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The Comparison of the Efficacy of the Antiseptic Solutions in Treatment of *Staphylococcus aureus* Infected Woven Vascular Graft *In Vitro* and *In Vivo*

SUMMARY OF DOCTORAL DISSERTATION

Medicine and Health Sciences
Medicine M 001

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Antiseptikų veiksmingumo palyginimas veikiant *Staphylococcus aureus* infekuotą austą kraujagyslės protezą *in vitro* ir *in vivo*

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CONTENT

THE LIST OF ABBREVIATIONS

CFU – colony forming units

IQR – interquartile range

MF – Faculty of Medicine

MLM – modified Lubbock's medium

NPWT – negative pressure wound therapy

OD – optical density

PBS – phosphate buffer solution

PMN – polymorphonuclears

PVGI – prosthetic vascular graft infection

SD – standard deviation

SEM – scanning electron microscopy

TSA – tryptic soy agar

TSB – tryptic soy broth

VU – Vilnius University

1. INTRODUCTION

1.1 Clinical relevance of the study

Prosthetic vascular graft infection (PVGI) is a rare but threatening complication in vascular surgery. It is estimated that PVGI occurs to 0.5-9.5% of the patients undergoing vascular graft implantation [1-8]. Regardless of the relatively low incidence rate, if left untreated, this complication often results in life-threatening hemorrhage or sepsis [9]. Despite all the measures of treatment, the outcomes of PVGI include high numbers of limb amputations (5-70% of all cases) and death (10-75% of all cases) [4, 9-14].

PVGI may develop perioperatively (due to breaches in asepsis) and postoperatively (mostly due to the infection of the surgical site or systemic bacteremia). The most common infectious agents include *Staphylococci, Escherichia, Enterococci and Salmonella* species. *Staphylococcus aureus* is responsible for the majority of PVGIs in the early postoperative period. Its tendency to form bacterial biofilms on the surface of the graft aggravates the course of treatment [2, 15-18].

There are no widely accepted guidelines or algorithms for the treatment of PVGI. An aggressive surgical approach, including removal of the infected graft, debridement of the infected perigraft tissues, systemic antibiotic therapy and forming an extra-anatomic bypass was considered a “golden standard” for a long time [4]. This approach is not suitable in some cases, for example, when a patient is critically ill and would not survive a major surgical intervention or the graft is connected to the aorta or other vitally important arteries. Therefore, in some cases PVGI is chosen to be treated conservatively. Successful case reports or reviews on small series of patients, who received conservative treatment, are published in scientific literature [19, 20]. If a conservative treatment approach is chosen, usually several treatment methods are applied together to achieve better results: long-term antibiotic therapy, negative pressure wound therapy (NPWT), local wound washings and drainage applying constant or

repetitive irrigations [1, 3, 9, 20]. The wounds can be washed with sterile saline [21], antibiotic or antiseptic solutions. Although antibiotics are a must in the treatment of PVGI, only some of them are capable of penetrating biofilms, formed by the bacteria on the graft. The formation of a bacterial biofilm on one of the most commonly used prosthetic vascular grafts – Dacron – usually leads to a disastrous scenario. Since Dacron is made of polyester microfilaments, which create an uneven graft surface and increase the area of adhesion for bacteria to cling and cluster, the accessibility of antimicrobials and the host's immune cells greatly decrease [16, 22].

Two main problems are faced when PVGI is treated with antibiotics. First, due to the formation of bacterial biofilms the penetration of antibiotics is reduced. Second, bacteria develop resistance to antibiotics, so alternative antimicrobials – antiseptic solutions – are becoming more appealing, because the bacteria's resistance to them remains minimal [23]. Even though antiseptics are usually used for prophylaxis [24], introducing them into the treatment of complicated infections wound allow limiting the use of ineffective antibiotic therapy.

1.2 Aim of the study

To evaluate the efficacy of routinely used antiseptic solutions in the treatment of *Staphylococcus aureus* infected woven vascular graft *in vitro* and *in vivo.*

1.3 Study objectives

1. To compare a biofilm-disrupting activity of 0.1% octenidine dihydrochloride, 10% povidone-iodine and 0.02% chlorhexidine digluconate on *S. aureus* biofilms *in vitro*.
2. To compare the antimicrobial efficacy of 0.1% octenidine dihydrochloride, 10% povidone-iodine and 0.02%

chlorhexidine digluconate on *S. aureus* biofilms in a simulated wound environment *in vitro.*

3. To design an experimental PVGI model *in vitro* and to compare the antimicrobial efficacy of 0.1% octenidine dihydrochloride, 10% povidone-iodine and 0.02% chlorhexidine digluconate on *S. aureus* biofilms, situated on the patches of vascular graft.
4. To design an experimental PVGI model *in vivo* for Wistar rats and to compare the efficacy of 0.1% octenidine dihydrochloride, 10% povidone-iodine and 0.02% chlorhexidine digluconate, treating *S. aureus* infected surgical wounds with patches of woven vascular graft.
5. To determine the duration of the treatment needed to eradicate *S. aureus* from the wounds with infected vascular grafts and to perform cytological and histological analysis of the perigraft tissues.

1.4 Statements to be defended

1. Antiseptic solutions are able to affect *S. aureus* biofilms *in vitro*.
2. Irrigations with antiseptic solutions are effective in treating *S. aureus* infected surgical wounds with patches of woven vascular graft in rats.

1.5 Novelty of the study

In the era of quickly developing bacterial resistance to antibiotics, the medical community is in constant search of alternative ways to treat various infections. A conservative method to treat PVGI – local wound irrigation with antiseptic solutions – is used from time to time, but is not a well established treatment method. After thoroughly reseaching this topic, it was discovered that there is a lack of representative, wound-simulating *in vitro* models, which would assist

studying an interaction of biofilm-producing *S. aureus* and routinely used antiseptic solutions on woven vascular grafts. No publications were found on *in vivo* studies where antiseptic solutions would be used for treatment of PVGI, but not for prophylaxis.

Therefore, a novel *in vitro* model, simulating the wound environment and PVGI, was designed to study antimicrobial efficacy of antiseptics on *S. aureus* biofilms. In order to test if wound irrigation with antiseptic solutions is an effective treatment method, a new experimental model of PVGI was developed for Wistar rats.

2. MATERIALS AND METHODS

The *in vitro* experiments, studying the efficacy of routinely used antiseptic solutions on *S. aureus* biofilms were performed at Vilnius University (VU) Faculty of Medicine (MF) Institute of Biomedical Science Department of Physiology, Biochemistry, Microbiology and Laboratory Medicine Division of Microbiology and Lithuanian Center of Non-formal Youth Education laboratory from May 2017 to October 2018. Three strong biofilm-producing *S. aureus* strains (215N, A7189 and ATCC 25923) were selected for the experiments. The biofilm-disrupting activity and antimicrobial efficacy of 0.1% octenidine dihydrochloride (Octenisept®, Schulke&Mayr GmbH, Germany), 10% povidone-iodine (Betadine®, EGIS Pharmaceuticals LTD, Hungary) and 0.02% chlorhexidine digluconate (Fresenius Kabi AG, Germany) on *S. aureus* biofilms were tested in three different *in vitro* settings. Phosphate buffer solution (PBS) was used for control groups.

The *in vivo* experiments, evaluating the efficacy of the antiseptics for treatment of infected surgical wounds with vascular grafts in rats were carried out in State Research Institute Centre for Innovative Medicine Department of Immunology, VU Life Sciences Center and VU MF Institute of Biomedical Science Department of Physiology, Biochemistry, Microbiology and Laboratory Medicine Division of Microbiology from February 2012 to July 2018. All researchers attended courses of Laboratory Animal Science prior to the experiments. The permissions to perform the studies were granted by State Food and Veterinary Service on January 4, 2012 and April 25, 2016 (permit no. 0222 and G2-43).

The experiments were performed on Wistar rats of both genders. All rats underwent a surgery when a patch of woven vascular graft was implanted under the skin. The wounds were inoculated with a biofilm-producing *S. aureus* 215N strain. A conservative method to treat PVGI – wound irrigation with antiseptic solutions (0.1% octenidine dihydrochloride, 10% povidone-iodine and 0.02% chlorhexidine digluconate) was tested. Sterile saline solution was used for the control

group. Microbiological tests of the wound washouts were performed repeatedly to evaluate the efficacy of the treatment.

2.1 Materials and methods for *in vitro* experiments

2.1.1 Qualitative and quantitative analysis of *S. aureus* biofilm production

In order to select strong biofilm-producers for the further experiments, ten different *S. aureus* strains were tested. The strains were selected randomly from the storage of VU MF Institute of Biomedical Science Department of Physiology, Biochemistry, Microbiology and Laboratory Medicine Division of Microbiology. Qualitative and quantitative evaluation of biofilm formation were performed according to *Rewatkar, Wadher* [25], *Darwish and Asfour* [26]. The cultures were cultivated on tryptic-soy agar (TSA, Liofilchem®, Italy) for 24 h at 35°C±2°C. After cultivation, one colony of each strain was transferred to Congo red agar and incubated for 24 h at 35°C±2°C. *S. aureus* strains, which produced black colonies on Congo red agar, were considered biofilm-producing strains [20, 190].

Quantitative selection of *S. aureus* isolates was performed using a modified crystal violet absorbtion assay [27]. 100 µL of each strain's suspension were transferred to a 96-well plate (Thermo Scientific™, Nunc ™) (Figure 1 A) and cultivated for 24 h at 35°C±2°C. After the incubation the medium was aspirated from the wells and they were washed three times with PBS to eliminate planktonic bacteria. Biofilms, which were stuck to the bottom of the wells, were fixed with 96% ethanol solution and colored with aqueous crystal violet dye. The well plate was washed again and left to dry. Finally, each well was filled with 300 µL of 30% acetic acid to extract crystal violet from the biofilms. After 30 minutes, 200 µL of each extract was transferred to a clean 96-well plate. The optical density (OD) of the solutions was determined using a spectrophotometer (Dynex MRX) at 595 nm.

The wells with tryptic soy broth (TSB) without bacteria served as a negative control. Each strain was tested 12 times. The mean value of OD was used to evaluate the mass of a produced biofilm.

According to the qualitative and quantitative biofilm-formation assays, three strains were found to be strong biofilm-producers: 215N, A7189 and ATCC 25923. They were used in the further experiments.

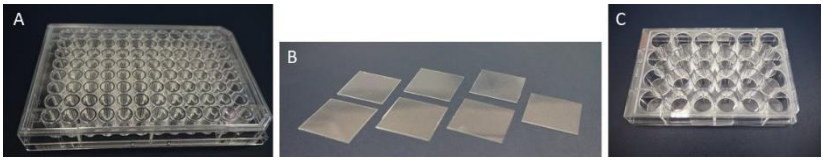

Figure 1. A – 96-well microplate; B – glass coverslips; C – 24-well plate.

