Medical and Biological Sciences, 2013, 27/2, 41-47

ORIGINAL ARTICLE / PRACA ORYGINALNA

Adrian Reśliński[1], Stanisław Dąbrowiecki[2], Katarzyna Głowacka[3], Jakub Szmytkowski[1]

THE INFLUENCE OF OCTENIDINE DIHYDROCHLORIDE ON BACTERIAL BIOFILM ON THE SURFACE OF A POLYPROPYLENE MESH

WPŁYW ROZTWORU DICHLOROWODORKU OKTENIDYNY NA BIOFILM WYTWORZONY NA POWIERZCHNI SIATKI POLIPROPYLENOWEJ

[1]Department of General and Endocrine Surgery, Ludwik Rydygier College of Medicine in Bydgoszcz, Nicolaus Copernicus University in Torun, Poland
Acting Head: Jacek Szopiński PhD
[2]Department of Nutrition and Dietetics, Ludwik Rydygier College of Medicine in Bydgoszcz, Nicolaus Copernicus University in Torun, Poland
Acting Head: Justyna Przybyszewska PhD
[3]Department of Plant Physiology, Genetics and Biotechnology, Warmia – Mazury University in Olsztyn, Poland
Head: Ryszard Górecki prof.

S u m m a r y

Deep surgical site infections (DSSI's) involving the implanted biomaterial remain an important issue in hernia surgery. The etiological factors include *Staphylococcus aureus* and *Escherichia coli*. The ability of these microorganisms to create a biofilm on the surface of the implant is considered to be one of the main reasons why successful treatment of DSSI's is not an easy task. It is widely agreed that an important element of a successful treatment plan for infections involving biofilm formation is the use of agents capable of penetrating the biofilm matrix, and displaying a satisfactory efficacy against the microorganisms present within. Antiseptic agents meet the above criteria.

The goal of this study was to evaluate the influence of a solution of octenidine dihydrochloride (Octenisept) on the biofilm present on a surface of a monofilament polypropylene mesh implant.

The study involved 140 bacterial strains from the collection of the Department of Microbiology Nicolaus Copernicus University Collegium Medicum in Bydgoszcz, Poland. The strains included 70 (50%) *S. aureus* isolates and 70 (50%) *E. coli* isolates. The influence of an antiseptic agent on the created biofilm was evaluated with the use of a qualitative and quantitative method, as well as by scanning electron microscopy (SEM).

In the qualitative assessment, the observed effect of octenidine was a diminished intensity of biomaterial staining after incubation with TTC in comparison to samples from the control group. In the quantitative study, the live cell counts of *S. aureus* and *E. coli* isolated from the biofilm present on the implant surface, after exposure to the antiseptic agent were found to be diminished. SEM studies have shown that exposure to octenidine hydrochloride decreases the number of bacteria adhering to the biomaterial surface.

The Octenisept octenidine dihydrochloride solution displays bactericidal activity against *S. aureus* and *E. coli* bacteria present in the biofilm created on the surface of monofilament polypropylene mesh.

S t r e s z c z e n i e

Głębokie zakażenie miejsca operowanego (GZMO) obejmujące wszczepiony biomateriał stanowi istotny problem w chirurgii przepuklin. Czynniki etiologiczne tego powikłania, wśród których wymienia się m.in.: *Staphylococcus aureus* i *Escherichia coli* tworzą biofilm na powierzchni implantów uznawany za jedną z głównych przyczyn trudności w leczeniu GZMO. Uważa się, że istotnym elementem leczenia zakażeń przebiegających z powstaniem biofilmu jest stosowanie substancji aktywnych wobec drobnoustrojów w biofilmie oraz charakteryzujących się zdolnością do przenikania przez jego macierz. Związkami chemicznymi posiadającymi wymienione właściwości są antyseptyki.

Celem pracy była ocena wpływu roztworu dichlorowodorku oktenidyny w postaci preparatu antyseptycznego Octenisept na biofilm wytworzony na powierzchni monofilamentowej siatki polipropylenowej.

