Experimental Grounds for Using Collagen-Based Anti-Adhesion Barrier Coated with Biocides for Prevention of Abdominal Surgical Infection

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The aim of the study was to evaluate adhesive properties of the surgical anti-adhesion barrier based on collagen in combination with 0.05% Chlorhexidine bigluconate solution and Prontosan in experiment performed *in vitro*.

Materials and Methods. The study was carried out using CollaGUARD adhesion barrier consisting of renatured horse type I collagen and reference strains of *Escherichia coli* ATCC®25922, *Klebsiella pneumoniae* ATCC®700603, *Pseudomonas aeruginosa* ATCC®27853, *Staphylococcus aureus* ATCC®25923. Bacteria adhesion to the barrier (membrane) untreated and combined with biocides was evaluated by determining the viable colony forming unit (CFU/cm^2) numbers and using atomic force microscopy after 24 h and 6 days.

Results. Cells of all bacterial strains adhered to the surface of the membrane within 24 h forming a biofilm of bacilli tightly adjacent to each other (*E. coli*, *P. aeruginosa*) or cocci (*S. aureus*) integrated between disorganized collagen fibers, or adherent bacteria were seen separately (*K. pneumoniae*). The *Sq* index characterizing the surface roughness of the biofilm formed by *S. aureus* bacteria was 221.3±38.6 nm and was 3.0, 3.8, and 3.6 times higher compared with that for *E. coli* (72.8±12.6 nm), *K. pneumoniae* (57.5±21.8 nm), and *P. aeruginosa* (60.8±22.1 nm), respectively, and also exceeded 4.4 times the *Sq* index for the membrane itself (50.3±26.3 nm). Collagen degradation was revealed in case of contamination by protease-producing bacteria with collagenase activity: *P. aeruginosa* for 24 h and *S. aureus* for 6 days. Adsorption of the biocide on the membrane surface after its short exposure to Chlorhexidine bigluconate solution or Prontosan led to inhibited growth and adhesion of cells of bacteria, except for *P. aeruginosa*. In experimental models, in agar medium and in suspension culture, Chlorhexidine bigluconate proved to be more effective than Prontosan. The difference between the biocides in inhibiting the adhesion of bacteria to the membrane surface is not associated with changes in its surface roughness.

Conclusion. Impregnation of a surgical membrane with antibacterial compounds just before the implantation can serve as an additional method for preventing abdominal surgical infection.

Key words: collagen anti-adhesion membrane; bacterial adhesion; Chlorhexidine bigluconate; Prontosan; abdominal surgical infection.

Introduction

During the post-surgical period, the frequency of the adhesion process following abdominal surgery remains high and amounts to 85% according to different authors [1, 2], which is accompanied by a decrease in the quality of life of patients due to chronic pain syndrome and dyspeptic phenomena. Besides, there is a possibility of developing acute adhesive intestinal obstruction which accounts for up to 90% of all acute

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intestinal obstruction cases [3]. Surgical treatment of this pathology is associated with a high risk of tissue trauma and mortality [4].

Adhesion prevention strategy involves minimizing accesses, specifically, by endoscopic techniques, careful handling of tissues during surgery, meticulous hemostasis and lavage of the abdominal cavity with neutral solutions, prevention of foreign body intrusions, the use of state-of-the-art areactive atraumatic suture material as well as delimiting or separating serous surfaces with anti-adhesion barriers (membranes) [1, 3, 5]. The mechanism of action of the latter is based on temporal separation of contiguous organs on the visceral and parietal areas of the abdominal cavity during the whole process of physiological tissue regeneration. This method is believed to have the highest preventive effect [2, 5, 6].

Anti-adhesion barriers are made on the basis of various substances (dextran solutions, hyaluronic acid, carboxymethylcellulose, collagen) and have liquid, gel or solid aggregate state. Great attention is paid to collagen as the base material of anti-adhesion barriers. Its advantages include weak antigenicity, non-toxicity, high biocompatibility, biodegradation ability and hemostatic properties [7, 8]. Another reason for the widespread use of collagen is the strength and stability of the fiber in the formation of cross-links under the action of glutaraldehyde [9]. The use of collagen simultaneously with allosteric implants has been shown to be more physiological increasing tolerability of synthetic prosthetic materials [10, 11].

