Formation of biofilms in traumatic injuries of the ocular adnexa

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Introduction. *Biofilms (microbial communities) can be formed by both pathogenic and non-pathogenic microorganisms. The formation of biofilms causes many problems in the clinical practice as they slow down the healing process and are often characterized by antibiotic resistance.*

Purpose. *To study the ability of microorganisms that were obtained from the wounds of patients with traumatic injuries to the ocular adnexa to form biofilms.*

Materials and Methods. *60 patients with traumatic ocular adnexal injuries were examined. The bacteriological examination of wound swabs was carried out with species composition and population levels of the microflora defined. The ability of microorganisms to form biofilms was further analyzed. The biofilm studies were performed to determine the amount of biomass generated [O'Toole and Kolter, 1998] by dyeing the biofilms with crystal violet. The method is based on the ability of the crystal violet dye to bind to cells and a matrix of biofilm. The results were interpreted according to the optical density of the dyed solvent.*

Results. *Seventy-five (75) strains of microorganisms were collected and identified from sixty (60) patients. Gram-positive and gram-negative microflora made up 73% (n=54) and 27% (n=20), respectively. The leading positions were taken by Staphylococcus aureus (n=21), Acinetobacter spp. (n=11), Klebsiella ozenae (n=8), Micrococcus spp. (n=8), Corynebacterium spp. (n=5), Artrobacter cuminsii (n=4), Staphylococcus epidermidis (n=3). The greatest ability for biofilm formation was manifested in Staphylococcus aureus, Acinetobacter spp. and Klebsiella ozenae, which are the representatives of the opportunistic flora.*

Staphylococcus aureus was most intensively forming a biofilm in the time interval from Day 1 to Day 3; Acinetobacter spp. was most active during the first day and Klebsiella ozenae – Day 3 to Day 7.

Conclusions. *Most clinically relevant microorganisms that had been collected from the wounds of patients with traumatic injuries of the ocular adnexa had an ability to form microbial biofilms. This ability was most evident in Staphylococcus aureus, Acinetobacter spp. and Klebsiella ozenae, which are the representatives of the opportunistic flora.*

Keywords:
biofilm, microorganisms, wound infection, ocular adnexa, crystal violet

Introduction

In the natural environments, microorganisms are known to exist mostly within microbial communities which are called biofilms. The current concept describes a biofilm as a continuous layer of bacterial cells which stick to each other and a surface and embedded with a biopolymer matrix [1, 4]. Such bacterial organizations can be formed by bacteria of one or several types. They can be comprised of active functioning cells or of "resting" nonculturable bacteria. Biofilms can be formed by nonpathogenic and pathogenic bacteria from the skin and mucosa flora [1, 8].

It has been proved than microorganisms can form biofilms on both biotic and abiotic surfaces [1,8,11]. Microbial biofilms colonize all implants which are used in the medical practice, such as suture material, drainage, contact lenses, intraocular lenses, stents, catheters, artificial valves etc. [1, 5, 11]. This deserves special attention since biofilms can develop on them as early as first days after being implanted. This mode of existence of bacteria, fungi, and microbial associations causes numerous problems in the surgical practice since microbial biofilm formation contributes to inflammation, making it chronic, and significantly slows down the wound healing [5, 7]. In addition, biofilm microorganisms are characterized by high rates of resistance to antibacterial drugs, antiseptics, disinfectants, antibodies, and phagocytes [5, 8].

Experiments have demonstrated that planktonic forms of bacteria and fungi in most cases induce acute inflammation and acute exacerbation of a chronic disease, which can be observed at biofilm rupture and dissemination of the pathogen. While microorganism of

biofilms can cause chronic diseases, among which are ocular inflammatory diseases (blepharitis, conjunctivitis, keratitis, scleritis, uveitis), infections of ocular adnexa soft tissues, chronic osteomyelitis etc. [1, 5, 8, 11].

Researchers, including microbiologists, pharmacists, and clinicians, pay much attention to studying biofilms since the ability of pathogenic bacteria to form biofilms creates problems in the clinical practice. Given all of the above and the relevance of this problem, **the purpose** of our paper was to study the ability of microorganisms that were obtained from the wounds of patients with traumatic ocular adnexal injuries to form biofilms.

