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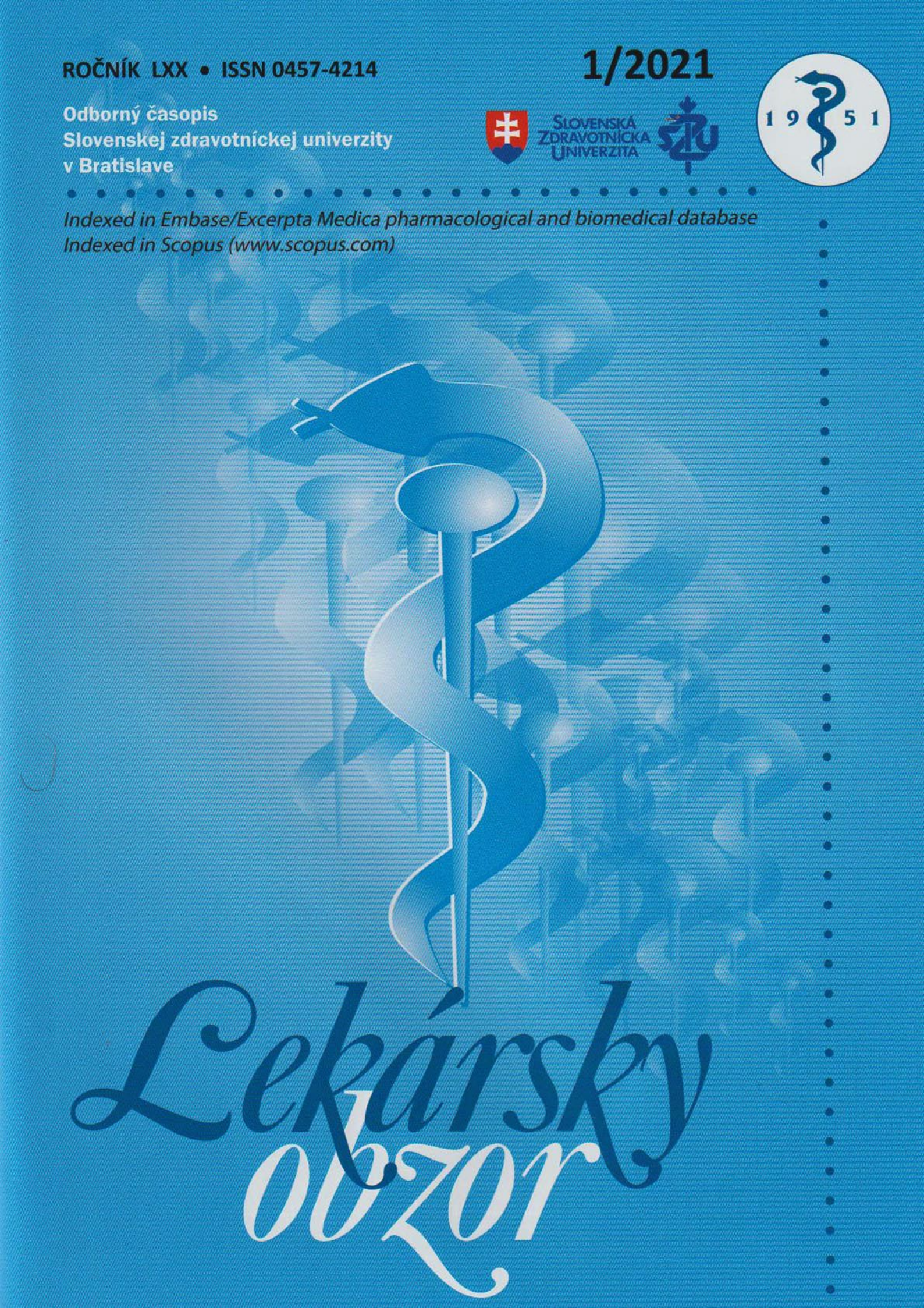

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LOCAL ANESTHETICS CONTRIBUTING THE STRUGGLING BIOFILM-FORMING ACTIVITY OF S. AUREUS COLONIZING SURFACES OF VENOUS CATHETERS. MULTICENTRER CLINICAL TRIAL

Vplyv lokálnych anestetík na tvorbu mikrobiálneho biofilmu S. aureus na povrchu venóznych katétrov. Multicentrická klinická štúdia