Given that the collagen membrane can play the role of a reservoir for drugs, impregnating the membrane with antibacterial substance solution immediately before implantation can prevent its interaction with the bacteria of the compromised biotope. Application of biopolymers with marked colonization resistance allows preventing surgical site infection. The possibility of using collagen membrane in patients operated on for acute surgical diseases of the abdominal cavity with a high risk of postoperative peritonitis remains understudied.

The aim of the study was to evaluate adhesive properties of a surgical anti-adhesion barrier based on collagen in combination with biocides in the experiment carried out *in vitro*.

Materials and Methods

The work was performed using reference strains of *Escherichia coli* ATCC®25922, *Klebsiella pneumoniae* ATCC®700603, *Pseudomonas aeruginosa* ATCC®27853, *Staphylococcus aureus* ATCC®25923 obtained from the National Collection of Pathogenic Microorganisms of the National Institute of Standardization and Control named after L.A. Tarasevich (now Scientific Center for Evaluation of Medical Products, Ministry of Health of the Russian Federation, Moscow).

The study was carried out using 0.5 mm thick CollaGUARD adhesion barrier (Innocoll, Germany) consisting of renatured horse type I collagen degradable in the macroorganism's tissues during 3–5 weeks [12].

At the preliminary stage, the technique of V. Pérez-Köhler et al. [13] was used. Suspensions of overnight bacterial culture cells standardized to 2 units according to McFarland standard and diluted 1:100 in Luria–Bertani broth (LB-broth) were inoculated with lawn (50 μl) on LB-agar. CollaGUARD anti-adhesion barrier fragments (≈10×10 mm) pre-immersed for 10 min in 0.89% NaCl (control), 0.05% aqueous Chlorhexidine bigluconate and Prontosan (0.1% undecylenic amidopropyl betaine; 0.1% polyaminopropyl biguanide — polihexanide) were laid out.

Chlorhexidine bigluconate concentration was chosen based on most frequent use in surgical practice. Culture dishes were incubated at 37°C for 24 h. Antibacterial activity was evaluated taking into account bacterial growth inhibition zones (GIZ) by *k* coefficient calculated as the area ratio in millimeters: $S_{GIZ}/S_{fragment}$.

At the second stage, the analogous membrane fragments were placed in the wells of 24-well flat-bottomed polystyrene culture plate (Corning, Belgium) containing suspension (1.0 ml) diluted 1:100 with standardized overnight culture of each bacterium. The dynamics of microbial population growth was monitored by measuring the optical density of cells with Benchmark Plus microplate reader (Bio-Rad Laboratories, USA) at 630 nm wavelength after 24 h and 6 days.

After exposure, barrier fragments were washed three times in 5 ml of 0.89% NaCl, put in 1.0 ml phosphate-buffered medium and ultrasonicated at 37 kHz 5 times during 1 min, the tablets placed in Elma Ultrasonic 30S sonication bath (Elma, Germany). Bacterial adhesion was evaluated by the number of living cells (colony forming units, CFU/ml) after inoculation of bacterial suspensions from consecutive decimal dilutions onto LB-agar.

Surface profiles and bacterial adhesion were studied using Asylum MFP-3D-BIO atomic force microscope (Asylum Research, USA) in the Laboratory of Atomic-Force and Confocal Microscopy hosted by Rhodococcus centre of E.A. Vagner Perm State National Research University (Russia). Scanning was performed in a tapping mode in air using AC240TS silicon cantilever (Asylum Research, USA) with 50–90 kHz resonant frequency and 0.5–4.4 N/m spring constant. To characterize the biofilm surface structure (roughness, *Sq*) and height, two- and three-dimensional topographical images of bacteria were obtained. The images were processed using Igor Pro 6.22A software (WaveMetrics, USA).

Statistical analysis of the data obtained was performed using Microsoft Office Excel 2016 and Statistica 10.0 software. The indices were presented as the arithmetic mean and its error (M±m). The significance of differences in the mean values was determined using paired Student's t-test, Mann–Whitney U-test was used to compare two independent samples.

The differences between the compared samples were considered statistically significant at $p<0.05$.