Materials and methods

In 2018-2019, we examined 60 patients with traumatic injuries of the ocular adnexa who applied to the emergency room of Eye Microsurgery Department, Sumy Regional Clinical Hospital. The examinations followed the standards of health care delivery and regulations of the ethics committee. The bacteriological examination of wound swabs was carried out to define the presence of microorganisms in the injured tissues and to study their species composition. The material studied was collected at the patient's first visit before primary surgical debridement and antibacterial therapy. Species composition and population levels of microflora in the injured tissues were defined following the modification requirements for counting the number of bacteria using the Urquhart and Gould plating method, at the Bacteriological Laboratory of the Sumy State University Medical Institute. Isolates with a bacterial count of 102 CFU/mL were considered diagnostically significant. Species were identified using classical methods for isolation and identification of microorganisms [3]. The microorganisms which were sown more frequently were, afterwards, analyzed for the ability to form biofilms. The amount of biomass generated was determined in the biofilms [O'Toole and Kolter, 1998] [1, 3, 9]. Thus, a mixture of the microorganisms studied was placed into a polystyrene plate and incubated in a nutrition broth for 1, 3, and 7 days at 37ºC. After culturing bacteria, the medium with planktonic cells was diluted from the plate and washed thrice in sterile phosphate buffer saline (PBS) at the same amount as the cultivation; PBS was also removed. Afterwards, 4 ml of 0.1% crystal violet were placed into the plate wells. Biofilms were incubated with dye for 10-15 minutes at room temperature. The dye was then removed from the wells which were then washed to remove the non-bonded dye. The plates were turned upside-down onto filter paper and dried. Thereafter, 4 ml of 95% ethanol solution were added to the wells. The solvent was collected and placed in clean flat-bottomed plates and the optical density was measured at a wavelength of 595 nm by a Multiscan FC Microplate Photometer (Thermo Scientific, ESW 1.01.16 version). The results were interpreted according to the optical density of the dyed solvent [1, 3, 9]. The results were processed statistically by the Graph Pad Quik Calcs program with the t-Student test.

Results and Discussion

60 patients with traumatic injuries of the ocular adnexa were examined, which revealed and identified 75 strains of microorganisms. A relative ratio of Gram-positive to Gram-negative flora was 73% (n=54) to 27% (n=20), respectively, which is demonstrated in Figure 1.

Table 1 shows that the leading positions were taken by Staphylococcus aureus (n=21), Acinetobacter spp. (n=11), Klebsiella ozenae (n=8), Micrococcus spp. (n=8), Corynebacterium spp. (n=5), Artrobacter cuminsii (n=4), Staphylococcus epidermidis (n=3). The rest of the strains were revealed infrequently.

Staphylococcus epidermidis, Micrococcus spp., coryneform bacteria (Corynebacterium spp., Artrobacter cuminsii) are commensal bacteria and cannot cause diseases if the skin is not damaged. Staphylococcus aureus, Acinetobacter spp. and Klebsiella ozenae are opportunistic pathogens which can induce various purulent inflammations, from soft tissue wound infection to panophthalmitis, and are often characterized by resistance to antibiotics (Fig. 2) [2, 7].

Considering the frequency of microorganism isolation and the ability to form biofilms, we revealed Staphylococcus aureus, Acinetobacter spp., Klebsiella ozenae, Micrococcus spp., Corynebacterium spp., Arthrobacter cuminsii, and Staphylococcus epidermidis.

Based on the data in Table 2, it can be concluded that most microorganisms form biofilms in vitro. Most of all, this ability is shown in Staphylococcus aureus, Acinetobacter spp. and Klebsiella ozenae, which are opportunistic pathogens. The rest of the strains are characterized by a weaker ability to form biofilms. The difference was considered to be statistically significant with $p<0.05$.Figure 3 demonstrates the changes in forming biofilms by opportunistic pathogens.

