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I. Horbachevsky Ternopil National Medical University

Microbiological substantiation of the use of xenografts saturated with silver nanocrystals for the treatment of burn wounds

The aim of the work: to study the antimicrobial efficacy of xenografts saturated by silver nanoparticles, to suggest their application in the treatment of burned wounds.

Materials and Methods. The antimicrobial efficacy of xenografts saturated with silver nanocrystals was investigated in vitro by diffusion into agar, in a liquid nutrient medium and by studying the adhesive activity using test cultures: *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027 and *Candida albicans* ATCC 885-653.

Results and Discussion. The antimicrobial properties of silver, which was saturated the pieces of cryolyophilized xenoskin, were not inferior to the effectiveness of modern dressings, which were used as a positive control (wound dressing applications Mepilex Transfer Ag (Mölnlycke, Sweden) та Atrauman Ag (Heidenheim, Germany)) in studies. Nanosilver had reduced a bioburden in infected wounds and the adhesive potential of microorganisms, which is important to prevent contamination of burn wounds.

Thus, the possibility of using xenografts saturated with silver nanocrystals it is considered for local treatment of burns in order to prevent purulent-inflammatory complications that may occur.

Key words: burns; xenograft; silver nanocrystals; antimicrobial properties.

Problem statement and analysis of recent research and publications. One of the main factors determining the prognosis of burn injuries severity is microbial contamination of the wound. It is known, at least 50 % of a mortality caused by burns are the result of wound infections, which is inevitable even with perfect compliance with the rules of asepsis and antiseptics. The critical number of microbes that determines the development of the inflammatory purulent process is ≥ 105 microbial cells in 1 g of wound tissue [1]. Invasion and colonization of the wound surface by microorganisms slow down a wound healing, lead to a deepening burn, and may lead to generalization of infection. Therefore, a treatment of local medicines that would prevent microbial contamination of burn wounds or reduce it below the critical level must be provided.

The elaboration of silver-containing antimicrobial applications is one of the modern nanotechnology and medicine fields. First of all, an advantage of silver nanoparticles use is due to the pharmacological effects of this metal: a wide antimicrobial range, lack a development of a resistance of most pathogenic microorganisms to silver containing medicines, their immunomodulatory properties, absence of data of hypersensitivity to them [2, 3]. The mechanisms of antimicrobial action of this metal are not yet studied well. However, it has been known that such action conditioned the interaction of positively charged silver ions with the electrostatic forces of the microbial cell, which have a negative charge; inhibition of transmembrane transport of Ca^{2+} and Na^{+}; formation of silver complexes with nucleic acids, which leads to disruption of DNA stability, or with a sulfur atom, which leads to the inactivation of proteins containing thiol groups, thereby inhibiting the viability of microorganisms [4, 5, 6]. In another study, it was found that silver ions exhibit bactericidal properties by inhibiting bacterial cell wall synthesis and affecting ribosome protein synthesis at the 30S subunit, or by inhibiting the activity of some transmembrane enzymes, thereby damaging the bacterial cell membrane structure [7, 8]. It is proved that silver, especially in nanocrystalline form, has fungicidal activity. The effect of silver is due to the irreversible binding of this metal to the cysteine residue, which contains a thiol group in the phosphomannose isomerase, interrupts the synthesis of cell walls and, in turn, leads to loss of essential nutrients and death of fungi, for instance *C. albicans* [9].

The antimicrobial properties of silver are significantly enhanced by its transition into nanoparticles [6]. Silver nanoparticles considered as the most promising as they contain remarkable antimicrobial efficacy due to their large surface area to volume ratio [10]. The use of silver as nanoparticles can reduce the concentration of the metal hundreds of times while maintaining all its bactericidal properties. It is proved that the use of silver medicines increases wound healing, in particular, burns due to the reduction of inflammatory processes in the wound, prevention of microbial contamination and modulation of fibrogenic cytokines [6, 11, 12]. Summarizing the current data of medicinal and antimicrobial properties of silver nanoparticles the new wound management standards based on the usage of silver can be suggested as potentional therapeutic choice.

The aim of the work: to study the antimicrobial efficacy of xenografts saturated by silver nanoparticles, to suggest their application in the treatment of burned wounds.