Results

The experiments in agar medium revealed that with CollaGUARD barrier without preliminary treatment with antiseptics, all bacterial cultures under study formed lawn growth around its fragments and in most cases also beneath it (Figure 1). Short-term exposure of the barrier to biocide solutions led to emergence of growth inhibition zones in all type strains of tested bacteria. Table 1 shows the data on *k* coefficient for CollaGUARD barrier in the control (of 0.89% NaCl) and test (antiseptic agents) variants of the experiment.

In case of CollaGUARD + Chlorhexidine bigluconate combination, the antibacterial effect was the most pronounced in *S. aureus* (k=3.69±1.28), but for other cultures, the difference from the control variant also proved to be significant. Statistically significant difference in this combination was revealed only in the pair of *S. aureus* and *E. coli* (U-test: p=0.0306). After exposure of a barrier fragment to Prontosan, *k* coefficient was significantly lower than in case of Chlorhexidine bigluconate for all studied cultures. When comparing antibacterial activities of the two antiseptic agents, it was revealed that the effect of Chlorhexidine bigluconate was more pronounced than that of Prontosan for all investigated strains, for *E. coli* and *S. aureus* it was even statistically significant.

The following data were obtained in the experiments on adhesion of bacteria to the surface of CollaGUARD barrier in suspension culture. After 24 h, the indices of optical density of *E. coli, K. pneumoniae,* and *S. aureus* cell suspensions were significantly lower in the presence of membrane fragments impregnated with antiseptics than in the control (Table 2). Bacterial growth of *P. aeruginosa* was not inhibited in any variant, with barrier fragments not even detected in three out of four independent experiments.

After 6 days, optical density of *E. coli* and *K. pneumoniae* cultures in variants with biocides was also lower than in the control one. By that time, the collagen membrane had fully "dissolved" in the control variant with *S. aureus* bacteria.

Cell adherence to the membrane surface was registered within 24 h for all microorganism representatives (for *P. aeruginosa* — data from one experiment). In combination with Chlorhexidine bigluconate, we revealed no colonization of the membrane by *E. coli* and *S. aureus* bacteria in this period, and adhesion of *K. pneumoniae* cells was statistically significantly lower than in the control. Prontosan proved to be less effective: the number of enterobacteria adhered to the barrier surface was 10^3–10^4 CFU/ml. Antibacterial activity of biocides regarding *S. aureus* was registered in both exposure periods.

When analyzing images obtained with atomic force microscopy, it was found that after 24 h a biofilm formed on the surface of the membrane fragment, which