In Figure 4, it can be seen that Staphylococcus aureus was forming a biofilm at the most intensive rate within the time period from Day 1 to Day 3.

Figure 5 demonstrates that the most intensive rate of biofilm formation for Acinetobacter spp. was on Day 1.

Figure 6 shows that Klebsiella ozenae was forming a biofilm most actively from Day 3 to Day 7.

Experiments have demonstrated that the ability of microorganisms to form a biofilm can be realized not only in vitro but also directly in wounds [5, 6, 7, 8]. It is also known that the planktonic forms of bacteria and fungi in most cases induce acute inflammation and acute exacerbation of a chronic disease, which can be observed at biofilm rupture and dissemination of the pathogen, while microorganism of biofilms can cause chronic diseases [4, 6, 7]. Since bacteria in biofilms are resistant to both antimicrobial drugs and nonspecific defences, one of the directions in treating wound infection must be the inhibition of microorganisms' ability to form a biofilm and lysis of existing biofilms [4, 7, 10].

Conclusions

Firstly, Staphylococcus aureus (n=21), Acinetobacter spp. (n=11), Klebsiella ozenae (n=8), Micrococcusspp. (n=8), Corynebacterium spp. (n=5), Arthrobacter cuminsii (n=4), Staphylococcus epidermidis (n=3) were sown more frequently in the patients with traumatic injuries of the ocular adnexa. Other strains were sown rarely ($p<0.05$).

Secondly, most clinically significant microorganisms obtained from the wounds of the patients with ocular adnexal injuries had an ability to form biofilms.

Thirdly, most of all this ability was manifested in Staphylococcus aureus, Acinetobacter spp. and Klebsiella ozenae, which are representatives of the opportunistic flora.

Finally, Staphylococcus aureus was most intensively forming a biofilm in the time interval from Day 1 to Day 3; Acinetobacter spp. was most active during the first day and Klebsiella ozenae – from Day 3 to Day 7.

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Table 1. Species of the microorganisms isolated in wounds of the patients with ocular adnexa traumatic injuries

No	Isolated strains of icroorganisms	The number of microorganisms isolated	
		Absolute count	Relative count (%)
1	Staphylococcus aureus	21	28.0%
2	Acinetobacter spp.	11	14.7%
3	Micrococcus spp.	8	10.7%
4	Corynebacterium spp.	5	6.7%
5	Propionobacterium freudenreichii	1	1.3%
6	Staphylococcus hominis	1	1.3%
7	Staphylococcus klosii	1	1.3%
8	Staphylococcus capitis	1	1.3%
9	Staphylococcus hyicus	1	1.3%
10	Staphylococcus cohnii	1	1.3%
11	Staphylococcus arlettae	1	1.3%
12	Clostridia fallax	1	1.3%
13	Arthrobacter cuminsii	4	5.4%
14	Aerococcus viridans	2	2.7%
15	Listeria monocytogenes	1	1.3%
16	Listeria innocua	1	1.3%
17	Candida spp.	1	1.3%
18	E. coli	1	1.3%
19	Dermabacter spp.	1	1.3%
20	Staphylococcus epidermidis	3	4.0%
21	Klebsiella ozenae	8	10.7%
Total		75	100.0%

Table 2. Values of the optical density of the strains studied

No	Clinical strain	Initiation	Day 1	Day 3	Day 7	Dye
1	Staphylococcus aureus	0.105	0.240*	0.412*	0.488*	Crystal violet
2	Micrococcus spp.	0.305	0.330	0.360	0.478*,**	Crystal violet
3	Klebsiella ozenae	0.187	0.270	0.308	0.578*	Crystal violet
4	Corynebacterium spp.	0.271	0.286*	0.313*,**	1.039*,**,***	Crystal violet
5	Acinetobacter spp.	0.182	0.375*	0.563*	0.770*,**,***	Crystal violet
6	Arthrobacter spp.	0.147	0.240*	0.330*,**	0.936*,***	Crystal violet
7	Staphylococcus epidermidis	0.232	0.240	0.248	0.477*,**,***	Crystal violet