2.1.2 Microscopic evaluation of the integrity of *S. aureus* biofilms

OBJECTIVE 1: to compare a biofilm-disrupting activity of 0.1% octenidine dihydrochloride, 10% povidone-iodine and 0.02% chlorhexidine digluconate on *S. aureus* biofilms *in vitro*

215N, A7189 and ATCC 25923 *S. aureus* strains were used for the experiment. The biofilms were grown in TSB on borosilicate glass coverslips (Figure 1 B) in Petri dishes for 48 h and then treated with 0.1% octenidine dihydrochloride, 10% povidone-iodine and 0.02% chlorhexidine digluconate solutions for 10 minutes. PBS was used for the control groups. The integrity of the biofilm was visualized by scanning the glass coverslips with a Scan Scope XT Slide Scanner (Leica Aperio Technologies, Vista, CA, USA). The actual area of the coverslips, covered with the biofilms, was analysed and quantified using Halo Area Quantification v1.0 algorithm and expressed in percent. The area of the coverslips, covered with the biofilms in control groups was compared to the area of the coverslips, covered in biofilms after the application of the antiseptics.

2.1.3 Antimicrobial efficacy of different antiseptic solutions on *S. aureus* biofilms

OBJECTIVE 2: to compare the antimicrobial efficacy of 0.1% octenidine dihydrochloride, 10% povidone-iodine and 0.02% chlorhexidine digluconate on *S. aureus* biofilms in a simulated wound environment *in vitro*

The methodology of the experiment was based on Lubbock's chronic wound pathogenic biofilm model (further – Lubbock's model), described by *Sun et al.* [28]. The essence of the model is to mimick a wound environment *in vitro*, using a medium, which consists of blood components and nutrients. We modified the model and used medium, which consisted of 50% of sheep blood plasma, 5% of cold lysed horse erythrocytes, 45% of brain-heart infusion and 1% of type B gelatin (further – modified Lubbock's medium, MLM).

The biofilms of 215N, A7189 and ATCC 25923 *S. aureus* strains were grown in a MLM in the 24-well plates (Thermo Scientific™, Nunc ™) (Figure 1 C). The biofilms were treated with 0.1% octenidine dihydrochloride, 10% povidone-iodine and 0.02% chlorhexidine digluconate solution for 10 minutes. PBS was used for control groups. After the time of the exposition, the colony forming units (CFU) of *S. aureus*, remaining in the biofilms, were evaluated microbiologically on Mannitol-salt agar (Liofilchem®, Italy) plates.

2.1.4 Antimicrobial efficacy of different antiseptic solutions on *S. aureus* biofilms on the vascular grafts

OBJECTIVE 3: to design an experimental PVGI model *in vitro* and to compare the antimicrobial efficacy of 0.1% octenidine dihydrochloride, 10% povidone-iodine and 0.02% chlorhexidine digluconate on *S. aureus* biofilms, situated on the patches of vascular graft

Lubbock's model [28] was modified and adapted to design an innovative experimental PVGI model *in vitro*. The biofilms of *S. aureus* 215N, A7189, ATCC 25923 strains were grown in MLM on the patches of a non-impregnated woven Dacron graft (Twillweave®, Vascutek Terumo) (Figure 2 A). The patches of the vascular graft, covered in biofilms (Figure 2 B) were treated with 0.1% octenidine dihydrochloride, 10% povidone-iodine, 0.02% chlorhexidine digluconate solution for 10 minutes. PBS was used for control groups. After the time of the exposition, the amount of *S. aureus* remaining in the biofilms on the vascular grafts, was evaluated microbiologically on Mannitol-salt agar plates.

For *in vitro* experiments, depicted in sections 2.1.2, 2.1.3 and 2.1.4 the standardised inocula of *S. aureus* strains were used (Table 1). Identification of *S. aureus* was based on latex agglutination, plasma coaguliation tests and light microscopy. *S. aureus* ATCC 29213 was used as a control strain.

Table 1. *S. aureus* inocula used for *in vitro* experiments

S. aureus strain	Inoculum for 2.1.2 experiment, CFU*/mL	Inoculum for 2.1.3 experiment, CFU/mL	Inoculum for 2.1.4 experiment, CFU/mL
215N	$1.49x10^6$	$8.18x10^7$	$1.49x10^6$
A7189	$7.63x10^7$	$7.63x10^7$	$7.63x10^7$
ATCC 25923	$6.91x10^7$	$6.91x10^7$	$6.91x10^7$

*CFU – colony forming units

Figure 2. A – a non-impregnated woven Dacron graft and its patches; B – a patch of Dacron graft, covered by *S. aureus* biofilm.

2.1.5 Scanning electron microscopy of the vascular grafts

A scanning electron microscope (SEM, HITACHI TM-1000, Japan, accelerating voltage 15 kV) was used to visualize the structure of a non-impregnated Dacron vascular graft. Three patches of clean and three patches of biofilm-covered grafts were scanned. Prior to scanning, patches of the vascular graft with *S. aureus* biofilms were fixed in 5% glutaraldehyde solution for 12 hours. All patches of the graft were gold-coated before scanning. Every patch was photographed 10 times to get a representative image of the graft with and without the biofilm.

2.2 Materials and methods for *in vivo* experiments

A new experimental rat model was designed to study the efficacy of antiseptics in the treatment of PVGI. A biofilm-producing *S. aureus* 215N strain, previously used for *in vitro* experiments, was chosen to induce the infection. The treatment of the wounds was performed by applying daily irrigations with either 0.1% octenidine dihydrochloride, 10% povidone-iodine, 0.02% chlorhexidine digluconate solution or sterile saline (for control group).

Wistar rats of both genders, 3-6 months of age and 239-450 g of body mass were used for the study. Prior to the main experiment, a pilot study was performed to estimate a proper *S. aureus* dose to cause a suppuration of a surgical wound, which would persist for at least two weeks.

The main study was performed in two stages. The first series of *in vivo* experiments was performed to test a newly designed rat model for the treatment of PVGI caused by *S. aureus* and to evaluate the efficacy of three different antiseptic solutions. The second series of *in vivo* experiments was performed with the most effective antiseptic solution to evaluate the repeatability of the newly designed model, to achieve *S. aureus* eradication from the wound washouts and to evaluate both cytological and histological wound findings.

2.2.1 The first series of *in vivo* experiments

OBJECTIVE 4: to design an experimental PVGI model *in vivo* for Wistar rats and to compare the efficacy of 0.1% octenidine dihydrochloride, 10% povidone-iodine and 0.02% chlorhexidine digluconate, treating *S. aureus* infected surgical wounds with patches of woven vascular graft

The rats (n=48) were placed into separate cages one week prior to the experiment. Balanced standardised food, tap water and same controlled conditions were ensured. The anaesthesia was induced using 10% ketamine 40 mg/kg ("Bio-ketan", Vetoquinol Biowet Sp. Zoo, Poland) and 2% xylazin solution 5 mg/kg ("Xylazin" 2%, "Bela-Pharm GmbH & Co. KG", Germany), injected intraperitoneally. A patch of a sterile non-impregnated woven Dacron graft was implanted under the skin for each rat on the right side of the spine and the wound was inoculated with 1 mL of *S. aureus* suspension, equivalent to 5 McFarland standard. After five days of incubation, wound washouts were taken for the microbiological evaluation. The rats were randomly grouped into four groups of 12 and daily wound irrigations were started with different antiseptic solutions (0.1% octenidine dihydrochloride, 10% povidone-iodine and 0.02% chlorhexidine digluconate solution or sterile saline (control group). The treatment was carried out for four days. To evaluate the dynamics, wound washouts were taken again on day eight and 10 of the experiment. After the termination of the experiment, the grafts were removed from the wounds and tested microbiologically. The detailed course of the first series of *in vivo* experiments is depicted in Figure 3.

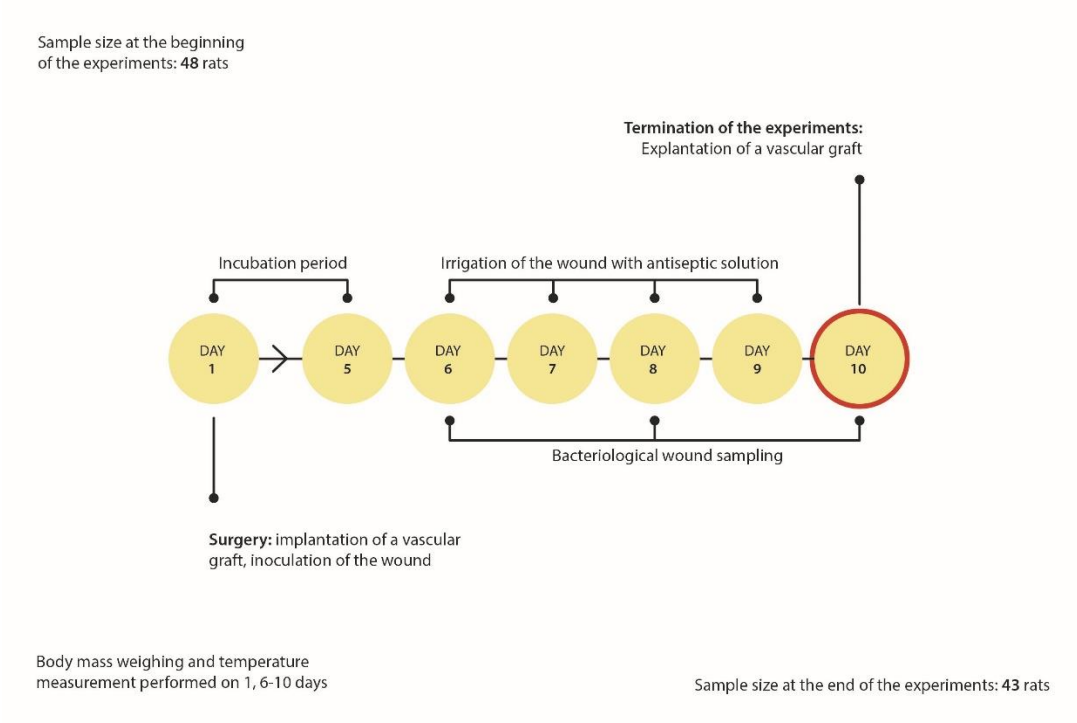

Figure 3. The first series of *in vivo* experiments.

2.2.2 The second series of *in vivo* experiments

OBJECTIVE 5: to determine the duration of the treatment needed to eradicate *S. aureus* from the wounds with infected vascular grafts and to perform cytological and histological analysis of the perigraft tissues

Using the same methodology (described in section 2.2.1) a group of 10 rats underwent a vascular graft implantation and wound inoculation with *S. aureus*. The suppurative wounds were treated with daily irrigations of 0.1% octenidine dihydrochloride for seven days. The cytological examinations of the wound washouts were performed along with the microbiological tests. For enumeration of *S. aureus* in the wound washouts, samples were plated on Mannitol-salt agar plates.

The second series of *in vivo* experiments were terminated on day 13. For microbiological evaluation the patches of vascular graft were removed from the wounds. Blood samples were taken via cardiopuncture. The cutaneous fistulas and perigraft tissues were resected for microbiological and histological analysis. The detailed course of the second series of *in vivo* experiments is depicted in Figure 4.

For both the first and the second series of *in vivo* experiments identification of *S. aureus* colonies was based on latex agglutination and plasma coaguliation tests and light microscopy. *S. aureus* ATCC 29213 was used as a control strain.