Badaniem objęto 140 szczepów bakterii z kolekcji Katedry i Zakładu Mikrobiologii Collegium Medicum im. L. Rydygiera w Bydgoszczy Uniwersytetu Mikołaja Kopernika w Toruniu, wśród których było 70 (50%) izolatów *S. aureus* oraz 70 (50%) *E. coli*. Ocenę wpływu antyseptyku na wytworzony biofilm wykonano metodą jakościową, ilościową oraz z użyciem skaningowego mikroskopu elektronowego (SEM).

W badaniu metodą jakościową efektem działania roztworu dichlorowodorku oktenidyny na wytworzony biofilm było zmniejszenie intensywności zabarwienia powierzchni biomateriału po inkubacji z TTC w stosunku do grupy kontrolnej. W badaniu ilościowym stwierdzono zmniejszenie liczby żywych komórek *S. aureus* i *E. coli* izolowanych z biofilmu na powierzchni implantu po ekspozycji na działanie antyseptyku. Natomiast badanie z użyciem SEM wykazało, że roztwór dichlorowodorku oktenidyny powoduje zmniejszenie liczby bakterii przylegających do powierzchni biomateriału.

Roztwór dichlorowodorku oktenidyny w postaci preparatu Octenisept wykazuje działanie bakteriobójcze wobec *S. aureus* i *E. coli* w biofilmie wytworzonym na powierzchni monofilamentowej siatki polipropylenowej.

Key words: octenidine hydrochloride, polypropylene mesh, biofilm, TTC, SEM

Słowa kluczowe: dichlorowodorek oktenidyny, siatka polipropylenowa, biofilm, TTC, SEM

INTRODUCTION

Deep surgical site infections (DSSI's) involving the implanted biomaterial remain an important issue in hernia surgery [1, 2]. One of the main factors responsible for the difficulties in the treatment of this complication is the creation of a bacterial biofilm on the implant surface [3]. The typical etiological factors of DSSI's with biofilm – forming capabilities include *Staphylococcus aureus* and *Escherichia coli* [4].

It is widely agreed that an important element of a successful treatment plan for infections involving biofilm formation is the use of agents capable of penetrating the biofilm matrix [5], and displaying a satisfactory efficacy against the microorganisms present within [6]. Antiseptic agents used in local treatment of chronic problem wounds and biomaterial infections meet the above – listed criteria [1, 7].

In theory, the ideal antiseptic agent should display a broad spectrum of antimicrobial activity immediately after application. Moreover, it cannot be toxic, teratogenic or carcinogenic, and should not negatively influence wound healing [7,8]. Such an ideal antiseptic has not yet been reported despite ongoing research [7]. One of the chemical compounds meeting most of the above criteria is [N,N'-(1,10-decanediyldi-1[4H]-pyridinyl-4-ylidene)-bis-(1-octanamine) dihydrochloride, or octenidine dihydrochloride [8, 9]. It is characterized by a particular affinity to the lipid components of cell membranes, such as cardiolipin; this property allows it to display good antimicrobial efficacy with a minimal cytotoxic effect on the living tissue [9]. In its commercially available form (the Octenisept antiseptic solution containing 0.1% octenidine dihydrochloride, 2.0% phenoxyethyl alcohol and additives) it has been demonstrated to exert a bactericidal effect on planktonic forms of *S. aureus* and *E. coli* after exposure of as little as one minute [10, 11]. A review of the available literature has not yielded any publications on the influence of a one-minute exposure to an octenidine dihydrochloride solution on the biofilm created by *S. aureus* and *E. coli* on the surface of biomaterial implants used in hernia surgery.

The aim of this study was to evaluate the influence of a solution of octenidine dihydrochloride on the biofilm present on a surface of a monofilament polypropylene mesh implant.

MATERIALS AND METHODS

Bacterial strains. The study involved 140 bacterial strains from the collection of the Departament of Microbiology of Nicolaus Copernicus University