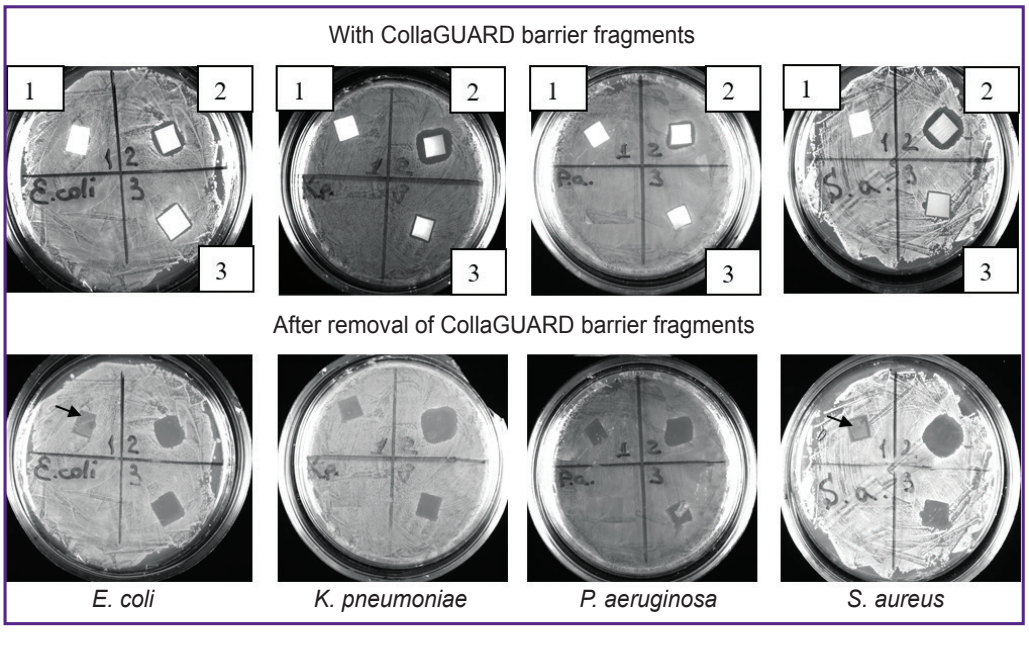

Figure 1. Zones of bacterial culture growth inhibition, created by fragments of CollaGUARD adhesion barrier in combination with biocides:
(*1*) 0.89% NaCl (control); (*2*) 0.05% Chlorhexidine bigluconate solution; (*3*) Prontosan; the images have been obtained using Gel Doc XR+ system (Bio-Rad Laboratories, USA) and represent each investigated bacterial culture at 24-hour lawn growth on LB-agar; *arrows* indicate the areas of bacterial colonization under the membrane fragments in the control variants

Table 1

_K_ coefficient characterizing bacterial growth inhibition in the diffusion test with the use of CollaGUARD barrier in combination with biocides

Culture	Variant of the experiment*	k, $S_{GIZ}/S_{fragment}$	Significance of differences (Student t-test)		
			p_1	p_2	p_3
E. coli	0.89% NaCl	1.02±0.03	0.0002	0.0650	0.0227
	Chlorhexidine bigluconate	2.37±0.35			
	Prontosan	1.54±0.56			
K. pneumoniae	0.89% NaCl	1.04±0.11	0.0495	0.0310	0.0875
	Chlorhexidine bigluconate	2.31±1.39			
	Prontosan	1.45±0.38			
P. aeruginosa	0.89% NaCl	1.0±0.0	0.0079	0.0205	0.0722
	Chlorhexidine bigluconate	2.59±0.04			
	Prontosan	1.69±0.06			
S. aureus	0.89% NaCl	1.0±0.0	0.0036	0.0210	0.0496
	Chlorhexidine bigluconate	3.69±1.28			
	Prontosan	2.26±0.93			

Notes. * n=6 for each variant; p_1 — differences between 0.89% NaCl and Chlorhexidine bigluconate; p_2 — differences between 0.89% NaCl and Prontosan; p_3 — differences between Chlorhexidine bigluconate and Prontosan.

Table 2

Optical density of bacterial suspension and number of viable cells adhered on the surface of CollaGUARD barrier in combination with biocides

Culture	Variant of the experiment*	Optical density of suspension (OD600)		Number of cells adhered (CFU /ml)	
		After 24 h	After 6 days	After 24 h	After 6 days
E. coli	0.89% NaCl	0.823±0.202	1.043±0.103	1.92E+05±2.20E+05	4.63E+05±6.53E+05
	Chlorhexidine bigluconate	0.276±0.167$^+$	0.464±0.399$^+$	0.00E+00±0.00E+00$^+$	4.00E+02±6.93E+02$^+$
	Prontosan	0.274±0.137$^+$	0.609±0.099$^+$	7.36E+03±5.37E+03$^+$	1.03E+02±1.41E+02$^+$
K. pneumoniae	0.89% NaCl	0.807±0.095	1.153±0.371	7.01E+06±1.13E+07	1.27E+05±1.67E+05
	Chlorhexidine bigluconate	0.161±0.065$^+$	0.177±0.018$^+$	4.32E+03±5.88E+03$^+$	4.08E+03±7.07E+03$^+$
	Prontosan	0.319±0.116$^+$	0.292±0.227$^+$	4.84E+04±1.76E+04$^+$	2.54E+03±8.31E+03$^+$
P. aeruginosa	0.89% NaCl	1.173±0.241	1.131±0.093	1.88E+06**	—
	Chlorhexidine bigluconate	1.063±0.685	1.002±0.103	1.18E+04**	—
	Prontosan	1.052±0.424	1.112±0.423	5.05E+06**	—
S. aureus	0.89% NaCl	0.999±0.101	0.988±0.613	3.27E+05±1.95E+05	1.14E+06**
	Chlorhexidine bigluconate	0.345±0.193$^+$	0.510±0.189	0.00E+00±0.00E+00$^+$	0.00E+00±0.00E+00$^+$
	Prontosan	0.461±0.372$^+$	0.440±0.211$^+$	0.00E+00±0.00E+00$^+$	0.00E+00±0.00E+00$^+$