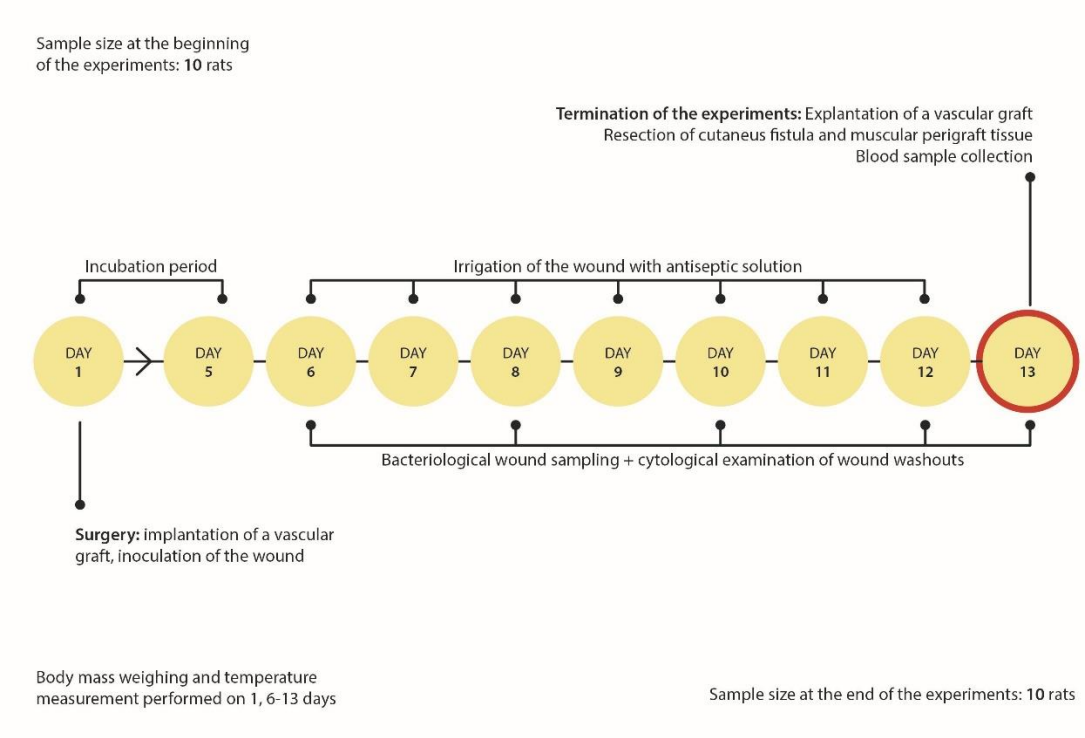

Figure 4. The second series of *in vivo* experiments.

2.3 Statistical analysis

The data were analyzed using parametric and non-parametric tests. Parametric tests were used when data were assumed to be normally-distributed (paired sample T test and independent samples T test). Non-parametric tests were used when data were assumed to be not normally distributed (Mann Whitney U, Kruskal Wallis H and Wilcoxon T tests). Correlation analysis was performed using Spearman's Rho test. The data were processed using SPSS 24.0 and Microsoft Excel 2016 programs. Statistical significance was assigned when p values were less than 0.05.

3. RESULTS

3.1 The results of *in vitro* experiments

3.1.1 Qualitative and quantitative evaluation of biofilm-producing *S. aureus* strains

Nine out of 10 *S. aureus* strains produced black colonies on Congo red agar and thus were considered biofilm-producing strains. Red colonies of *S. aureus* were produced by I-1242 strain, therefore it was considered a non-biofilm producer (Figure 5).

Quantitative evaluation of *S. aureus* biofilm production was assessed by a spectrophotometric crystal violet absorbtion assay (Table 2) [27]. Three *S. aureus* strains, which showed signs of strong biofilm production (215N, A7189 and ATCC 25923) were selected for further *in vitro* experiments.

Figure 5. Differentiation of biofilm-producing *S. aureus* strains on Congo red agar.

Table 2. Quantitative evaluation of the biofilm production by different *S. aureus* strains

S. aureus strain	Mean OD_{595} nm (SD)*	Biofilm production
215N	**0.73**±0.04	**Strong**
A1152	0.47±0.14	Weak
A4192	0.49±0.08	Weak
A6132	0.49±0.13	Weak
A7189	**0.69**±0.12	**Strong**
I-1242	0.15±0.04	None
I-1717	0.51±0.09	Moderate
I-1867	0.57±0.05	Moderate
I-1975	0.53±0.09	Moderate
ATCC 25923	**0.86**±0.04	**Strong**
Control well	0.04±0.02	-

*OD – optical density; SD – standard deviation

3.1.2 Evaluation of the biofilm-disrupting activity of different antiseptic solutions on *S. aur*eus biofilms

S. aureus biofilms successfully formed on the glass coverslips in all experimental groups. The area of the coverslips, covered by a biofilm, was homogenous in the control groups of all three *S. aureus* strains (215N, A7189 and ATCC 25923) (Kruskal Wallis H test, p=0.33) (Table 3).

Table 3. The area of the glass coverslips, covered by a biofilm for different *S. aureus* strains

S. aureus strain	The area of the glass coverslips, covered by *S. aureus* biofilm in the control groups, %				p value
	Minimum	Maximum	Median	IQR*	
215N	71.60	76.80	72.85	3.57	0.33
A7189	74.20	86.18	82.18	7.30	
ATCC 25923	67.66	85.65	70.32	12.43	

*IQR – interquartile range

A significant biofilm-disrupting activity for all three *S. aureus* strains was observed only after application of chlorhexidine digluconate (Kruskal Wallis H test, 215N strain $p=0.02$, A7189 strain $p<0.001$, ATCC 25923 strain $p=0.02$ (Figure 6).

The effect of the antiseptics (octenidine dihydrochloride, povidone-iodine and chlorhexidine) did not differ between different *S. aureus* strains (Kruskal Wallis H test, $p=0.23$, $p=0.99$, $p=0.09$, respectively) (Figure 7).

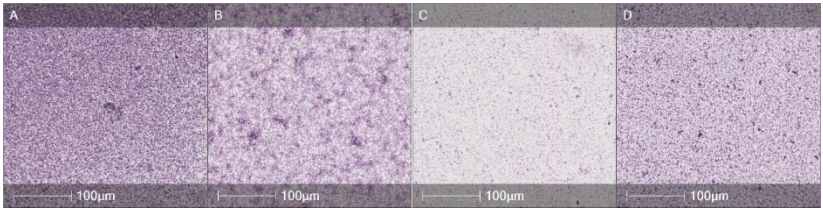

Figure 6. Scanned images of the glass coverslips covered by the biofilms of *S. aureus* 215N strain after application of the antiseptics (Scan Scope XT scanner, one representative image for a group): A – control group; B – octenidine dihydrochloride group; C – chlorhexidine digluconate group; D – povidone-iodine group.

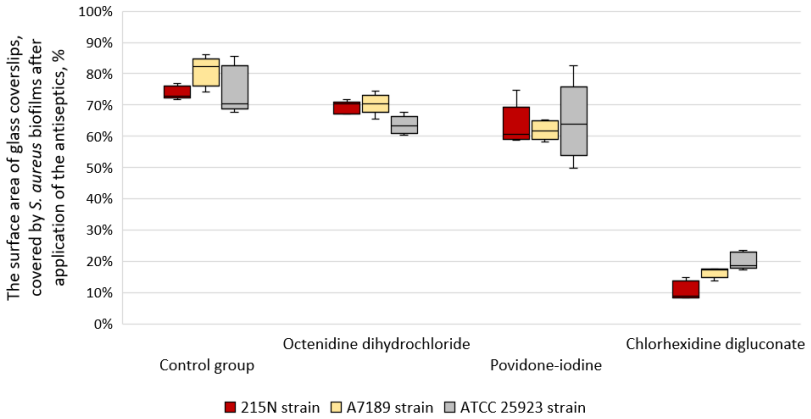

Figure 7. The biofilm-disrupting activity of different antiseptics on *S. aureus* biofilms, formed on the glass coverslips. The area of the biofilms on the coverslips did not change significantly after the application of octenidine dihydrochloride ($p=0.2$) and povidone-iodine ($p=0.4$), but decreased significantly after the application of chlorhexidine digluconate ($p=0.02$, $p=0.02$ and $p<0.001$, respectively for strains 215N, A7189 and ATCC 25913).

3.1.3 Evaluation of the antimicrobial efficacy of different antiseptic solutions on *S. aureus* biofilms

In a MLM all *S. aureus* strains (215N, A7189 and ATCC 25923) produced biofilms which were similar in texture and mass ($p=0.7$). The CFUs of *S. aureus* in the biofilms were enumerated for the control groups of all the strains (Table 4) and then compared to the number of the CFUs, left in the biofilms after the application of the antiseptics.

Table 4. The number of *S. aureus* CFUs in the biofilms, produced in a modified Lubbock's medium

S. aureus strain	*S. aureus* in the biofilm, CFU/mL			
	Minimum	Maximum	Median	IQR*
215N	$3.50x10^7$	$7.20x10^9$	$4.57x10^8$	$2.56x10^9$
A7189	$1.00x10^6$	$2.00x10^{12}$	$6.00x10^{11}$	$1.29x10^{12}$
ATCC 25923	$4.00x10^{10}$	$7.00x10^{14}$	$2.00x10^{14}$	$4.99x10^{14}$

*IQR – interquartile range

The application of the antiseptic solutions on the biofilms, produced by *S. aureus* 215N strain, resulted in:

- a decrease of *S. aureus* CFUs in the biofilms by three orders of magnitude after application of 0.02% chlorhexidine digluconate solution (showing median (M) and interquartile range (IQR) (from $M=4.57x10^8$, $IQR=2.56x10^9$ to $M=2.77x10^5$, $IQR=4.86x10^5$ CFU/mL, p=0.002);
- no *S. aureus* was detected after the application of 0.1% octenidine dihydrochloride and 10% povidone-iodine (p=0.002).

The application of the antiseptic solutions on the biofilms, produced by *S. aureus* A7189 strain, resulted in:

- a decrease of *S. aureus* CFUs in the biofilms by five orders of magnitude (from $M=6.00x10^{11}$, $IQR=1.29x10^{12}$ to $M=1.00x10^6$, $IQR=7.00x10^5$ CFU/mL, p=0.02) after the application of 0.02% chlorhexidine digluconate solution and nine orders of magnitude (from $M=6.00x10^{11}$, $IQR=1.29x10^{12}$ to $M=1.05x10^2$, $IQR=2.09x10^2$ CFU/mL, p=0.002) after the application of 10% povidone-iodine;
- no *S. aureus* was detected after the application of 0.1% octenidine dihydrochloride (p=0.002).

The application of the antiseptic solutions on the biofilms, produced by *S. aureus* ATCC 25923 strain, resulted in:

- no significant change of the number of *S. aureus* CFUs in the biofilms after the application of 0.02% chlorhexidine digluconate (decreased from M=2.00x10^{14}, IQR=4.99x10^{14} to M=4.50x10^{13}, IQR 2.92x10^{14} CFU/mL, p=0.82);
- no *S. aureus* was detected after the application of 0.1% octenidine dihydrochloride (p=0.002); after the application of 10% povidone-iodine no *S. aureus* was detected in five out of six biofilms, 7 CFU/mL were detected in one out of six biofilms (p=0.002).