Collegium Medicum in Bydgoszcz, Poland. The strains included 70 (50%) *S. aureus* isolates and 70 (50%) *E. coli* isolates. The microorganisms had been isolated in the period 2008–2009 from wound smears and pus samples collected from various patients hospitalized at the Departments of: General and Endocrine Surgery, General and Vascular Surgery and General Surgery and Transplantology of the Dr A. Jurasz University Hospital No.1 in Bydgoszcz, Poland. Initial identification of the *S. aureus* strains was based on colony morphology on Columbia Agar with 5% sheep blood (Becton Dickinson, Sparks, USA) and the presence of clumping factor and / or free coagulase. Species requiring verification were identified using the ID32 Staph (bioMérieux S.A. RCS Lyon, France) test for staphylococci. In the identification of *E. coli* strains, colony morphology on the MacConkey Agar (Becton Dickinson) was considered along with ID32 E test results (bioMérieux). The ID32 Staph and ID32 E tests were performed according to the manufacturer's instructions and the results were read after a 24 – hour incubation in the oxygen – containing atmosphere at 37ºC in the ATB Expression system (bioMérieux), with the use of the V 2.8.8 database. The isolates included in the study material had different chromosomal DNA patterns within one species, which was verified in the initial stage of the study by pulsed – field gel electrophoresis (PFGE). The isolates were stored in brain heart infusion, (BHI; bioMérieux) with an addition of 15% glycerol (Polskie Odczynniki Chemiczne, POCH) at a temperature of -70ºC.

Evaluation of the influence of octenidine hydrochloride solution on biofilm. The testing was performed using a qualitative [12,13] and quantitative method [14]; scanning electron microscopy was also performed [15]. The investigated implant was composed of a monofilament polypropylene mesh, as this type of biomaterial is the most widely used in hernia surgery [4].

In the quantitative method, utilizing the property of metabolically active microorganisms to reduce colorless 2,3,5-triphenyltetrazolium ***chloride*** (TTC; POCH) to red formazan [12,13], sterile fragments of the implant, measuring 2 x 1 cm, were incubated in 4 ml of tryptic soy broth (TSB, Becton Dickinson, Sparks, USA) containing a bacterial inoculum equivalent to the 0.5 McFarland turbidity standard. Implant incubation at 37ºC continued for 48 hours in the oxygen – containing atmosphere, with a change of the growth medium to sterile substrate after 24 hours. The biomaterial samples were then rinsed with 0.9% phosphate buffered saline (PBS) at a pH of 7.2 and placed in 4 mL of the antiseptic agent – a commercially available octenidine dihydrochloride solution (Octenisept, containing 0.1% octenidine dihydrochloride, 2.0% phenoxyethyl alcohol and additives; Schülke & Mayr). After one minute, the implant samples were transferred to a neutralizing solution, containing: 3% Tween 80 (Sigma), 3% saponin ((Fluka, Steinheim, Germany), 0.1% histidine (Sigma) and 0.1% lecithin – 0.1 % (Sigma) [16]; this solution does not influence bacterial cells [10]. After rinsing with PBS (pH 7.2) and placing in 4 mL of sterile TSB with the addition of 20 μL of a 1% TTC solution, the samples were further incubated for 24 hours at 37ºC in the oxygen – containing atmosphere. The degree of TTC reduction to red formazan, indicating the presence of live bacteria in the biofilm, was graded on a following scale: 0 – no TTC reduction; 1 – slight pink dotting of the mesh surface, 2 – solid pink coloring of the entire surface, 3 – red coloring of the mesh surface, clouding and red coloring of the substrate (very strong TTC reduction). For each isolate, the test was repeated three times. The control group consisted of implant fragments incubated with the bacterial inoculum without subsequent exposure to the antiseptic solution.

In the quantitative assessment, the monofilament mesh fragments were coated with bacterial biofilm using the same procedure as described above. Samples were rinsed with 0.9% phosphate buffered saline (PBS) at a pH of 7.2 and placed in 4 mL of the antiseptic agent. After one minute, the implant samples were transferred to the neutralizing solution [16]. Next, they were rinsed with PBS (pH 7.2) and shaken (1100 rpm) in 1 ml of 0.5% saponin for 2 minutes [17]. Serial 10-fold dilutions of the suspension thus obtained were performed, with subsequent inoculation of three Petrie dishes containing trypticase soy agar for every dilution; 100 μl of the solution were used per each Petrie dish. After 24 hours of incubation of the implant fragments at 37ºC, the result (average of three measurements) was recorded as the number of colony-forming units (CFU's) per one milliliter of suspension (CFU/ml). Again, the control group consisted of implant fragments incubated with the bacterial inoculum without subsequent exposure to the antiseptic solution.