Notes. * n=3 for each variant; $^+$ statistical significance of differences as compared to the corresponding control (0.89% NaCl); ** one experiment data are shown.

had a structure of bacilli tightly adjacent to each other (*E. coli, P. aeruginosa*), or cocci (*S. aureus*) integrated between disorganized collagen fibers or separately adhered bacteria (*K. pneumoniae*) (Figure 2 (a)). Groups of staphylococci cells (the bacteria diameter was 1.10±0.08 μm) were integrated between disorganized collagen fibers. Indeed, *Sq* index characterizing the biofilm surface formed by *S. aureus* bacteria equaled 221.3±38.6 nm and was 3.0, 3.8, and 3.6 times higher compared to that for *E. coli* (72.8±12.6 nm), *K. pneumoniae* (57.5±21.8 nm), and *P. aeruginosa* (60.8±22.1 nm) and exceeded the same index in the

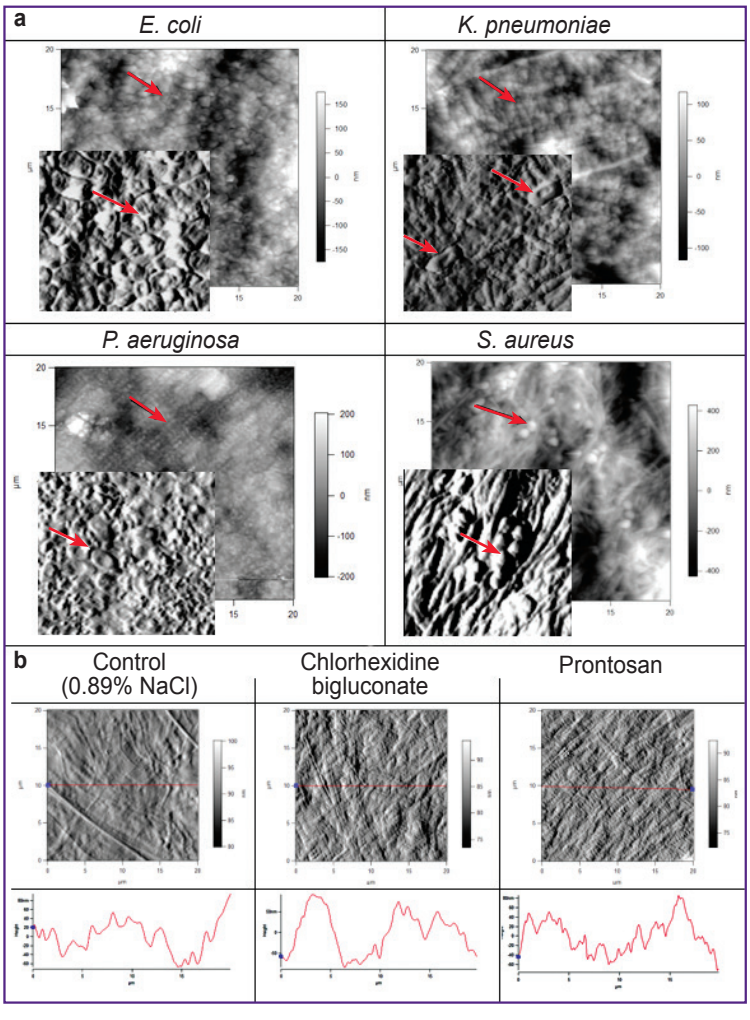

Figure 2. Images of CollaGUARD adhesion barrier fragment obtained by atomic force microscopy:
(a) after 24-hour exposure to bacterial suspensions of reference strains (*arrows* indicate bacterial cells); (b) after 10-minute exposure to biocide solutions; the diagrams show the results of surface profilometry in the direction indicated by the red line in the photo

control by 4.4 times (50.3±26.3 nm). No significant changes in surface roughness ($p>0.05$) were found after short-term exposure of collagen fibers to Chlorhexidine bigluconate (*Sq*=47.1±19.3 nm) and Prontosan (*Sq*=52.9±7.4 nm) (Figure 2 (b)).