The results of this experiment are represented in Figure 8.

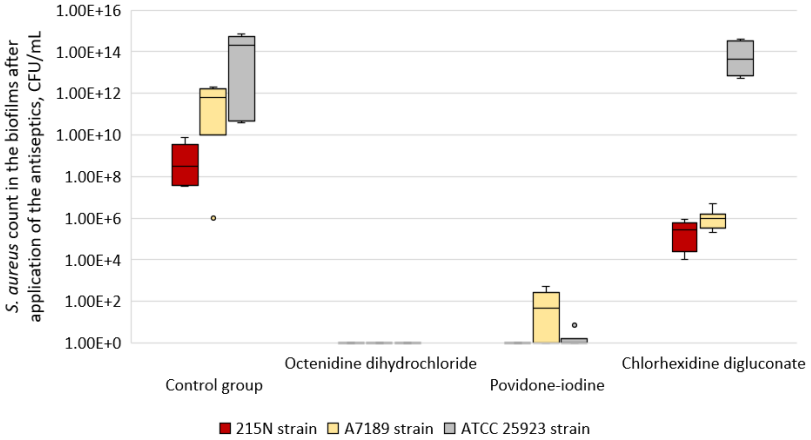

Figure 8. The antimicrobial efficacy of different antiseptic solutions on *S. aureus* biofilms, grown in a MLM. After the application of octenidine dihydrochloride the bacteria of all three strains of *S. aureus* were eradicated (p=0.002). After the application of povidone-iodine, no bacteria of 215N strain were found (p=0.002); the number of bacteria of A7189 and ATCC 25923 strains significantly decreased (p=0.002). After the application of chlorhexidine digluconate the number of bacteria of 215N and A7189 strains significantly decreased (p=0.002 and p=0.02, respectively), but did not change for ATCC 25923 strain (p=0.82).

3.1.4 PVGI on scanning electron microscopy

Due to the components of the medium, which resemble a wound *in vivo*, a modified Lubbock's model was used to design an innovative model of infected vascular graft *in vitro*. After the time of incubation, the patches of a non-impregnated woven vascular prosthesis (Figure 9 A) were successfully colonized by *S. aureus* biofilms (Figure 9 B, C and D) and treated with antiseptic solutions.

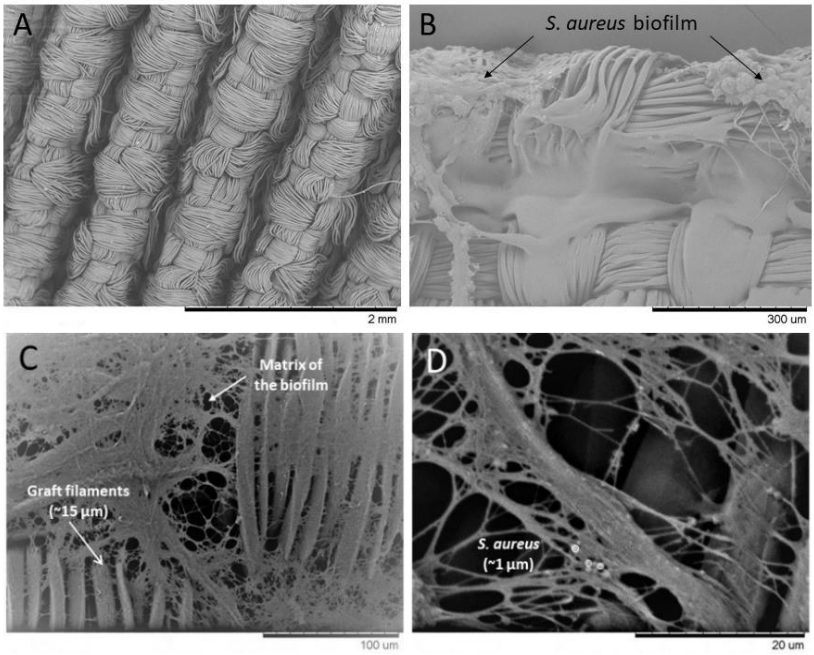

Figure 9. Images of a non-impregnated woven vascular graft (Dacron) by SEM. A – image of a vascular graft at 50x; B – image of a vascular graft with *S. aureus* biofilm at 150x; C – matrix of the biofilm at 800x; D – cocci at 5000x.

3.1.5 Evaluation of the antimicrobial efficacy of different antiseptic solutions on *S. aureus* biofilms in PVGI model *in vitro*

The CFUs of *S. aureus* in the biofilms on the vascular grafts were enumerated for the control groups of all the strains (Table 5) and then compared to the number of CFUs left in the biofilms after the application of the antiseptics.

Table 5. *S. aureus* CFUs in the biofilms, produced in a MLM on the vascular grafts

S. aureus strain	*S. aureus* in the biofilms on the vascular grafts, CFU/mL, $x10^{14}$			
	Minimum	Maximum	Median	IQR*
215N	1.20	8.00	3.04	4.10
A7189	1.00	9.00	4.50	6.50
ATCC 25923	3.00	8.00	7.50	1.00

*IQR – interquartile range

After the application of antiseptic solutions on the grafts with *S. aureus* biofilms, the results were as follows:

- in comparison with the control group, all three antiseptic solutions demonstrated a similar antimicrobial efficacy in a newly designed PVGI model *in vitro* for all three *S. aureus* strains (p=0.002);
- *S. aureus* counts in the biofilms on the vascular grafts decreased by seven orders of magnitude after application of all three antiseptic solutions;
- the efficacy of 0.1% octenidine dihydrochloride, 10% povidone-iodine and 0.02% chlorhexidine digluconate was similar for *S. aureus* strains A7189 and ATCC 25923 (p>0.05);
- 0.1% octenidine dihydrochloride was more effective than 10% povidone-iodine (p=0.02) and 0.02% chlorhexidine dihydrochloride (p=0.04) for *S. aureus* 215N strain. The efficacy of povidone-iodine and chlorhexidine digluconate was similar (p=0.94);

- octenidine dihydrochloride was more effective for *S. aureus* 215N strain than for ATCC 25923 strain (Mann Whitney U test, p=0.02). The results of the experiment are represented in Figure 10.

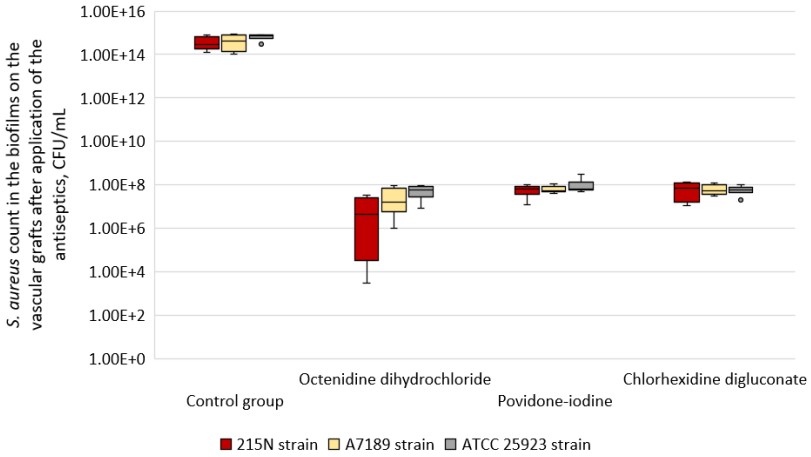

Figure 10. The antimicrobial effect of different antiseptic solutions on *S. aureus* biofilms, grown in a MLM on the vascular grafts. In comparison with control group, all antiseptic solutions demonstrated similar antimicrobial effect in a newly designed PVGI model *in vitro* (p=0.002).

3.2 The results of *in vivo* experiments

3.2.1 The final sample size

One rat, which was randomly assigned to the control group, did not recover from anaesthesia (possible anaesthetic overdose). Two rats, which were randomly assigned to povidone-iodine group and one rat, which was assigned to chlorhexidine's group did not reach adequate level of suppuration (based on the results of microbiological tests on day six of the experiment before starting the treatment) and were excluded from the study. One rat, which was assigned to chlorhexidine's group, died on day eight of the experiment. The loss of appetite and body mass (from 355 g to 188 g), reduced physical activity and pus around the eye area distinguished this rat from the other rodents.

The final sample size in the first series of experiments was 43 rats:

- 12 rats in octenidine dihydrochloride's group;
- 10 rats in povidone-iodine's group;
- 10 rats in chlorhexadine digluconate's group;
- 11 rats in control group.

In the second series of *in vivo* experiments the final sample size was 10 rats.

3.2.2 Macroscopic findings in the first series of *in vivo* experiments

During the time of the first series of experiments the fluctuations of mean body temperature for rats remained in the normal range (35.8-37.5°C) and did not differ significantly between the study groups. The mean body mass for the rats on day one of the experiment was 317.6±48.1 g. The experimental groups were homogenous according to the body mass (dispersion analysis, $p=0.97$). The changes in rats' body mass during the course of the study are represented in Table 6.

On day six of the experiments all rodents presented with a hardened swelling at the incision site (Figure 11). There were no suture leaks or

eritema. Some rats had developed crusts at the incision site and cutaneous fistulas with purulent discharge. Once wound irrigations with antiseptic solutions were started, all rats developed crusts at the site of the abscess and cutaneous fistulas.

Table 6. The changes in rats' body mass, related to the antiseptic used for the treatment of PVGI

Antiseptic solution	Octenidine dihydrochloride	Povidone-iodine	Chlorhexidine digluconate	Control group
Mean body mass at day 1, g	318.5±40.1	323.1±58.2	312.5±48.6	316.2±52.0
Mean body mass at day 10, g	299.3±44.1	306.2±60.6	276.4±54.3	313.9±50.0
Mean change of body mass, g	-19.3±59.6	-16.9±83.9	-36.1±72.9	-2.3±72.1
Effect's size, Cohen's d	-0.32	-0.20	-0.49	-0.03
Mean change of body mass, %	-5.9	-5.6	-11.5	-0.1
p value	0.05	0.004	0.04	0.57

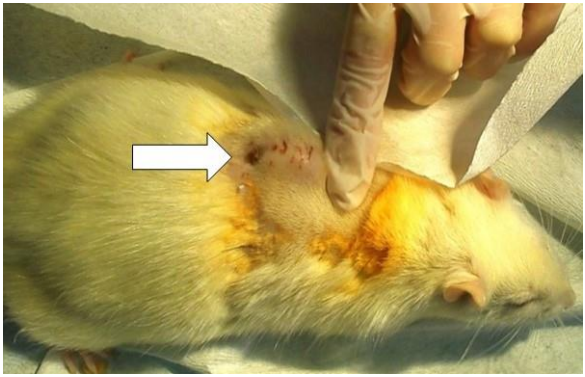

Figure 11. A hardened swelling and cutaneous fistula with purulent discharge at the site of a vascular graft implantation on day six of the experiment (before starting the treatment).