The influence of octenidine dihydrochloride on the biofilm was also evaluated by scanning electron microscopy (SEM). Random fragments of the biomaterial, coated with a biofilm in a procedure identical to that described above, were rinsed with PBS (pH 7.2) and placed in 4 mL of the antiseptic agent. After one minute, the implant samples were transferred to the neutralizing solution [16], rinsed with PBS (pH 7.2) and fixed in a 2.5% glutaraldehyde solution (POCH, Gliwice, Poland) in a 0.1 M phosphate buffer at a pH of 7.4 for 48 hours at 4^0C. After fixation, the material was rinsed for 2x 20 min in phosphate buffer at room temperature. The samples were then dehydrated in a graded series of ethanol concentrations: 30, 50, 70, 80, 96%, 10 minutes in each solution, and twice for 30 minutes in 99.8% ethanol. After dehydration, the implant fragments were placed in a solution of hexamethyldisilazane (HMDS; Polysciences) for 45 minutes, and dried at room temperature. The dried material was placed on copper tables and sputter – coated with gold in an atmosphere of argon in an ionic coater (Fine Coater, JCF-1200, JEOL, Tokyo, Japan). The sputter-coated material was placed in a SEM column (JSM-5310LV, JEOL, Tokyo, Japan) and analyzed at a voltage of 15 -20 kV [15]. The results were analyzed and registered using the NSS Version 3.0 software package (Thermo Fisher Scientific). Like in two previous cases, the control group consisted of implant fragments incubated with the bacterial inoculum without subsequent exposure to the antiseptic solution.

Statistical analysis. The statistical analysis of the results was performed using the Statistica 10.0 software package (StatSoft Poland). The codependence of qualitative variables was tested using the nonparametric χ^2 test. The statistical analysis of non – normally distributed quantitative variables was performed using the nonparametric Wilcoxon test for codependent samples. The *adopted* significance level was $p \leq 0.05$.

RESULTS

All of the investigated *S. aureus* and *E. coli* strain created a biofilm on the surface of the monofilament polypropylene mesh implant. The results of the qualitative analysis of the influence of the antiseptic agent on the biofilm are presented in Table I. After exposure of *S. aureus* biofilm to the solution of octenidine dihydrochloride for one-minute, no TTC reduction was observed in 2.9% of the strains; none of the strains exhibited very strong TTC reduction. A statistically significant ($p<0.0001$) increase was observed in the percentage of strains with a weak TTC reduction: from 4.3% (controls) to 90.0% (strains exposed to antiseptic solution). In the case of *E. coli* , a statistically significant ($p<0.0001$) reduction of the percentage of strains exhibiting a strong TTC reduction from 81.4% (controls) to 27.1% (study group) was observed. None of the *E. coli* strains exhibited very strong TTC reduction after exposure to octenidine dihydrochloride.

Table I. *The influence of the antiseptic solution on TTC reduction by S. aureus (n=70) and E. coli (n=70) present within biofilm*

TTC reduction	*S. aureus*				*E. coli*			
	control		antiseptic		control		antiseptic	
	N	%	N	%	N	%	N	%
None	0	0.0	2	2.9	0	0.0	0	0.0
Weak	3	4.3	63	90.0	11	15.7	51	72.9
Strong	60	85.7	5	7.1	57	81.4	19	27.1
Very strong	7	10.0	0	0.0	2	2.9	0	0.0

(N – number of strains, % – percentage of strains)

In the quantitative study, a one-minute exposure of *S. aureus* biofilm to octenidine hydrochloride resulted in a statistically significant ($p<0.0001$) decrease of the median value of CFU x ml^{-1} isolated from the biofilm, from 1.3 x 10^6 (control group) to 4.5 x 10^4 (study group; Fig.1). In the case of *E. coli* , a statistically significant ($p<0.0001$) decrease of the median value of CFU x ml^{-1} isolated from the biofilm was observed, from 1.5 x 10^6 (control group) to 3.0 x 10^5 (study group; Fig.1).

Upon SEM examination, in the control group a biofilm was observed, composed of microorganisms adhering to one another and to the biomaterial surface (Fig. 2 and 4). After a one-minute exposure to the antiseptic agent, no multilayered bacterial aggregations were observed (Fig. 3 and 5).