Discussion

In contrast to synthetic implants, surgical anti-adhesion barriers based on natural substances, particularly collagen, are biocompatible, display high surface activity and biodegradation ability. Being the main protein of connective tissue, "exogenous" collagen implanted during laparotomy incorporates into the surrounding tissues to ensure healing, reconstruction and restoration of mechanical and functional integrity of the abdomen [9]. The currently used cross-linking technology stabilizing three-dimensional fiber structure determines membrane resistance to tissue collagenase of the macroorganism [14]. CollaGUARD adhesion barrier consisting of renatured horse type I collagen belongs to this type of barriers. The study carried out by the manufacturer has proved that application of CollaGUARD membrane lowers the risk of adhesion formation in surgical patients by more than six times and also statistically significantly reduces their incidence and severity when assessed *in vivo* [12]. There have been demonstrated the results of successful application of this membrane in abdominal and pelvic surgery in Russia [10, 15].

Collagen fibers are known to have a positive effect on adhesion of microbial cells and to promote biofilm formation [16–18]. Adherence of Staphylococcus aureus cells to collagen, for example, is mediated by collagen-binding protein (Cna), which is a MSCRAMM-factor (microbial surface components recognizing adhesive matrix molecules) [19]. *P. aeruginosa* adheres to any surface, especially through type IV pili, but adhesion to collagen can occur via a mechanism unrelated to pili [20]. On the surface of *Pseudomonas* cells there is a common collagen-binding receptor which is blocked by sugars, inhibiting adhesion of *P. aeruginosa* to type I and type II collagen [21]. There has been described a variant of type I mannose-sensitive fimbriae whose FimH adhesin contains Ala62 amino acid residue necessary for adhesion of meningitis-associated *E. coli* to type

I and type IV collagen [22]. *K. pneumoniae* type III pili are consist of basic protein subunit (MrkA) and adhesin (MrkD) which mediates cell binding to type IV and/or type V collagen [23]. Given that bacterial adhesion to the surface of the implanted natural polymer which becomes a source of nutrient substrates for microorganisms is a key stage of biofilm formation, coating of the implant with an antimicrobial drug is a promising strategy to prevent implant-associated infection [13, 18]. In clinical practice of some countries, it has been proposed to impregnate or put an allosteric implant in an antibiotic solution just before use [24–26]. However, the efficacy of this method in reducing the risk of bacterial complications when using anti-adhesion barriers made of natural fibers has not been proved.

The present experimental study is devoted to in vitro evaluation of *E. coli, K. pneumoniae, P. aeruginosa, S. aureus* adhesion to CollaGUARD adhesion membrane untreated and combined with Chlorhexidine bigluconate and Prontosan, the biocides most commonly used in surgical practice. The selected microbial spectrum has been determined by the data provided by numerous multicenter national and international studies where the main etiopathogens of secondary peritonitis are identified [27–29]. The concentration of bacteria used in the experiments on modeling of bacterial colonization conditions was 10^6 cells/ml, which exceeded significantly the bacterial load required for surgical site infection [30] — this model is a common practice in studying bacterial adhesion *in vitro*.

Preliminary results obtained on agar medium confirmed the antibacterial effect of the tested biocides held by the membrane fibers.

It should be noted that the effect of Chlorhexidine bigluconate on all bacterial cultures was more pronounced than that of Prontosan, the difference being statistically significant for bacteria *E. coli* and *S. aureus*. Indeed, *k* coefficient, characterizing the degree of bacterial growth inhibition was the lowest for *E. coli* and *K. pneumoniae* in the variant with Prontosan, which was consistent with the experiment results in suspension culture: bacterial colonization of surfaces was recorded just after a day. The absence of *S. aureus* cell adhesion on the membrane surface impregnated with both biocides was not without reason: growth inhibition zones and, accordingly, *k* coefficient were the highest on agar. We should notice a significant decrease in the optical density of cell suspension, registered in variants with biocides for all cultures, except *P. aeruginosa*. However, after a day, even fragments of CollaGUARD barrier, including those impregnated with antiseptics, were not found in three of the four experiments. Given that *P. aeruginosa* produces several proteases, including elastases (LasA and LasB), alkaline protease (AprA), new proteases described recently — protease IV and large exoprotease (LepA) — complete dissolution of the membrane in a short time is understandable [31–33]. Besides, collagen decay is believed to occur mainly at a neutral pH level, in which LasB Zn-bound metalloprotease affecting many proteins including collagen is functionally active [34].