3.2.3 Microbiological findings of the first series of *in vivo* experiments

The efficacy of the antiseptics in treatment of *S. aureus* infected wounds with implanted patches of vascular grafts for rats was evaluated by comparing the changes of *S. aureus* CFUs in the wound washouts before the beginning of treatment and after the course of treatment. In four days of treatment, the number of *S. aureus* CFU/mL in the wound washouts significantly decreased in all treatment groups, but insignificantly increased in control group (Table 7, Figure 12). The best results were achieved by treatment with octenidine dihydrochloride – the count of bacteria in the wound washouts decreased by 99.98% ($p<0.001$).

Table 7. The results of microbiological tests from rats' wound washouts

Antiseptic solution	CFU/mL before the treatment, M (IQR)*	CFU/mL after the treatment, M (IQR)*	Change in CFU, %	p value
Control group	$1.13x10^7$ ($1.63x10^7$)	$9.70x10^6$ ($1.61x10^7$)	↑19.72	0.77
Octenidine dihydrochloride	$1.77x10^7$ ($1.17x10^7$)	$1.14x10^3$ ($2.91x10^3$)	↓99.98	<0.001
Povidone-iodine	$1.59x10^7$ ($1.41x10^7$)	$9.39x10^4$ ($2.97x10^5$)	↓90.73	0.002
Chlorhexidine digluconate	$1.22x10^7$ ($2.19x10^7$)	$2.16x10^6$ ($3.17x10^6$)	↓65.97	0.004

*M – median; IQR – interquartile range

In comparison with the control group, only the treatment with octenidine dihydrochloride significantly reduced *S. aureus* CFU counts in the wound washouts ($p=0.02$). The efficacy of povidone-iodine came close to the limit of statistical significance ($p=0.06$). The efficacy of chlorhexidine digluconate did not differ from sterile saline in the control group ($p=0.13$).

Total eradication of *S. aureus* from the wound washouts was achieved for two rats, treated with octenidine dihydrochloride and one rat, treated with povidone-iodine. High counts of *S. aureus* (variating from $1.08x10^2$ to $8.05x10^7$ CFU/mL) were detected on the patches of vascular grafts removed from the wounds. No correlation was found

between *S. aureus* cell counts found on the grafts and in the wound washouts for the same rat.

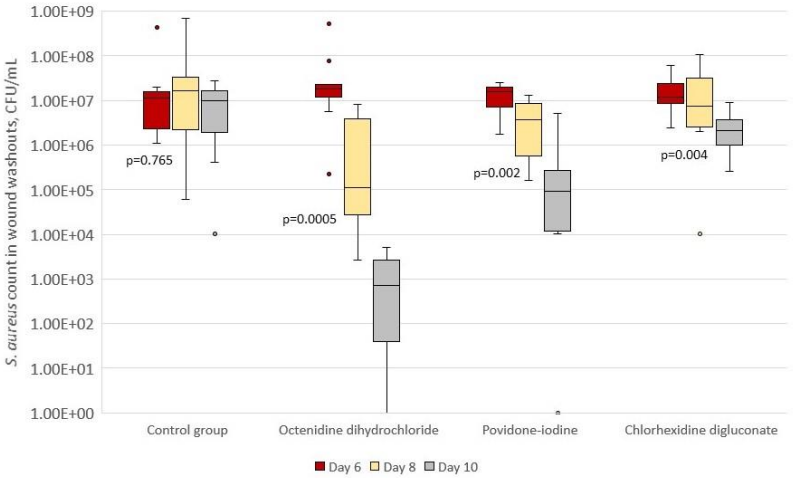

Figure 12. The changes in *S. aureus* counts in the wound washouts during the time of treatment. In four-day treatment course the count of *S. aureus* CFUs decreased for rats, treated with octenidine (n=12, p<0.001), povidone-iodine (n=10, p=0.002) and chlorhexidine (n=10, p=0.004). A nonsignificant increase of *S. aureus* CFUs was registered in the control group (n=11, p=0.77).

3.2.4 The second series of *in vivo* experiments

An additional series of experiments was run with a group of 10 rats. All of them were treated with octenidine dihydrochloride. All rodents survived the surgery. No significant fluctuations in body temperature were registered.

On day six of the experiment a hardened swelling was observed at the site of the incision for all rodents. There was no eritema around the suture line. For most of the rats there was a crust at the incision site. Two rats presented with cutaneous fistulas. From day 10 of the experiment all rodents presented with crusts of varying sizes and

purulent fistulas (Figure 13). At day 12 of the experiment a massive excretion of purulent discharge was noticed for all rodents through the fistulas.

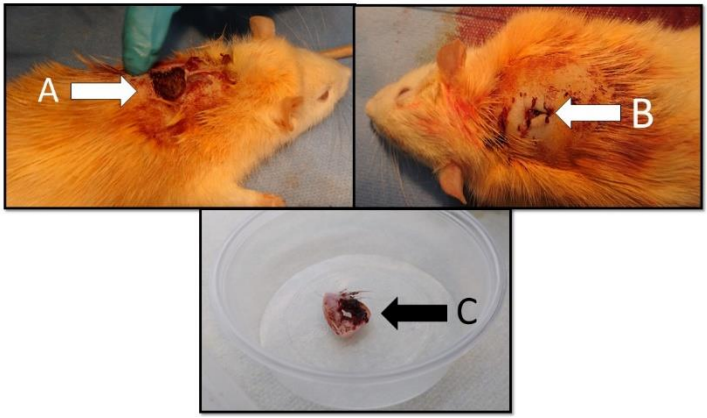

Figure 13. Macroscopic findings for a rat, treated with octenidine dihydrochloride (day 13 of the experiment): A – a crust at the incision site; B – a cutaneous fistula at the incision site; C – a cutaneous fistula, resected for histological and microbiological evaluation.

A significant reduction of the body mass by 9.27% was registered during the course of the experiment (paired samples T test, $p<0.001$). The mean body mass at day one of the experiment was 267.3±19.6 g, and at day 13 – 242.2±18.3 g.

Before starting wound irrigations with octenidine dihydrochloride the median of *S. aureus* CFU/mL in the wound washouts was 1.50×10^6 (IQR=6.17×10^6 CFU/mL). After completing a seven-day treatment course, *S. aureus* was eradicated from the wound washouts for nine out of 10 rats. For one rat 15 CFU/mL were detected in the wound washouts (Figure 14). The results of the treatment showed high statistical significance (Wilcoxon signed-rank test, $p=0.002$).

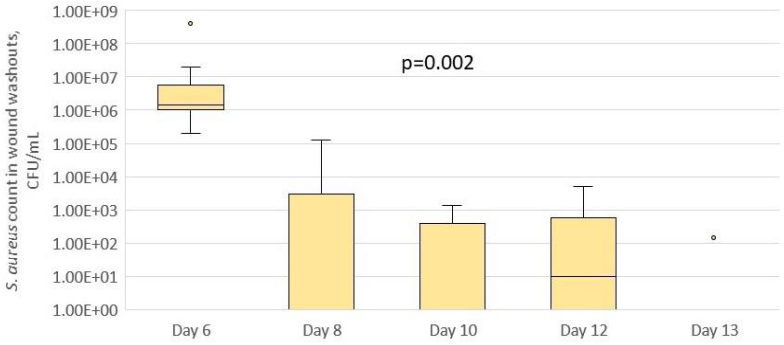

Figure 14. The results of the second series of *in vivo* experiments. A seven-day treatment course resulted in a significant decrease of *S. aureus* CFUs in the wound washouts (n=10, p=0.002). *S. aureus* was eradicated for nine out of 10 rats. For one rat 15 CFU/mL of *S. aureus* were detected in the wound washouts on day 13.

At the end of the experiment (day 13) the patches of vascular graft were explanted for seven rats. For three other rats the patches of vascular graft had been expelled from the wounds through the cutaneous fistulas before the termination of the experiment, and thus were not microbiologically evaluated. For one rat, no *S. aureus* was found on the graft. For six other rats the counts of *S. aureus* on the graft varied from 20 CFU/mL to $2x10^7$ CFU/mL ($M=8.00x10^1$, $IQR=8.33x10^6$ CFU/mL).

A digital analysis of Gram-stained cytological smears (Figure 15) revealed that higher number of inflammatory cells in the wound washouts on day 13 significantly correlated with shorter *S. aureus* eradication time from the wound washouts (number of days, taken for *S. aureus* CFU/mL in the wound washouts to decrease to zero) (Spearman's correlation, r=-0.74, p=0.01, Figure 16).

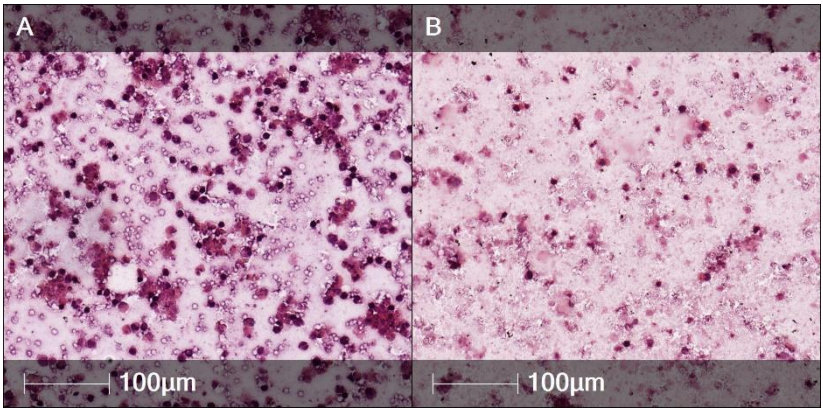

Figure 15. Gram-stained cytological smears of the wound washouts. A – dense infiltration of inflammatory cells; B – moderate density of inflammatory cells.

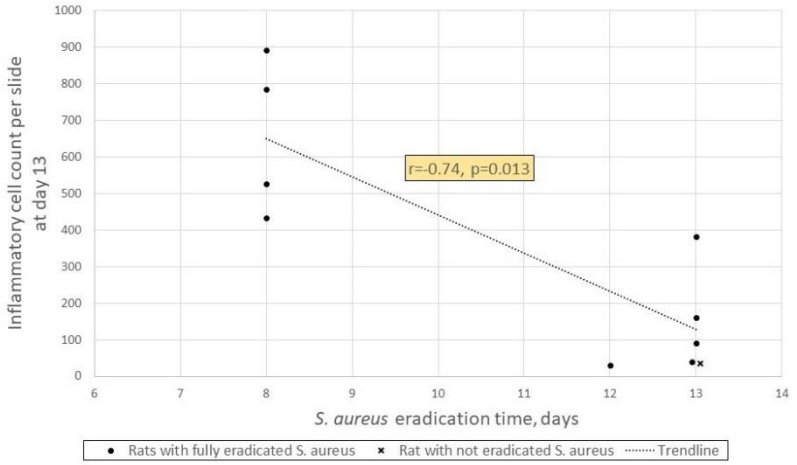

Figure 16. Correlation between high inflammatory cell count in the wound smears and faster *S. aureus* eradication time from the wound washouts. Spearman's correlation, r=-0.74, p=0.01.

No dissemination of *S. aureus* into the bloodstream was detected. *S. aureus* was detected in nine out of 10 samples of the cutaneous fistulas (M=1.20x10^3, IQR=2.85x10^4 CFU/mL/g).