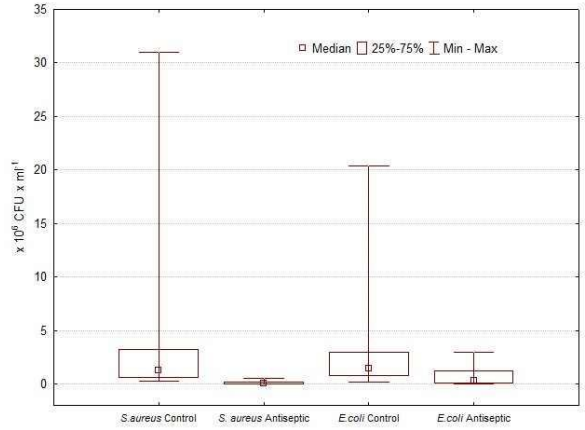

Fig. 1. *The influence of the antiseptic solution on the biofilm created by S. aureus strains (n=70) and E. coli strains (n=70) – a quantitative analysis*

Ryc. 1. *Wpływ roztowru antyseptyku na biofilm wytworzony przez szczepy S. aureus i E. coli (n=70) – ocena metodą ilościową*

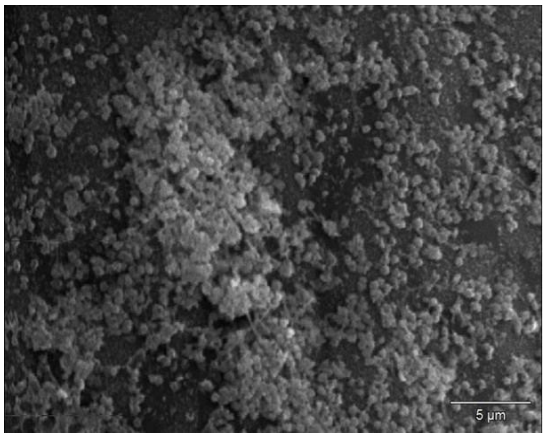

Fig. 2. *Biofilm of S. aureus (control group) on the surface of a monofilament polypropylene mesh; SEM – magnification 3500x*

Ryc. 2. *Biofilm S. aureus (kontrola) na powierzchni monofilamentowej siatki polipropylenowej; SEM – powiekszenie 3500x*

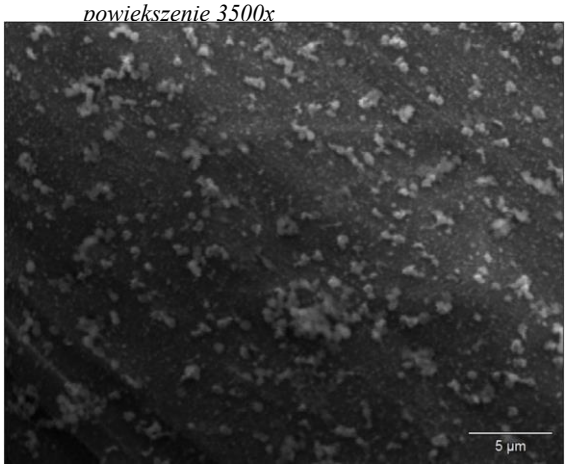

Fig. 3. *The effect of octenidine dihydrochloride on the biofilm of S. aureus; SEM – magnification 3500x*

Ryc. 3. *Efekt działania dichlorowodorku oktenidyny na biofilm S. aureus; SEM – powiększenie 3500x*

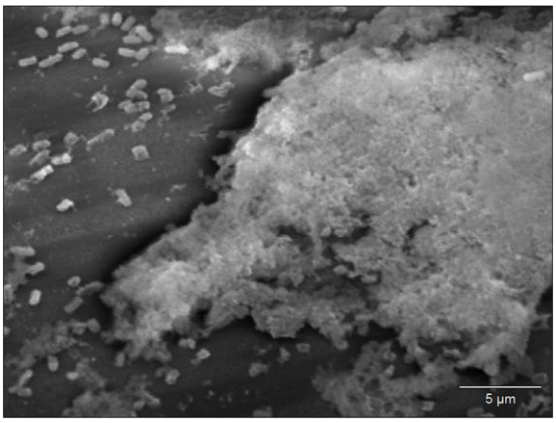

Fig. 4. *Biofilm of E. coli (control group) on the surface of a monofilament polypropylene mesh; SEM – magnification 3500x*