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SUMMARY
Background: The aim of the study was to investigate the influence of local anesthetics on biofilm-forming properties of clinical isolates *S. aureus* and their biofilm-formation on venous catheters made of different polymers.
Materials and methods: The study was based on investigating 62 clinical strains of *S. aureus* isolated from surgical patients. Biofilm-forming properties of clinical strains *S. aureus* were assessed by using the Christensen's spectrophotometric method. *S. aureus* biofilms were cultivated on polyurethane and teflon catheters by immersion in broth medium inoculated by suspension of bacterial culture. Also biofilm-formation on the surfaces of various catheters was observed by microscopic examination after their removal from the culture medium, washing with sterile saline and staining with crystal violet. In the study we used bupivacaine 0.125 %, 0.25 % and lidocaine 0.5 %, 1.0 %.
Results: The findings obtained demonstrated that clinical isolates *S. aureus* have high biofilm-forming potential, their optical density unit (ODU) was 0.244 ± 0.06. Bupivacaine in concentration 0.125 % contributed to a decrease in the optical density of the biofilms of the investigated species in 0.7 times ($p = 0.001$), compared with their indices without the presence of the anesthetics. There was a decrease in the film-forming properties of investigated isolates in the presence of lidocaine in concentrations 0.5 % and 1.0 % in a 1.13 time and 1.15 times respectively. The ODU values of the film forms obtained by us correlate with the results of microscopic examination.
Conclusion: 0.5 % and 1.0 % lidocaine and 0.25 % bupivacaine significantly inhibit the biofilm-production of *S. aureus* clinical isolates and formation of biofilms on the surfaces of catheters from different polymers.
Key words: biofilm, local anesthetics, polymers, resistance, venous catheters.
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Introduction

At present postoperative infectious complications are reported worldwide to be the commonest in patients at surgical and intensive care units. According to WHO reports the incidence of infectious complications among patients in high-income countries reaches 7 – 10 %, while in low-income countries, this figure reaches up to 20 % (15, 10). A significant threat is

catheter-associated infections (CAI) due to features of microorganisms, that cause them. The traditional antibacterial therapy is less effective (4, 5), because of biofilm-formation by pathogens (1, 8). Recent studies have proved that microorganisms in biofilm are more resistant to antibacterial preparations then their planktonic forms in hundred´s times (12). The role of biofilms in the development of more than 60 % of chronic diseases, in the pathogenesis of which microbial pathogens are involved, is established (3). Therefore, special attention is now being paid to the diagnostic control of antimicrobials, which should be tested on adhesive microorganisms. Moreover, the effective doses should be considered as effective at those concentrations, that are bactericidal against most bacteria in biofilms rather than to their planktonic forms (14).

Catheter-associated bloodstream infections rank the third place among the main nosocomial infections and the first place among the dominant causative agents of bacteraemia, including around 10 % of all infections in hospitalized patients, 20 % of nosocomial infections and up to 87 % of primary bacteraemia (11, 15). The spectrum of microorganisms, which cause CAI, depends on extensive range of factors such as the severity of the patient's status, catheter type, type of unit, the path of infection, etc. Gram-positive cocci have been found out as the commonest causative agents for CAI, moreover coagulase-negative staphylococci and *Staphylococcus aureus* have been isolating in 49 % and 17 % cases respectively. Infections related with staphylococcal biofilms as usual are difficult to treat with antimicrobial therapy leading to the need for replacing the contaminated device (2, 7, 15).

In such circumstances, considerable attention is paid to the prevention of infectious complications. Currently powerful antimicrobial activity of antiseptics are known. However, along with antimicrobials in surgical practice, local anaesthetics, which in addition to the direct analgesic effect, have a bacteriostatic effect in monotherapy and increase the bactericidal effect in combination with antiseptics.

The aim of the study was to investigate the influence of local anesthetics on biofilm-forming properties of clinical isolates *S. aureus* and their biofilm-formation on venous catheters made of different polymers.

Materials and methods

The study was based on investigating 62 clinical strains of *S. aureus* isolated from surgical patients staying at the Vinnytsia Regional Clinical Hospital and Vinnytsia Regional Endocrinology Clinical Center. The research has been carried out according to the Helsinki Declaration on the ethical principles for medical research involving human subjects and approved by the Bioethics Committee of Pirogov Memorial National Medical University (Vinnytsia, Ukraine). To investigate the microflora, the material was taken from the surface of the infected venous catheters, followed by cultivation on Blood agar and Yolk-Salt agar for 48 hours. The final identification was carried out in accordance with the standard methodology for morphological, tectorial and biochemical properties and by applying automatic bacteriological analyser Vitec-2 compact bio Mérieux (France).