Notes: * - this parameter is statistically significantly different ($p<0.05$) from that at initiation ** this parameter is statistically significantly different ($p<0.05$) from that at Day 1 *** this parameter is statistically significantly different ($p<0.05$) from that at Day 3

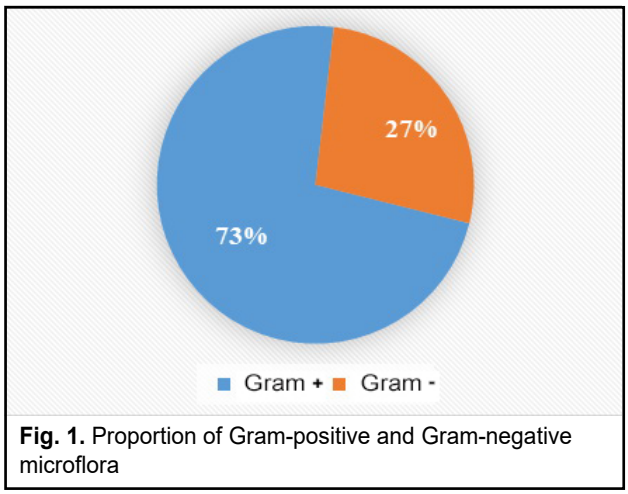

Fig. 1. Proportion of Gram-positive and Gram-negative microflora

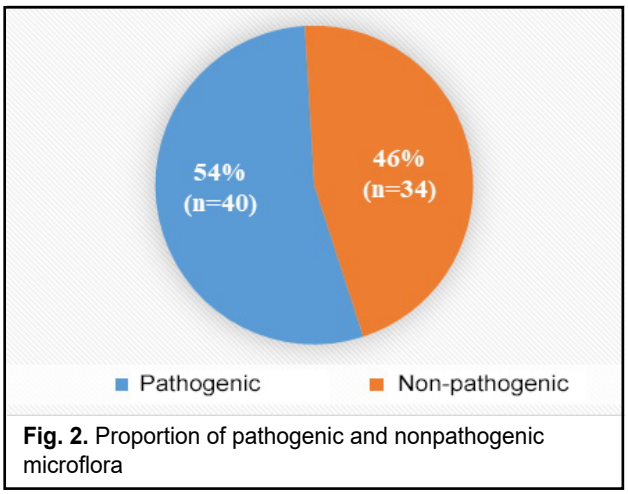