Ryc. 4. *Biofilm E. coli (kontrola) na powierzchni monofilamentowej siatki polipropylenowej; SEM – powiększenie 3500x*

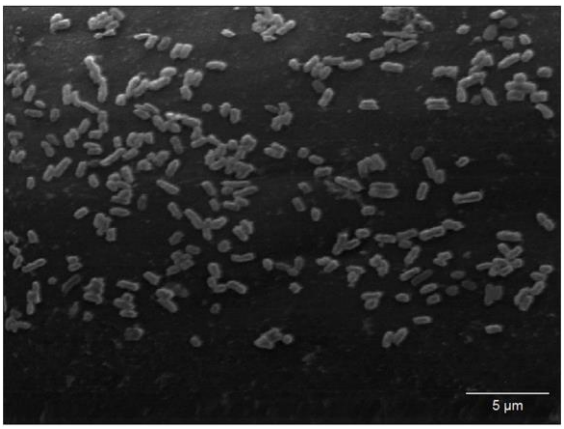

Fig. 5. *The effect of octenidine dihydrochloride on the biofilm of E. coli; SEM – magnification 3500x*

Ryc. 5. *Efekt działania dichlorowodorku oktenidyny na biofilm E. coli; SEM – powiększenie 3500x*

DISCUSSION

The influence of a solution of octenidine dihydrochloride (Octenisept) on the biofilm present on a surface of a monofilament polypropylene mesh implant was evaluated in this study. Based on the results of a quantitative and qualitative evaluation and scanning electron microscopy we have ascertained that it exerts a bactericidal effect on *S. aureus* and *E. coli* present within a biofilm.

According to Chen and Wen [5], proper treatment of infections associated with biofilm formation must include agents which are efficacious against microorganisms protected by the biofilm. Our own results, as well as the observations of other authors

[16,18], indicate that octenidine dihydrochloride displays such properties. The antiseptic agent used in our study showed bactericidal properties against *S. aureus* and *E. coli* after an exposure of as little as one minute, which is indicative of its ability to penetrate into the biofilm. Other researchers have confirmed the good penetration of octenidine dihydrochloride solutions through the biofilm matrix [16,18].

In our qualitative study, exposure to octenidine dihydrochloride resulted in a decreased intensity of the color reaction on the surface of the biofilm – coated implant after incubation with TTC, compared to the control group. This effect corresponded with a decrease in TTC reduction by the bacteria within the biofilm; other authors also report this finding [16].

In our quantitative study, decreased live bacterial cell counts were found in isolates from biofilm – coated polypropylene mesh fragments after exposure to a solution of octenidine dihydrochloride. Similar outcomes have been reported by Bartoszewicz et al. [16], who studied the effect of octenidine hydrochloride on bacterial biofilm covering catheters and suturing material.

In their SEM studies, Bartoszewicz et al. [16] showed octenidine dihydrochloride to decrease the number of bacteria adhering to the biomaterial surface. Our findings are similar.

The results presented here need *in vivo* verification, since *in vitro* conditions cannot accurately replicate a clinical situation. However, one may assume that octenidine dihydrochloride solutions may be efficacious in local therapy of DSSI's involving an implanted biomaterial; it has been documented that neither the presence of blood nor plasma proteins decreases the efficacy of this agent [19]. Moreover, no resistance to octenidine dihydrochloride has been so far reported, even among MRSA strains [9]. The well – documented synergy of this compound with antibiotics may also positively influence treatment outcomes [9].

CONCLUSIONS

A solution of octenidine dihydrochloride, commercially available as Octenisept, displays bactericidal properties against *S. aureus* and *E. coli* within a biofilm present on the surface of a monofilament polypropylene hernia mesh.

The study funded by research grant NCU 06/2009.

ACKNOWLEDGEMENTS

The authors wish to thank Professor Eugenia Gospodarek, Head of the Department of Microbiology of Nicolaus Copernicus University Collegium Medicum in Bydgoszcz for making this study possible.