Biofilm-forming properties of clinical strains *S. aureus* were assessed by using the *Christensen's* spectrophotometric method (MtP-test "microtiter plate test"). Biofilms were modelled in liquid nutrient medium in wells of a sterile, flat-bottom 96-well polystyrol tablet (Corning, USA) and stained with 1% solution of crystalline violet. The properties of microorganisms to form a biofilm were measured by absorbance of the dye in units of optical density (ODU) using a spectrophotometer with a wavelength of 570 nm. The ability of microorganisms to form biofilms was evaluated as low (ODU < 0.120), average (ODU = 0.121 – 0.239) and high (ODU > 0.240) (11). The influence of the anaesthetics on *S. aureus* film-formation was assessed by the reproduction of the biofilms according to the above-described procedure with adding anaesthetics in clinical concentrations and the subsequent spectrophotometric ODU assessment.

Furthermore, *S. aureus* biofilms were cultivated on polyurethane and teflon catheters by immersion in 5 ml of meat-peptone broth inoculated by daily suspension of bacterial culture. The bacterial suspension, at a concentration of 10^6 – 10^7 cells per 1 ml, was introduced into the culture medium at the rate of 0.1 ml of bacterial suspension per 1 ml of broth. Further inoculated culture media with catheter fragments, 12 – 13 mm long, were incubated in a thermostat at 37 °C. In the same time, studies were conducted on the effect of anaesthetics on the film formation process. For this purpose, incubation of staphylococcus film-forming strains and catheter fragments was performed in a nutrient medium with anaesthetic in a 1 : 3 ratio. Biofilm-formation on the surfaces of various catheters was observed by microscopic examination (magnification ×900, ×1500) after their removal from the culture medium, washing with sterile saline and staining with crystal violet for 3 minutes.

To compare the dynamics of biofilm-formation, microscopic studies were performed on the second-fourth days from the beginning of cultivation, taking into account the number of adhesion loci of microorganisms, signs of colonization (reproduction of adhesive bacteria) and formation of biofilm (formation of trajectories between micro-colonies of adhesive microorganisms, as well as films, formation of extracellular bio-matrix with uneven distribution of microorganisms).

In the study there were used bupivacaine 0.125 %, 0.25 % and lidocaine 0.5 %, 1.0 %.

The statistical analysis of the findings obtained was performed by using the standard SPSS Statistics 23 and Microsoft Excel 2010 software packages. We calculated the arithmetic mean (M), the mean error of the arithmetic mean (± m), and the criterion of the reliability of the differences (p). The differences between the indica-

tors studied were evaluated according to Student's t-test.

Results

The findings obtained demonstrated, that clinical isolates *S. aureus* had high biofilm-forming potential, and their ODU was 0.244 ± 0.06 (Tab. 1).

Tab. 1. Characteristics of biofilm-forming properties of *S. aureus* clinical isolates in the presence of anaesthetics (n=62, ODU, M±m).

Micro-organism	Control	Bupivacaine		Lidocaine	
		0.125%	0.25%	0.5%	1.0%
S. aureus	0.244 ± 0.06	0.237 ± 0.06	0.212 ± 0.01*	0.216 ± 0.04*	0.212 ± 0.01*

* – the reliability of the difference between the values of ODU in presence of anaestheticrelative to the values of ODU of control (without anaesthetic), $p < 0.01$. Source: Authors' own processing

The study showed, that anaesthetics in different concentrations inhibit the biofilm-formation of clinical isolates *S. aureus*. It should be noted that bupivacaine in concentration 0.125 % contributed to a decrease in the optical density of the biofilms of the investigated species in 0.7 times ($p = 0.001$), compared with their indices without the presence of the anesthetics. However, its concentration 0.25% has led to inhibition of film-formation by *S. aureus* in 1.15 times reliability. Similarly, there was a decrease in the film-forming properties of investigated isolates in the presence of lidocaine in concentrations 0.5 % and 1.0 % in a 1.13 time and 1.15 times respectively, compared with baseline without any anaesthetic ($p = 0.001$).

Microscopically, there was a significant number of adhesive cells focuses consisting of 5 – 10 cells on the surfaces of polyurethane catheters after 48 hours of cultivation. *S. aureus* colonized surfaces forming clusters of more than 100 cells (Fig. 1). In the same time, microscopy the fragments of teflon catheters showed significantly less number of adhesive focuses. Single areas consisted of less than 10 cells.

72 hours later, the formation of thick multi-layered microcolonies that are interconnected, was indicated on the polyurethane devices. Some microfilms revealed the onset of matrix formation around microorganisms, indicating the maturation of the biofilm. Teflon catheters determined the signs of colonization, the formation of focuses of adhesive microorganisms' reproduction. We found that biofilms that *S. aureus* strain began to form were predominantly at the stage of microcolonies (colonization), single-layer structures that started expanding on the surface.