It should be noted that CollaGUARD membrane fragments completely degraded in the control variant with *S. aureus* after six days. Evidently, colonization of the membrane surface by *S. aureus* cells and subsequent production of extracellular cysteine proteases such as staphopain A and B (ScpA and SspB) [35] led to its complete degradation.

Proteases with collagenolytic activity are also described for *E. coli* (U32 family with unknown catalytic mechanism) and *K. pneumoniae*, but these enzymes either have low affinity for the substrate (substrate specificity) or are active at different temperature and pH optima [36, 37].

Previously, we showed the negative effect of Chlorhexidine bigluconate and Prontosan on the sessile forms of *S. aureus* and *P. aeruginosa* bacteria in mixed and mono-species variants, which manifested itself in biofilm biomass reduction, cell polymorphism and loss of cell viability [38]. Biocides affected ready-made biofilms and the advantage of Prontosan was due to the action of amidopropyl betaine displaying the properties of a surfactant that destroys biofilm structure, which resulted in high efficiency of the bactericidal component. In this work, we have revealed no superiority of Prontosan as it was expected, though surfactants are known [39] to inhibit bacterial adhesion to different surfaces. Concentrating on the interphase surfaces (interfaces), amphoteric amidopropyl betaine is most likely to form a mono- or polymolecular layer which makes collagen fiber surface inaccessible to adsorption of another substance — polyhexanide exhibiting antibacterial activity. After exposure to Chlorhexidine bigluconate, anti-adhesion effect appeared to be higher, though it was not associated with changes in the surface roughness, since the topography and *Sq* index had no statistically significant differences.

The fundamental possibility of applying anti-adhesion barriers in conditions of bacterial contamination of surgical intervention site was shown earlier [40], but the feasibility of this approach in relation to collagen membranes and membranes combined with collagen seems ambivalent. Collagen is known to stimulate adhesion of both bacteria and host cells to the membrane surface and adjacent tissues, which is called “race on the surface” [41]. Besides, abdominal infection initiates synthesis of matrix metalloproteinases (interstitial collagenase MMP-1, neutrophil collagenase MMP-8, etc.) in most connective tissue cells, fibroblasts and macrophages, which promotes enzymatic degradation of collagen [9]. Collagen degradation is increased in case of contamination by protease-producing bacteria with collagenase activity. These and other factors determine contraindications for the use of collagen membranes in conditions of surgical site infection. Effectiveness of antimicrobial coating of anti-adhesion barriers (including

collagen-based or other natural surgical membranes) in controlling bacterial infections is considered to be proved insufficiently.

The results obtained in the study showed that *E. coli, K. pneumoniae, P. aeruginosa, S. aureus* which are the leading pathogens of the abdominal surgical infection adhered to the surface of CollaGUARD adhesion barrier during 24 h. The use of atomic force microscopy has made it possible to assess morphology and structural properties of biofilms formed by representatives of different taxons on the collagen membrane. Adsorption of a biocide on the membrane surface after its short exposure to Chlorhexidine bigluconate and Prontosan solution provides inhibited growth and adhesion of type strain cells of bacteria (except for *P. aeruginosa*). The absence of antibacterial/anti-adhesive action of antiseptics against *P. aeruginosa* is associated with the production of numerous proteases with collagenase activity. The difference between the biocides in inhibiting bacterial adhesion to the membrane is not associated with changes in its surface roughness.

The experimental model *in vitro* provides no possibility to predict how collagen membranes covered with antiseptics will behave *in vivo* in conditions of abdominal surgical infection. It is necessary to carry out further experimental and clinical studies to assess the feasibility of using anti-adhesion barriers based on natural substances in combination with biocides for prevention of adhesive disease in patients at risk of peritonitis and also to find out the role of antimicrobial coating in the processes of membrane integration and tissue regeneration.

Conclusion

Collagen fibers with antiseptics absorbed intraoperatively may promote release of biocides into the surrounding tissues in the postoperative period and eradication of infectious agents. Consequently, impregnation of a surgical membrane with antibacterial compounds just before the implantation is a promising strategy to prevent surgical site infections.

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Conflict of interests. The authors have no conflict of interests to disclose.

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