The area of necrosis in the samples of cutaneous fistulas varied from 1.87% to 49.07% (M=17.33%, IQR=17.14%) without significant relation to the rats' level of suppuration or response to treatment. Two samples of fistulas presented with no inflammation, two – with minor polymorphonuclear (PMN) infiltration, four – with mild PMN infiltration and two – with dense PMN infiltration. The grade of inflammation did not correlate with the area of necrosis in the fistula tissues (Spearman's correlation, r=-0.07, p=0.84). Histological images of the cutaneous fistulas are shown in Figure 17.

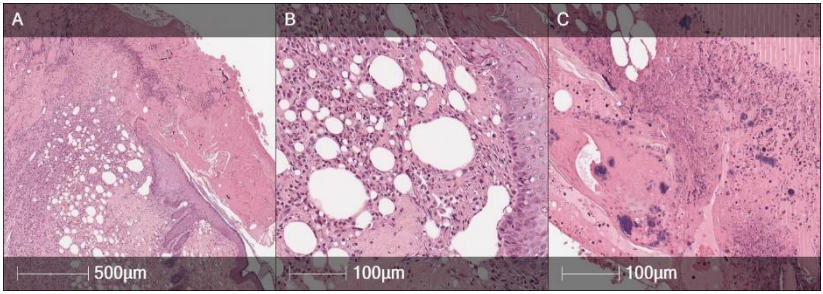

Figure 17. Images of hematoxylin and eosin-stained cutaneous fistulas. A – epidermis with a crust; B – PMN infiltration in the derma; C – clusters of bacteria in the crust.

The area of tissue necrosis in soft tissue specimens varied from 0 to 22.48% (M=1.50%, IQR=11.59%). According to the type and severity of the inflammation four rats had developed xanthogranulomatous inflammation, three rats had mild, one had minor and two had no PMN infiltration (Table 8, Figure 18).

Table 8. Histological evaluation of the soft tissue samples for rats treated with octenidine dihydrochloride

Rat number	The area of necrosis in soft tissue specimen, %	Type of inflammation in soft tissue specimen
1	1.90	Minor PMN
2	0	Xanthogranulomatous
3	11.58	Xanthogranulomatous
4	0	Xanthogranulomatous
5	1.17	None
6	20.77	Minor PMN
7	0	Xanthogranulomatous
8	0	None
9	22.48	Mild PMN
10	10.04	Minor PMN

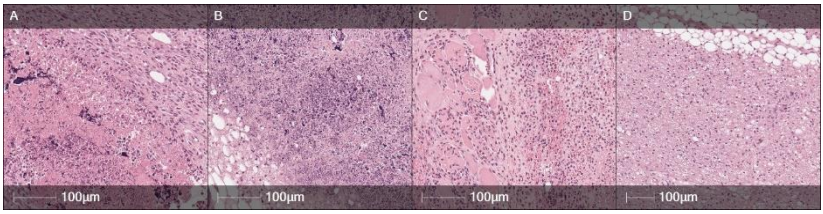

Figure 18. Images of hematoxylin and eosin-stained soft tissue specimens. A – soft tissue necrosis (left), PMN infiltration, proliferation of fibroblasts (right); B – soft tissue necrosis; C – PMN infiltration in the fibers of skeletal muscle (left), red blood cells and PMN infiltration (right); D – infiltration of macrophages (xanthogranulomatous inflammation).

DISCUSSION

Despite all efforts to treat PVGI, it still remains a disastrous complication in vascular surgery. This research was designed to investigate one of PVGI's conservative treatment options – wound irrigation with antiseptic solutions, as its role in the treatment of PVGI is not yet defined. The efficacy of three commonly used antiseptic solutions was tested in an innovative *in vitro* setting, mimicking PVGI and in an *in vivo* setting for rats with implanted patches of vascular graft. The biofilm-producing strains of *S. aureus* were chosen to induce the infection, as bacterial biofilms are recognized as one of the main reasons of ineffective antimicrobial treatment and infection recurrence. In clinical cases, bacterial biofilms are frequently found to be situated on implants, such as vascular grafts [24, 25]. The formation of a biofilm on a vascular prosthesis is one of the main hassles in the treatment of PVGI, because bacteria in a biofilm are more resistant to the host's immune system and antimicrobials.

In this research Lubbock's chronic wound pathogenic biofilm model [28] was successfully modified and used to design a PVGI model *in vitro.* Although all of the tested antiseptics were able to suppress the multiplying of bacteria on the surface of the vascular graft, their effectiveness was much lower than in the experiments, performed in the same media on *S. aureus* biofilms without vascular prostheses. The results of this study confirm the hypothesis that the presence of the vascular graft in a wound and in an *in vitro* setting as well increases the surface area for bacterial adhesion and worsens the susceptibility of the antiseptic.

Further research on antiseptics' ability to fight biofilm-producing *S. aureus* on a vascular graft was continued in an *in vivo* setting on Wistar rats. We succeded in producing a flourishing infection of a surgical wound, which was confirmed by clinical findings (formation of subcutaneous abscesses and cutaneous fistulas with purulent discharge), microbiological and cytological tests (*S. aureus* and inflammatory cells in the wound washouts) and histological analysis

of cutaneous and perigraft tissues (infiltration of inflammatory cells – PMN leukocytes as signs of acute inflammation and/or foamy macrophages as signs of xanthogranulomatous inflammation, clusters of bacteria in the crusts and tissue necrosis).

Despite an outstanding effect of octenidine dihydrochloride which managed to eradicate 99-100% of *S. aureus* CFUs from the wound washouts in a seven-day treatment course, eradication of the infection from the graft itself was only detected for one rat out of 10. Using the same methodology and octenidine dihydrochloride as an irrigation solution, we have managed to sterilize one more vascular graft for a rat in the previous studies [29].

The duration of our study was limited due to rats' tendency to heal on their own. Nevertheless, high counts of inflammatory cells for rats in the control group remained in the wound washouts until the end of the experiment, so we are certain that rats did not start to recover during the course of the experiment.

It is important to note that experimental models *in vitro* and animal models as well cannot clearly represent a clinical situation of PVGI, so drawing conclusions about antiseptics' efficacy in treatment of human PVGI based solely on our findings would be inaccurate. In order to establish this as an effective treatment method of PVGI clinical studies are necessary.

CONCLUSIONS

1. A significant biofilm-disrupting activity on *S. aureus* biofilms *in vitro* was observed exclusively after the application of 0.02% chlorhexidine digluconate solution.
2. In a simulated wound environment, containing blood components, the strongest antimicrobial efficacy on *S. aureus* biofilms was observed by 0.1% octenidine dihydrochloride solution. The efficacy of 10% povidone-iodine was almost as great as octenidine's, but the effect of chlorhexidine digluconate was poor.
3. Lubbock's chronic wound pathogenic biofilm model was modified and adapted to design a novel PVGI model *in vitro* with woven Dacron graft and biofilm-producing strains of *S. aureus*. The formation of the biofilms on the vascular grafts was confirmed by SEM. All three tested antiseptics presented with similar antimicrobial efficacy in a newly developed model of PVGI.
4. A new *in vivo* model of PVGI was designed for Wistar rats, using a biofilm-producing *S. aureus* 215N strain and a woven Dacron graft. 0.1% octenidine dihydrochloride solution, applied by daily irrigation, proved to be the most effective antiseptic solution, treating suppurative surgical wounds with *S. aureus* infected patches of Dacron vascular grafts in rats.
5. A seven-day treatment course with 0.1% octenidine dihydrochloride was sufficient to eradicate *S. aureus* from the wound washouts for nine out of 10 rats and one vascular graft. Cytological and histological analysis of the perigraft tissues confirmed active inflammation in the perigraft tissues.

PRACTICAL RECOMMENDATIONS

1. For researchers studying the effectiveness of local antiseptics and antibiotics on bacterial biofilms we recommend using our newly-designed experimental models *in vitro* and *in vivo.*
2. For physicians treating *S. aureus* infected wounds with a fair amount of organic matter (leucocytes, detrite) we recommend applying wound irrigations with 0.1% octenidine dihydrochloride or 10% povidone-iodine solutions.
3. For vascular surgeons treating a PVGI, caused by *S. aureus,* we recommend applying wound irrigations with 0.1% octenidine dihydrochloride solution. Once the wound is sterilised (based on microbiological tests from the wound washouts), infected vascular graft may be replaced with another vascular substitute.

FUTURE PERSPECTIVES

Based on the results and repeatability of our experiments, our newly-designed *in vitro* and *in vivo* models for studying antiseptics' efficacy on the treatment of PVGI proved to be successful. Since curing PVGI remains a challenge, we believe that it would be useful to continue our research further.

Studying the efficacy of antiseptic solutions on *S. aureus* biofilms revealed strong biofilm-disrupting activity of chlorhexidine digluconate and outstanding antimicrobial efficacy of octenidine dihydrochloride and povidone-iodine. Given the possibility that antiseptics could act synergically, it would be useful to try using a combination of these antiseptics on biofilm-covered vascular grafts *in vitro*.

Since a single application of antiseptics suppressed the multiplying of bacteria, but was not sufficient to eradicate *S. aureus* biofilms from the surface of the vascular grafts, we believe that it would be useful to carry out a new series of *in vitro* experiments, mimicking PVGI, and apply antiseptics in a repetitive mannier to achieve bacterial eradication from the surface of the vascular grafts.

During *in vivo* experiments, outstanding results were achieved using octenidine dihydrochloride solution for irrigation of infected surgical wounds with patches of vascular graft. A seven-day treatment course resulted in nine out of 10 sterile wound cultures from the wound washouts. Despite this, only one vascular graft was found sterile. Therefore, we believe that repeating the experiment and increasing the sample size wound be beneficial. First, it would reveal if sterilising one vascular graft was a single case success, or the number of sterilised grafts increases with the sample size. Second, after obtaining negative cultures from the wound washouts it would be useful to resect all perigraft tissues and to perform their microbiological analysis. After making sure there were no residing bacteria, it would be possible to implant a new sterile patch of a vascular graft (bare, silver-coated or rifampicin-impregnated) *in situ*

and observe, if it stays uninfected. Once wound irrigation with octenidine dihydrochloride is acknowledged to be able to sterilise the wound with a vascular graft and it is safe to implant a new vascular substitute *in situ*, we suggest starting a clinical study of PVGI and using octenidine dihydrochloride for wound irrigations.

In order to evaluate octenidine dihydrochloride's cytotoxic effect for graft surrounding tissues, it would be useful to form a control group of rats which wound be treated with sterile saline. After the treatment course it would be possible to compare histological analysis of the tissues treated with octenidine and sterile saline and distinguish the tissue damage caused by the infection from the cytotoxic effect of an antiseptic. It would allow us to evaluate the antiseptic and define its role in the treatment of PVGI.

SANTRAUKA LIETUVIŲ KALBA

Įvadas

Kraujagyslės protezo infekcija (KPI) yra gana reta, tačiau viena iš sunkiausiai gydomų komplikacijų kraujagyslių chirurgijoje. Ją patiria nuo 0,5 proc. iki 9,5 proc. visų ligonių, kuriems implantuojami sintetiniai kraujagyslių pakaitalai [1-8]. Negydant ši infekcija dažnai komplikuojasi gyvybei pavojingu kraujavimu ar sepsiu [9]. Tačiau net ir gydant neišvengiama pačių blogiausių išeičių – galūnių amputavimo (5-70 proc. visų atvejų) bei mirties (10-75 proc.) [4, 9-14].