Fig. 2. Proportion of pathogenic and nonpathogenic microflora

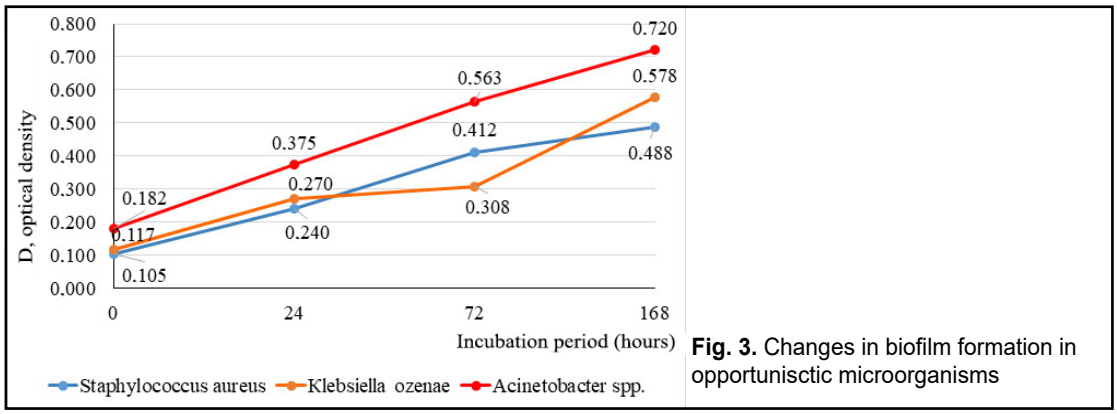

Fig. 3. Changes in biofilm formation in opportunisctic microorganisms

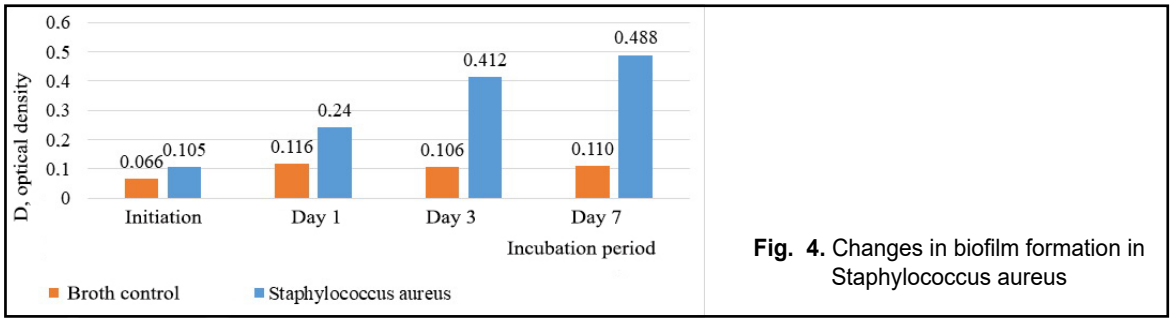

Fig. 4. Changes in biofilm formation in Staphylococcus aureus

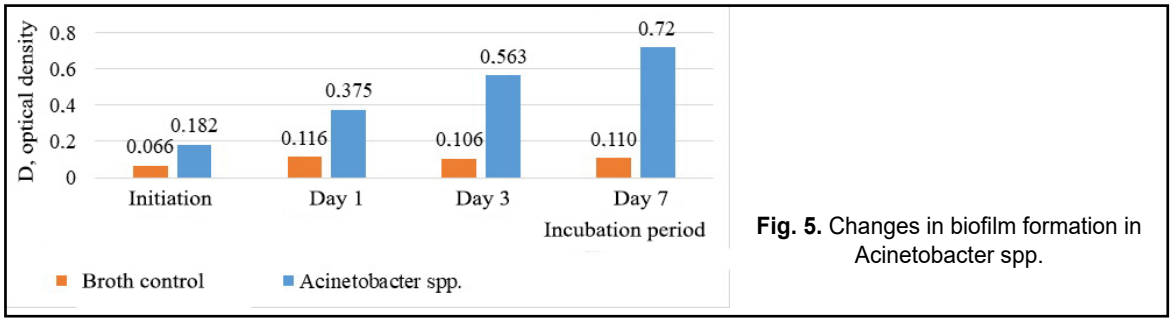

Fig. 5. Changes in biofilm formation in Acinetobacter spp.

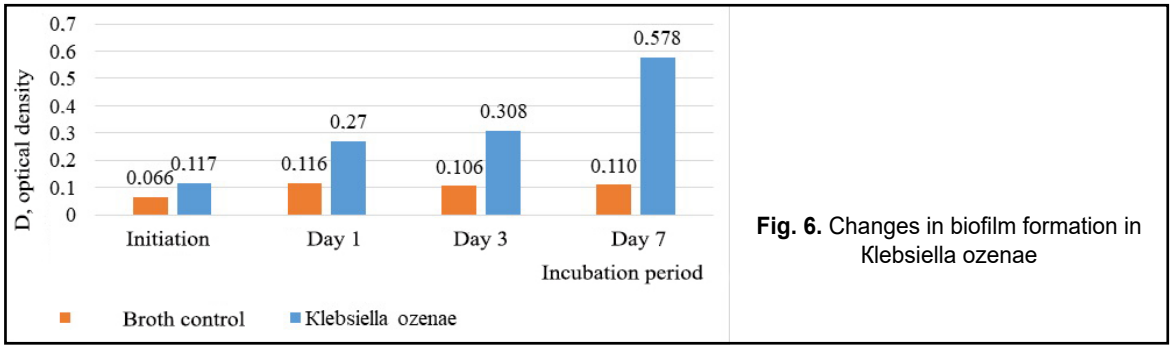

Fig. 6. Changes in biofilm formation in Klebsiella ozenae