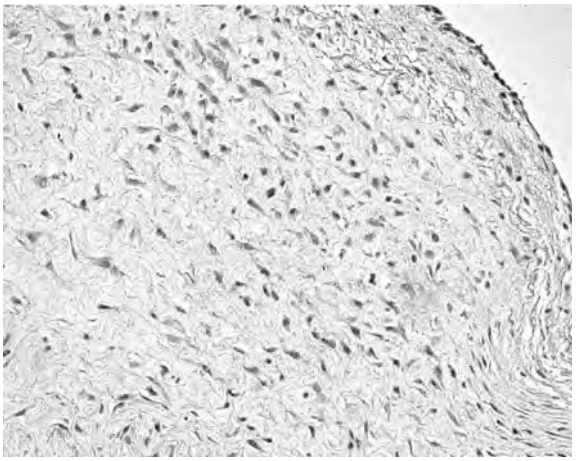

Fig. 3. Sample A, sterile, β-defensin 2, 2-week exposure (×250)

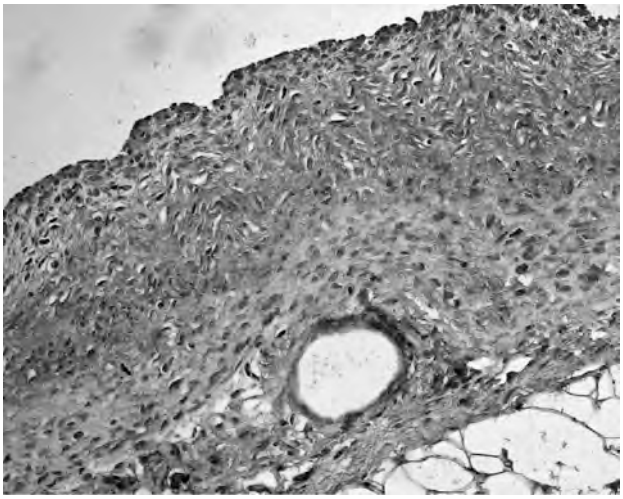

Fig. 4. Sample A, *S. epidermidis* (10^2 CFU/mL), TNF-α, 2-week exposure (×200)

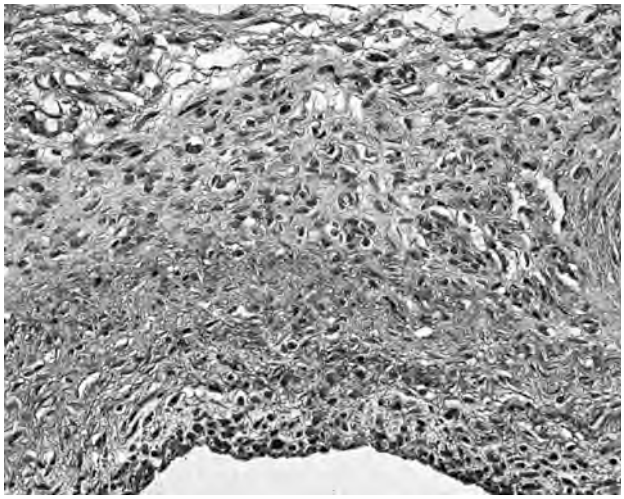

Fig. 5. Sample A, *S. epidermidis* (10^2 CFU/mL), IL-10, 2-week exposure (×250)

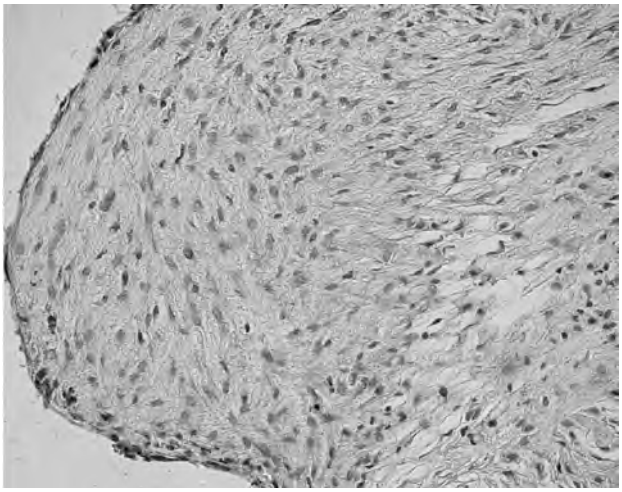

Fig. 6. Sample A, *S. epidermidis* (10^2 CFU/mL), β-defensin 2, 2-week exposure (×250)