On the fourth day from the beginning of incubation (96 hours) in microscopic examination of staphylococcal biofilms on polyurethane catheters, we discovered mature biofilms with immersed in polysaccharide matrices microorganisms, while in some formations the proportion of microorganisms was less than quantity of the polysaccharide matrix. The vast majority of films had a multilayer structure, with a large number of microorganisms in the matrix, well-expressed compounds in the structure of formation. After 96 hours of incubation, *S. aureus* formed biofilms on the teflon catheters, which had been maturing: the focuses of the adherent microorganisms (microcolonies) had been growing, trabeculae had been forming between them, the overwhelming number of formations was single-layered.

Fig. 1. Microscopy of colonization of *S. aureus* clinical isolates on the surface of polyurethane catheter. 1 – 48 hours; 2 – 72 hours; 3 – 96 hours (magnification ×1500). Source: Authors' own processing.

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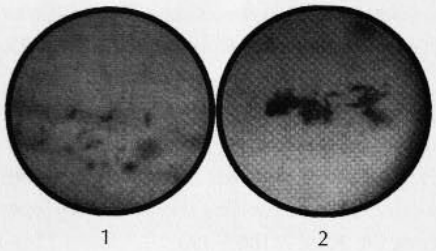
Local anaesthetics used in the study have not been found out to reliably suppress the biofilm-forming properties of *S. aureus* to the surface of polyurethane catheters as well as teflon ones during 48 hours. Bupivacaine 0.125 % demonstrated no effect on the biofilm-formation of clinical isolates to the polyurethane surfaces at the second and third days too in contrast of its action in concentration 0.25 %. Both of used anaesthetics – bupivacaine 0.25 % and lidocaine 0.5 %, 1.0 %, suppressed formation of films by *S. aureus* on polyurethane catheters from the 72 hours of cultivation. Formations were single-layered with a minor matrix production. On the fourth day of experiment, maturation of biofilms on the polyurethane surfaces were seen, however, there was less quantity of matrix.

Biofilms on the teflon catheters were more susceptible to the presence of anaesthetics in the second-third days of cultivation. All concentrations of bupivacaine and lidocaine, that were used, inhibited biofilm-production of *S. aureus* on the 72 hour of cultivation. Microcolonies and single-layer structures, that were formed, had no tendency to fusion. After 96 hours, microscopic signs of glycocalyx formation were not observed in biofilms formed by *S. aureus* on the surfaces of the teflon catheters (Fig. 2).

Fig. 2. Microscopy of colonization of *S.aureus* clinical isolates on the surface of polyurethane catheter in presence of 1) lidocaine 1.0 %; 2) bupivacaine 0.25 %, 96 hours, (magnification ×1500). Source: Authors' own processing.



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Discussion

The results we obtained point out the significant efficacy of anaesthetics on film forms of *S. aureus* clinical isolates and their formation on venous catheters. The ODU values of the film forms obtained by us correlate with the results of microscopic examination. Thus, presence of 0.125 % bupivacaine does not supress biofilm-forming properties of *S. aureus*. In the same time, increase of its concentration twice leads to significant suppression of biofilm-production of investigated isolates as well as formation films on venous catheters from different polymers. Lidocaine in concentrations 0.5% and 1.0 % show higher efficiency. It declines biofilm-forming activity of *S. aureus* on polyurethane and teflon devices from 72 to 96 hours of cultivation.

Bupivacaine and lidocaine are the most commonly used local anaesthetics for postoperative analgesia and surgical anaesthesia. It is known that those local anaesthetics have antimicrobial effect potential in addition to their anaesthetic, analgesic, antiarrhythmic, and anti-inflammatory effects (13). There are many studies over the past 30 years in which lidocaine demonstrated bactericidal actions on *Pseudomonas aeruginosa, Escherichia coli*, and *S. aureus* in surgical wound infections (9, 10). However, CAI as usual are caused by film forms of bacteria and determination of inhibiting action of local anaesthetics are extremely important.

Conclusions

0.5 % and 1.0 % lidocaine and 0.25 % bupivacaine significantly inhibit the biofilm-production of *S. aureus* clinical isolates ($p = 0.001$) and formation of their biofilms on the surfaces of catheters with different polymer structure. Moreover, suppression of this process by local anaesthetics has been showing on the teflon catheters better than polyurethane ones.*

Conflict of interest: Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article.

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