REFERENCES

1. Jezupors A, Mihelsons M. The analysis of infection after polypropylene mesh repair of abdominal wall hernia. World J Sur 2006; 30: 2270-2278
2. Stremitzer S, Bachleitner-Hofmann T, Gradl B et al. Mesh graft infection following abdominal hernia repair: risk factor evaluation and strategies of mesh graft preservation. A retrospective analysis of 476 operations. World J Surg 2010; 34: 1702-1709
3. Sanchez VM, Abi-Haidar YE, Itani KM. Mesh infection in ventral incisional hernia repair: incidence, contributing factors, and treatment. Surg Infect (Larchmt) 2011; 12: 205-210
4. Engelsman AF, van der Mei HC, Ploeg RJ et al. The phenomenon of infection with abdominal wall reconstruction. Biomaterials 2007; 28: 2314-2327
5. Chen L, Wen YM. The role of bacterial biofilm in persistent infections and control strategies. Int J Oral Sci 2011; 3: 66-73
6. Donlan RM. Biofilm elimination on intravascular catheters: important considerations for the infectious disease practitioner. Clin Infect Dis 2011; 52: 1038-1045
7. White RJ, Cutting K, Kingsley A. Topical antimicrobials in the control of wound bioburden. Ostomy Wound Manage 2006; 52: 26-58
8. Krzemiński M, Bartoszewicz M, Czarniak E et al. The use of octenidine dihydrochloride in the treatment of musculoskeletal infections. Adv Clin Exp Med 2010; 19: 631–636
9. Hübner NO, Siebert J, Kramer A: Octenidine dihydrochloride, a modern antiseptic for skin, mucous membranes and wounds. Skin Pharmacol Physiol 2010; 23: 244-258
10. Müller G, Kramer A. Biocompatibility index of antiseptic agents by parallel assessment of antimicrobial activity and cellular cytotoxicity. J Antimicrob Chemother 2008; 61: 1281-1287
11. Koburger T, Hübner NO, Braun M et al. Standardized comparison of antiseptic efficacy of triclosan, PVP-iodine, octenidine dihydrochloride, polyhexanide and chlorhexidine digluconate. J Antimicrob Chemother 2010; 65: 1712-1719
12. Gallimore B., Gagnon RF, Subang R et al. Natural history of chronic *Staphylococcus epidermidis* foreign body infection in a mouse model. J Infect Dis 1991; 164: 1220-1223

13. Różalska B, Sadowska B, Więckowska M et al. Wykrywanie biofilmu bakteryjnego na biomateriałach medycznych. Med Dośw Mikrobiol 1998; 50: 115-122
14. Saygun O, Agalar C, Aydinuraz K et al.: Gold and gold-palladium coated polypropylene grafts in a *S. epidermidis* wound infection model. J Surg Res 2006; 131: 73-79
15. Araujo JC, Téran FC, Oliveira RA et al. Comparison of hexamethyldisilazane and critical point drying treatments for SEM analysis of anaerobic biofilms and granular sludge. J Electron Microsc (Tokyo) 2003; 52: 429-433
16. Bartoszewicz M, Rygiel A. Biofilm jako podstawowy mechanizm zakażenia miejsca operowanego - metody prewencji w leczeniu miejscowym. Chir Pol 2006; 8: 171–178
17. Kwiecińska-Piróg J, Bogiel T, Gospodarek E. Porównanie dwiema metodami tworzenia biofilmu przez pałeczki *Proteus mirabilis* na powierzchni różnych biomateriałów. Med. Dośw Mikrobiol 2011; 63:131-138
18. Bartoszewicz M, Rygiel A et al. Penetration of a selected antibiotic and antiseptic into a biofilm formed on orthopedic steel implants. Ortop Traumatol Rehabil 2007; 9: 310-318
19. Pitten FA, Werner HP, Kramer A. A standardized test to assess the impact of different organic challenges on the antimicrobial activity of antiseptics. J Hosp Infect 2003; 55: 108-115

Address for correspondence:
Adrian Reśliński PhD
Department of General and Endocrine Surgery
Nicolaus Copernicus University in Torun
Ludwik Rydygier College of Medicine in Bydgoszcz
M. Skłodowskiej-Curie 9 Str.
85-094 Bydgoszcz, Poland
tel. 00 48 52 585-47-30
fax 00 48 52 585-40-16
email: bigar@wp.pl

Received: 26.03.2013
Accepted for publication: 28.05.2013