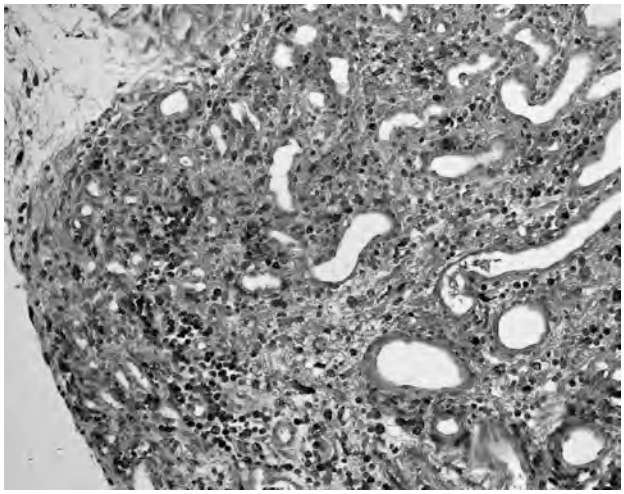

Fig. 7. Sample A, *P. aeruginosa* (10^2 CFU/mL), TNF-α, 2-week exposure (×200)

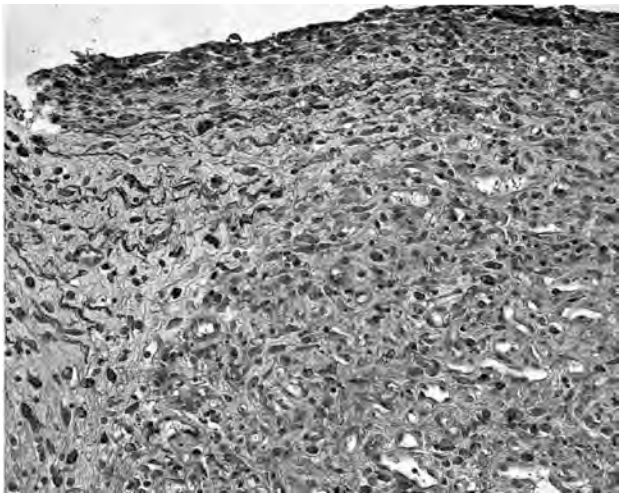

Fig. 8. Sample A, *P. aeruginosa* (10^2 CFU/mL), IL-10, 2-week exposure (×250)

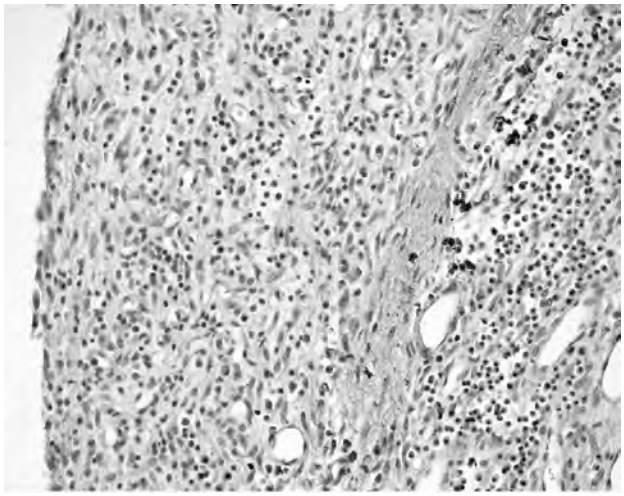

Fig. 9. Sample A, *P. aeruginosa* (10^2 CFU/mL), β-defensin 2, 2-week exposure (×250)

The expression of β-defensin 2 in macrophages and fibroblasts surrounding the biomaterial B was low both after 2- and 4-week exposition in the tissue of the rabbit. A more pronounced change in the difference of expression of β-defensin 2 between 2- and 4-week samples was observed for the sample B+ – this cytokine was expressed moderately after 2-week exposure while only few positive structures were observed after 4 weeks. The expression of IL-10 around all 3 samples was high after the exposition of both 2 and 4 weeks; a trend toward a small decrease for the aforementioned cytokine was observed in the samples after 4-week exposition.

Cytokine Expression in Tissues Surrounding the Test Samples Contaminated with S. epidermidis. In the tissue samples surrounding the biomaterial A (Figs. 4–6), contaminated with bacteria at the concentration of 10^2 CFU/mL, moderate expression of TNF-α, very high expression of IL-10, and low expression of β-defensin 2 in macrophages and fibroblasts was observed after 2 weeks. The tissues surrounding the biomaterials B and B+ showed higher expression of the aforementioned TNF-α and β-defensin 2. Very high expression of TNF-α and IL-10 and low expression of β-defensin 2 were observed particularly in the capsule of connective tissue surrounding all biomaterial samples contaminated with bacteria at the concentration of 10^3 CFU/mL.