Dažniausiai KPI sukelia fakultatyviniai anaerobai: stafilokokai, žarnyno lazdelės, enterokokai, salmonelės ir kt. Stafilokokai yra ypač dažni – jie sudaro nuo 24 iki 50 proc. visų KPI tiek ankstyvuoju, tiek vėlyvuoju pooperaciniu laikotarpiu [11, 15-18]. Kasdienėje kraujagyslių chirurgijos praktikoje ypač aktualus yra *Staphylococcus aureus* sukeltos KPI gydymas, nes ši bakterija ne tik dažniausiai sukelia KPI ankstyvuoju pooperaciniu laikotarpiu, bet ir yra linkusi formuoti bioplėveles, kurios dar labiau apsunkina gydymo eigą [2, 15-18].

Gydymo būdo, efektyvaus visais KPI atvejais, nėra. Įprastai rekomenduojama agresyvi chirurginė taktika: pašalinti infekcijos apimtus audinius kartu su pačiu kraujagyslės protezu, suformuoti ekstraanatominį šuntą, apeinant infekuotą vietą, skirti sisteminę antibiotikų terapiją didelėmis dozėmis [4]. Tačiau ši gydymo taktika ne visada įmanoma, ypač kai infekcijos apimtas protezas yra įsiūtas į aortą ar kitas gyvybiškai svarbias arterijas, kai ligonis išsekęs nuo gretutinių ligų ir neatlaikytų didelės apimties operacijos arba kai infekcijos sukėlėjas yra atsparus daugeliui antimikrobinių medžiagų. Dėl šių priežasčių tam tikrais atvejais bandoma taikyti konservatyvius gydymo metodus, leidžiančius išsaugoti protezą: ilgalaikę sisteminę antibiotikų terapiją, neigiamo slėgio žaizdų terapiją ir vietinį žaizdos drenažą nuolatiniais arba ciklišKais plovimais [1, 3, 9, 20]. Žaizdos

gali būti plaunamos steriliu 0,9 proc. natrio chlorido tirpalu [21], antibiotikų arba antiseptikų tirpalais.

Nors antibiotikai yra neatsiejama KPI gydymo dalis, taikant sisteminę antibiotikų terapiją susiduriama su keliomis problemomis: pirma, dauguma antibiotikų sunkiai prasiskverbia pro bakterijų suformuotas bioplėveles, antra – vis dažniau pasitaiko antibiotikams atsparių bakterijų padermių. Šiandien pranašesnės tampa kitos antimikrobinės medžiagos – antiseptikai, nes bakterijų atsparumas jiems išlieka minimalus [23].

Darbo tikslas

Įvertinti įprastai klinikinėje praktikoje naudojamų antiseptinių tirpalų efektyvumą, veikiant *Staphylococcus aureus* užkrėstą austą kraujagyslės protezą *in vitro* ir *in vivo*.

Darbo uždaviniai

1. Palyginti skirtingų *S. aureus* padermių bioplėvelių struktūrą ardantį 0,1 proc. oktenidino dihidrochlorido, 10 proc. povidono-jodo ir 0,02 proc. chlorheksidino digliukonato poveikį *in vitro.*
2. Palyginti 0,1 proc. oktenidino dihidrochlorido, 10 proc. povidono-jodo ir 0,02 proc. chlorheksidino digliukonato antimikrobinį veiksmingumą skirtingų *S. aureus* padermių suformuotoms bioplėvelėms *in vitro* sąlygomis imituojant žaizdos aplinką.
3. Sukurti eksperimentinį infekuoto austo kraujagyslės protezo modelį *in vitro* ir palyginti 0,1 proc. oktenidino dihidrochlorido, 10 proc. povidono-jodo ir 0,02 proc. chlorheksidino digliukonato tirpalų antimikrobinį veiksmingumą skirtingų *S. aureus* padermių bioplėvelėms ant kraujagyslės protezo lopinėlių.

4. Sukurti eksperimentinį infekuoto austo kraujagyslės protezo modelį *in vivo* Wistar klono žiurkėms ir palyginti 0,1 proc. oktenidino dihidrochlorido, 10 proc. povidono-jodo ir 0,02 proc. chlorheksidino digliukonato tirpalų veiksmingumą, gydant *S. aureus* užkrėstas žiurkių žaizdas su kraujagyslės protezo lopinėliais.
5. Įvertinti žiurkių gydymo trukmę, reikalingą pašalinti *S. aureus* iš kraujagyslės protezo aplinkos, atlikti citologinę bei histologinę žaizdos audinių analizę.

Ginamieji teiginiai

1. Antiseptiniai tirpalai *in vitro* sąlygomis geba paveikti *S. aureus* bioplėveles.
2. Žaizdų plovimas antiseptikais yra efektyvus metodas, gydant *S. aureus* sukeltą žaizdos infekciją su kraujagyslės protezu.

Tyrimo naujumas

Sparčiai besivystant bakterijų atsparumui antimikrobinėms medžiagoms, ieškoma alternatyvių būdų, kaip kovoti su infekcijomis. Konservatyvus KPI gydymo metodas – vietinis žaizdos plovimas antiseptiniais tirpalais – vis labiau diegiamas į praktiką, tačiau visuotinai neįsitvirtinęs kovos su KPI būdas. Išnagrinėjus šia tema paskelbtas publikacijas pastebėta, kad trūksta reprezentatyvių, žaizdai artimų *in vitro* modelių, kurie padėtų įvertinti antiseptikų poveikį bioplėveles formuojančiam *S. aureus* ant kraujagyslės protezų. Taip pat nerasta paskelbtų tyrimų, kur antiseptiniai tirpalai *in vivo* sąlygomis būtų panaudojami KPI gydymui, o ne profilaktikai.

S. aureus bioplėveles ardančio bei antimikrobinio antiseptikų poveikio vertinimui sukurtas naujas *in vitro* modelis, artimas žaizdai ir KPI. Tam, kad būtų įvertintas dar plačiai klinikinėje praktikoje netaikomas konservatyvus KPI gydymo metodas – žaizdų plovimas

antiseptiniais tirpalais – sukurtas naujas eksperimentinis modelis, panaudojant Wistar klono žiurkes.

Darbo metodika

In vitro eksperimentai, tyrinėjantys antiseptikų efektyvumą veikiant *S. aureus* bioplėveles, atlikti VU MF Biomedicinos mokslų instituto Fiziologijos, biochemijos, mikrobiologijos ir laboratorinės medicinos katedros Mikrobiologijos skyriuje ir Lietuvos mokinių neformaliojo švietimo centro Jaunojo tyrėjo laboratorijoje 2017 m. gegužės – 2018 m. spalio mėn. Eksperimentams atrinktos trys gausiai bioplėveles formuojančios *S. aureus* padermės: 215N, A7189 ir ATCC 25923. Bioplėvelėms susiformuoti ir antiseptikų veiksmingumui testuoti sukurtos skirtingos aplinkos sąlygos: mitybinė terpė, žaizdos aplinką imituojanti terpė su kraujo komponentais ir žaizdos aplinką imituojanti terpė su kraujo komponentais bei austo kraujagyslės protezo lopinėliais. Bioplėvelės veiktos 0,1 proc. oktenidino dihidrochlorido, 10 proc. povidono-jodo ir 0,02 proc. chlorheksidino digliukonato tirpalais. Kontrolinei grupei naudotas fosfatinio buferio tirpalas (FBT). Eksperimentų metu vertintas antiseptikų poveikis bioplėvelių struktūrai ir bakterijų gyvybingumui.

In vivo eksperimentai, tyrinėjantys antiseptikų veiksmingumą gydant infekuotas chirurgines žiurkių žaizdas su kraujagyslės protezais, vykdyti Valstybinio mokslinių tyrimų instituto Inovatyvios medicinos centro Imunologijos departamente, VU Gyvybės mokslų centre ir VU MF Fiziologijos, biochemijos, mikrobiologijos ir laboratorinės medicinos katedros Mikrobiologijos skyriuje 2012 m. vasario mėn. – 2018 m. liepos mėn. Prieš pradedant eksperimentą, visi dalyvavę tyrėjai išklausė vieno semestro trukmės Laboratorinių gyvūnų mokslo kursus ir gavo leidimus dirbti su laboratoriniais gyvūnais. 2012 m. sausio 4 d. ir 2016 m. balandžio 25 d. gauti Valstybinės maisto ir veterinarijos tarnybos leidimai (Nr. 0222 ir G2-43) tyrimams atlikti.

Eksperimentui naudotos Wistar klono abiejų lyčių žiurkės. Visoms žiurkėms po oda implantuoti austo poliesterinio kraujagyslės protezo lopinėliai. Žaizdos infekuotos bioplėvelę formuojančia *S. aureus* paderme. Tyrimo metu įvertintas konservatyvaus KPI gydymo būdo – žaizdų plovimo antiseptiniais tirpalais veiksmingumas. Tyrimui pasirinkti 0,1 proc. oktenidino dihidrochlorido, 10 proc. povidono-jodo ir 0,02 proc. chlorheksidino digliukonato tirpalai. Kontrolinei grupei naudotas sterilus 0,9 proc. natrio chlorido tirpalas.

Identifikuojant *S. aureus* kolonijas remtasi latekso agliutinacijos, plazmakoaguliazės testais, šviesine mikroskopija. Kontroline *S. aureus* paderme pasirinkta ATCC 29213.

Statistinė duomenų analizė

Duomenys išanalizuoti naudojant parametrinius ir neparametrinius statistinius testus. Parametriniai testai naudoti tais atvejais, kai buvo galima taikyti normaliojo duomenų pasiskirstymo prielaidą – taikytas dviejų suporuotų imčių t-testas bei dviejų atskirų imčių t-testas. Kitais atvejais naudoti neparametriniai testai: Wilcoxon t-testas analizuojant dvi suporuotas imtis, Mann – Whitney U testas analizuojant dvi nesuporuotas imtis ir Kruskal – Wallis H testas analizuojant daugiau negu dvi imtis tarpusavyje. Duomenų koreliacija įvertinta Spearman's Rho testu. Skaičiavimai atlikti naudojantis SPSS 24.0 bei Microsoft Excel 2016 programomis. Pasirinkta statistinio reikšmingumo riba – $p < 0,05$.

Rezultatai

In vitro eksperimentų rezultatai

Remiantis kokybine ir kiekybine (spektrofotometrine) skirtingų *S. aureus* padermių bioplėvelių formavimo analize, gausiai bioplėveles formuojančios padermės (215N, A7189 ir ATCC 25923) buvo atrinktos tolimesniems *in vitro* tyrimams.

Ant dengiamųjų stikliukų mitybinėje terpėje susiformavus trijų skirtingų padermių *S. aureus* bioplėvelėms ir paveikus jas antiseptiniais tirpalais, reikšmingas bioplėvelę ardantis poveikis stebėtas tik po veikimo chlorheksidinu (Kruskal – Wallis H testas, 215N padermei $p = 0,02$, A7189 padermei $p < 0,001$, ATCC 25923 padermei $p = 0,02$).