In the samples after the 4-week exposition, moderate expression of TNF-α was observed in the tissues surrounding all biomaterial samples contaminated with bacteria at both concentrations. The expression of IL-10 was found to be very high in macrophages and fibroblasts in all samples contaminated with bacteria at both concentrations. The expression of β-defensin 2 in macrophages and fibroblasts was not very pronounced – only a few positive cells were observed.

Cytokine Expression in Tissues Surrounding the Test Samples Contaminated With P. aeruginosa. In the tissue samples surrounding the biomaterial A (Figs. 7–9), contaminated with bacteria at the concentration of 10^2 CFU/mL, moderate expression of TNF-α, high expression of IL-10, and low expression of β-defensin 2 in macrophages and fibroblasts were observed after 2 weeks, while tissues surrounding biomaterials B and B+ showed high expression of TNF-α and IL-10 and low expression of β-defensin 2. High expression of TNF-α, very high expression of IL-10, and low expression of β-defensin 2 were documented in the tissues surrounding all biomaterial samples contaminated with bacteria at the concentration of 10^3 CFU/mL.

In the samples after an exposition of 4 weeks, very high expression of TNF-α and IL-10 was observed in the capsule of connective tissue surrounding all biomaterials, but the expression of β-defensin 2 was low.

Discussion

The biomaterials used for implantation have reactogenecity, i.e., a capability to cause a response reaction of various intensity of the macroorganism (1). The response reaction itself always has the same qualitative characteristics – inflammation and the restriction of the foreign body with a capsule of connective tissue, if it is not possible to degrade or push out the body (1). The reactogenecity can be adjusted by many factors, for example, the structure of the biomaterial (8), the properties of the macroorganism (8, 9), as well as the "third" force – microorganisms (2, 10). Many microorganisms, often the representatives of normal flora, have affinity for artificial objects implanted in organism (1, 10). In our study, the impact of the creation technology for originally synthesized bioceramic materials on the abilities for adhesion of bacteria was determined in vitro. Based on the observations by SEM in previous studies and results of microbiological cultures (11, 12), we must presume that the technology of creation has a very big impact on the adhesion and colonization of microorganisms on biomaterials, for example, crystalline or amorphous phase. The intensity of adhesion was higher for both *S. epidermidis* and *P. aeruginosa* used in the study on materials, whose raw material and product were crystalline (sample A). The bacteria attached less intensively to biomaterials created from amorphous raw materials (B), while the use of acid dipping to reduce the amorphous phase on the biomaterial (sample B+) could increase the adhesion of bacteria on the biomaterials.

In the in vivo part of our study, 2 concentrations (10^2 and 10^3 CFU/mL) to contaminate the biomaterial samples were chosen as it was shown by the in vitro tests that the adhesion of bacteria starts at the aforementioned concentrations. Duration of the experiment (2 and 4 weeks) corresponds to the time when an acute implant infection develops – typically up to 3 months (2, 13, 14). It was important in the study to model early implant infection with the originally synthesized samples of biomaterials used in the experiment, therefore determining the intensity of bacterial colonization under in vivo conditions as well as the intensity of inflammation by determining important cytokines: TNF-α, IL-10, and β-defensin 2. In our study, the expression of TNF-α and IL-10 in the tissues surrounding the implanted biomaterials was moderate in the control samples, which were not contaminated. A similar picture was observed also in the biomaterial samples contaminated with *S. epidermidis*. The most intense expression of both TNF-α and IL-10 was observed around

the samples contaminated with *P. aeruginosa*. The increased concentration of IL-10 around the biomaterial may show that the macroorganism try to decrease the reactivity of the immune system around the implant and that such a reaction can be a cause for risk of opportunistic infection (14).

Moreover, in the studies that did not use biomaterials contaminated with bacteria, an increase in inflammatory cytokines levels with various dynamics depending on the type of biomaterial used has been documented. The authors reported increased levels of IL-10 while TNF-α levels were found to be varying (13, 14). In the literature, there are scarce data on the influence of biomaterials colonized by bacteria on the production of β-defensin 2 (15).

Conclusions

The study showed that *P. aeruginosa* more intensively colonized biomaterials in the in vitro and in vivo study compared with *S. epidermidis*. Il-10 is a regulatory cytokine, which reduces the intensity of inflammatory cell activity, thus reducing nonspecific resistance of the organism. The expression of TNF-α and IL-10 was not found to be affected by short biomaterial implantation (2 and 4 weeks). Pronounced cytokine expression in tissues around implanted biomaterials contaminated with *P. aeruginosa* was observed.

Acknowledgments

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Statement of Conflict of Interest

The authors state no conflict of interest.

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