Trijų skirtingų *S. aureus* padermių bioplėvelėms susiformavus modifikuotoje Lubbock'o terpėje ir paveikus jas antiseptikais nustatyta, kad po bioplėvelių ekspozicijos oktenidino dihidrochloridu, 215N, A7189 ir ATCC 25923 padermių *S. aureus* bakterijų nerasta ($p = 0,002$). Po ekspozicijos povidono-jodu 215N padermės bakterijų nerasta ($p = 0,002$), A7189 ir ATCC 25923 padermių bakterijų žymiai sumažėjo ($p = 0,002$). Po ekspozicijos chlorheksidino digliukonatu 215N ir A7189 padermių bakterijų žymiai sumažėjo (atitinkamai $p = 0,002$ ir $p = 0,02$), o ATCC 25923 padermei bakterijų skaičius nepakito ($p = 0,82$).

Modifikavus ir adaptavus Lubbock'o modelį sukurtas inovatyvus infekuoto austo kraujagyslės protezo modelis *in vitro*. Bioplėveles ant kraujagyslės protezų lopinėlių paveikus antiseptiniais tirpalais, lyginant su kontroline grupe, visoms *S. aureus* padermėms visi trys testuoti antiseptikai pademonstravo efektyvų antimikrobinį veikimą – visų naudotų antiseptikų atveju *S. aureus* bakterijų ant kraujagyslės protezo sumažėjo septyniomis eilėmis ($p = 0,002$).

In vivo eksperimentų rezultatai

Dėl anestetikų perdozavimo, nepasiekus adekvataus supūliavimo lygio ar nugaišus iki eksperimento pabaigos į galutinę analizę dalis žiurkių neįtrauktos. Galutinis imties dydis pirmojo etapo eksperimentuose – 43 žiurkės: 12 žiurkių oktenidino dihidrochlorido, 10 – povidono-jodo, 10 – chlorheksidino digliukonato ir 11 – kontrolinėje grupėje. Antrojo etapo eksperimentuose, kur testuotas vien oktenidino dihidrochlorido veikimas, galutinis imties dydis – 10 žiurkių.

Antiseptikų veiksmingumas, gydant *S. aureus* infekuotas žiurkių žaizdas su kraujagyslės protezo lopinėliais vertintas lyginant *S. aureus* kolonijas formuojančių vienetų (KFV) skaičiaus pokyčius žaizdų nuoplovose prieš pradedant gydymą ir baigus gydymo kursą. Per keturias gydymo antiseptikais dienas KFV/ml žaizdų nuoplovose reikšmingai sumažėjo visose antiseptikais gydytų žiurkių grupėse, tačiau kontrolinėje grupėje – nereikšmingai padidėjo. Geriausių rezultatų pasiekta gydant oktenidino dihidrochloridu – bakterijų skaičius žaizdų nuoplovose gydymo kurso metu sumažėjo 99,98 proc.

Lyginant su kontroline grupe, tik gydymas oktenidino dihidrochloridu statistiškai reikšmingai sumažino *S. aureus* KFV skaičių žaizdų nuoplovose ($p = 0,02$). Povidono-jodo veiksmingumas buvo arti statistinio reikšmingumo ribos ($p = 0,06$), o gydymo chlorheksidinu rezultatai nesiskyrė nuo kontrolinės grupės ($p = 0,13$).

Visiškas *S. aureus* išnaikinimas iš žaizdų nuoplovų buvo mikrobiologiškai patvirtintas dviem žiurkėms, gydytoms oktenidinu, ir vienai žiurkei, gydytai povidono-jodu. Dideli *S. aureus* KFV kiekiai (svyruojantys nuo $1,08 \times 10^2$ iki $8,05 \times 10^7$ KFV/ml) buvo aptikti po gydymo kurso pašalintuose kraujagyslės protezų lopinėliuose. Nerasta ryšio tarp *S. aureus* kiekio, rasto ant kraujagyslės protezų lopinėlių, ir tos pačios žiurkės *S. aureus* kiekio žaizdų nuoplovose.

Atlikus papildomą analogiškų eksperimentų seriją, gydymui naudojant vien oktenidino dihidrochloridą, po septynių gydymo dienų devynioms iš dešimties žiurkių *S. aureus* bakterijos buvo išnaikintos iš žaizdų nuoplovų. Vienai žiurkei po gydymo kurso rasta 15 KFV/ml Gydymo rezultatai – aukšto statistinio patikimumo (Wilcoxon t-testas, $p = 0,002$).

Pabaigus eksperimentą (13-ąją dieną), septynioms žiurkėms iš žaizdų pašalinti kraujagyslės protezo lopinėliai. Kitoms trims žiurkėms kraujagyslės protezo lopinėliai išpūliavo ir iškrito pro fistules dar nepasibaigus eksperimentui, todėl nebuvo galimybės juos mikrobiologiškai ištirti.

Vienos žiurkės kraujagyslės protezo lopinėlyje nebuvo rasta nei vienos *S. aureus* bakterijos. Kituose šešiuose protezo lopinėliuose

KFV skaičius svyravo nuo 20 KFV/ml iki 2 x 10^7 KFV/ml (nurodoma mediana, M ir tarpkvartilinis plotis, TKP) (M = 8,00 x 10^1, TKP = 8,33 x 10^6 KFV/ml).

Skaitmeninė Gramo būdu dažytų neskiestų žaizdų nuoplovų citologinė analizė atskleidė, kad didesnis uždegiminių ląstelių skaičius 13-ąją eksperimento dieną reikšmingai koreliavo su trumpesniu *S. aureus* išnaikinimo laiku (dienų skaičiumi, užtrukusiu pašalinti *S. aureus* iš žaizdų nuoplovų iki 0) (Spearman'o koreliacija, r = -0,74, p = 0,01).

S. aureus diseminacijos kraujyje nerasta nei vienai žiurkei.

Bakterijų rasta devyniose iš dešimties odos-poodžio fistulių. *S. aureus* KFV skaičiaus mediana fistulėse buvo 1,20 x 10^3 (TKP = 2,85 x 10^4) KFV/ml/g.

Histologiškai nekrozės plotai odos-poodžio fistulių mėginiuose varijavo nuo 1,87 proc. iki 49,07 proc. (M = 17,33 proc., TKP = 17,14 proc.) be reikšmingo ryšio su žiurkės supūliavimo laipsniu ar atsaku į gydymą.

Histologiškai įvertinus fistulių bioptatus, dviejuose uždegimo nenustatyta (0 laipsnis), dviejuose rasta pavienė PMN infiltracija (I laipsnis), keturiuose – vidutinio tankio PMN infiltracija (II laipsnis), dviejuose – didelio tankio PMN infiltracija (III laipsnis). Uždegimo laipsnis nekoreliavo su nekrozės plotu fistulių audiniuose (Spearman'o koreliacija, r = -0,07, p = 0,84).

Nekrozės plotas minkštųjų audinių mėginiuose varijavo nuo 0 iki 22,48 proc. (M = 1,50 proc., TKP = 11,59 proc.). Pagal uždegimo tipą ir laipsnį, keturioms žiurkėms nustatytas ksantogranuliomatozinis uždegimas, trims – vidutinė, vienai – reta, dviem – jokios PMN infiltracijos.

Išvados

1. *In vitro* sąlygomis reikšmingas *S. aureus* bioplėvelių struktūrą ardantis poveikis nustatytas veikiant 0,02 proc. chlorheksidino digliukonato tirpalu.
2. *In vitro* sąlygomis imituojant pūlingą žaizdą su *S. aureus* bioplėvele stipriausias antimikrobinis efektas nustatytas veikiant 0,1 proc. oktenidino dihidrochlorido tirpalu. Labai stipriu antimikrobiniu aktyvumu pasižymėjo 10 proc. povidono-jodas, tuo tarpu 0,02 proc. chlorheksidino digliukonato efektyvumas buvo menkas.
3. Eksperimentinis infekuoto austo kraujagyslės protezo modelis *in vitro* sukurtas modifikavus ir adaptavus literatūroje aprašytą Lubbock'o lėtinės žaizdos patogeninių bioplėvelių modelį, panaudojant austo dakrono lopinėlius ir bioplėveles formuojančias *S. aureus* padermes. Bioplėvelių susiformavimas ant kraujagyslės protezo lopinėlių patvirtintas skenuojančiu elektroniniu mikroskopu. KPI modelyje *in vitro* visi naudoti antiseptikai pasižymėjo panašiu antimikrobiniu efektu.
4. Panaudojant bioplėvelę formuojančią 215N *S. aureus* padermę sukeltas chirurginių žaizdų su austo kraujagyslės protezo lopinėliais supūliavimas Wistar klono žiurkėms. Kasdieniais plovimais gydant pūlingas žiurkių žaizdas su austo kraujagyslės protezo lopinėliais efektyviausiu pripažintas 0,1 proc. oktenidino dihidrochlorido tirpalas.
5. Plaunant žaizdas 0,1 proc. oktenidino dihidrochloridu, septynių dienų gydymo kursas buvo pakankamas sterilizuoti devynių iš 10 žiurkių protezo aplinkos audinius ir vieną kraujagyslės protezą. Citologiniai žaizdų nuoplovų tepinėliai ir audinių preparatų histologinė analizė patvirtino aktyvų uždegiminį procesą protezo aplinkos audiniuose.

Praktinės rekomendacijos

1. Eksperimentatoriams: tiriant vietiškai veikiančių medžiagų (antiseptikų, antibiotikų) efektyvumą gydant infekcijas rekomenduojame pasinaudoti mūsų sukurtais eksperimentiniais *in vitro* ir *in vivo* modeliais.
2. Gydytojams: *S. aureus* infekuotas žaizdas, kuriose gausu baltyminių produktų (leukocitų, detrito), rekomenduojama plauti su 0,1 proc. oktenidino dihidrochlorido arba 10 proc. povidono-jodo tirpalais.
3. Kraujagyslių chirurgams: gydant *S. aureus* sukeltą žaizdos infekciją su implantuotu kraujagyslės protezu rekomenduojame žaizdą plauti su 0,1 proc. oktenidino dihidrochlorido tirpalu, o žaizdą sanavus (remtis mikrobiologinių pasėlių rezultatais iš žaizdų nuoplovų) – infekuotą protezą šalinti ir pakeisti nauju.

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THE LIST OF PRESENTATIONS

1. Poster presentation at MAC 2016 in Munich, Germany (December 1-3, 2016) “Animal experiment: conservative treatment of prosthetic vascular graft infection caused by *Staphylococcus aureus*“.
2. Poster presentation at “Evolutionary medicine: health and diseases in changing environment” Vilnius, Lithuania (June 5-8, 2018) “*In vitro* evaluation of antiseptics against *Staphylococcus aureus* biofilms formed in different surroundings”.
3. Poster presentation at ESVS 2018 Valencia, Spain (September 25-28, 2018) “*In vitro* evaluation of several antiseptic solutions against *Staphylococcus aureus* biofilms: Antiseptics are able to fight *Staphylococcus aureus* biofilms on vascular grafts”.
4. Oral presentation at a conference of Lietuvos žaizdų gydymo asociacija „Holistinis požiūris į opų ir žaizdų gydymą” (April 26, 2019) „Antiseptikų poveikio *Staphylococcus aureus* suformuotai bioplėvelei palyginimas”.

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