Materials and Methods. The antimicrobial efficacy of xenografts saturated with silver nanoparticles was studied in vitro by diffusion in an agar and in a liquid nutrient medium according to standard laboratory methods [13]. The following ATCC strains of microorganisms were used in the experiments as: *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027 and *Candida albicans* ATCC 885-653. The method of diffusion in an agar was standardized by the ensuring thickness of the Mueller-Hinton nutrient medium (10 mm), the area of xenograft flaps (1 cm^2) at a concentration of 0.5 on the McFarland turbidity standard. Xenograft flaps were pre-moistened with sterile sodium chloride saline. In a Petri dish on the surface of nutrient medium a xenograft flap saturated with silver nanoparticles and also samples of Mepilex Transfer Ag (Mölnlycke, Sweden) and Atrauman Ag (Heidenheim, Germany) silver-containing dressings (positive control) and a sterile xenograft flap were placed. The bacterial cultures were incubated at 37 °C during 24 hours. The results were carried out by measuring the diameter of the zone of growth inhibition of microorganisms around the samples. Evaluation of antimicrobial activity, taking into account the size of the flaps, was performed according to the following criteria: an absence of microbial growth inhibition around the flap or presence of a growth inhibition zone up to 16 mm in diameter was assessed test-microorganisms as not susceptible to the sample; the zone of growth inhibition with a diameter of 16–19 mm was evaluated as low susceptibility of a test-strain to the sample; the zone of growth inhibition with a diameter of 19–29 mm was evaluated as sufficient susceptibility of microorganisms to the sample; more than 29 mm – as high susceptibility of test-strains.

Antibacterial and antifungal effects on test-strains of microorganisms were also determined using a liquid nutrient medium. Xenograft flaps saturated with and without silver nanoparticles, and flaps of Mepilex Transfer Ag (Mölnlycke, Sweden) and Atrauman Ag (Heidenheim, Germany) dressings sized 10x10 mm were put in test tubes with sterile sugar meat-peptone broth (MPB). Then 0.1 ml of standardized suspension of daily cultured test-strain at a concentration of 0.5 on the McFarland standard was added to each tube. After that test tubes were incubated at 37 °C for 1 hour, 24, 48 and 72 hours. The presence or absence of microbial growth was visually assessed, and the contents of the tubes were inoculated on a sugar meat-peptone agar (MPA) in Petri dishes by a streak method using a 2 mm diameter bacterial loop and the concentration of the microbial cells in each test tube was determined by the Gold method. Each of the experiment was performed 10 times. Statistical analysis of the obtained data was performed using the software package Statistic 10.0 and Microsoft Office Excel.

The adhesive properties of test-strains of microorganisms were studied on formalized human erythrocytes 0 (I) blood group, Rh (+) according to the Brillis method [14]. To assess the effect of silver nanoparticles on the adhesive activity of test-strains the determination the index of adhesiveness of microorganisms (IAM) was done. Test-microorganisms were considered non-adhesive if IAM <1.75, low-adhesive if IAM=1.76–2.5, medium-adhesive (IAM=2.5-4.0), high-adhesive (IAM>4.0). If a difference between the indexes of adhesiveness in the experiment and control was at 20 % or more the changes of an adhesive potential of bacteria or yeast were considered significant. The experiments were performed three times. The obtained data were displayed as arithmetic means with standard deviation ($x \pm SD$), subjected to statistical processing using Microsoft Excel 2003.

Results and Discussion. Estimation of antimicrobial activity of xenografts and dressings samples by agar diffusion method is shown in Table 1. According to the data obtained, the xenograft saturated with silver nanoparticles was not inferior to the effectiveness of the Mepilex Transfer dressing, and it was found to be better than Atrauman Ag dressing. According to the obtained zones of growth retardation of test cultures on solid nutrient medium, gram-positive bacteria *S. aureus* and yeast *C. albicans*, non-fermenting gram-negative rods of *P. aeruginosa* showed low sensitivity to silver nanoparticles. Susceptibility to the studied samples of *E. coli* was assessed as sufficient.

As a result of incubation of standardized suspension of pure cultures of test microorganisms (*Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* and yeasts *Candida*) in sugar MPB in the presence of given flaps a distinct antimicrobial effect of silver nanoparticles was found (Table 2). After 1 hour of culturing the test-strains in the presence of xenograft fraps saturated with silver nanoparticles, also Mepilex Transfer Ag and Atrauman Ag dressings, no growth of gram-negative bacteria was detected, but very weak growth of *Staphylococcus aureus* and *Candida* was observed. Very weak growth was detected in test tubes with xenograft fraps without silver nanoparticles too. The concentration of the microbial suspension in these tubes was decreased, apparently due to the adsorption properties of cryolyophilized xenograft. The most effective influence of silver nanoparticles was found on test-strains of *E. coli* and *C. albicans*. Even after 48 hours the MPB in test-tubes remained sterile, and after 72 hours very weak growth of these microorganisms was observed. The growth of single colonies of test-

Table 1. Determination of antimicrobial activity of cryolyophilized xenograft saturated with silver nanoparticles by agar diffusion method

No.	Micro-organism	Xenograft flap saturated with silver nanoparticles		Control					
				xenograft flap without silver nanoparticles		flap of Mepilex Transfer Ag dressing		flap of Atrauman Ag dressing	
		zone of microbial growth inhibition, mm	level of susceptibility	zone of microbial growth inhibition, mm	level of susceptibility	zone of microbial growth inhibition, mm	level of susceptibility	zone of microbial growth inhibition, mm	level of susceptibility
1	*S. aureus*	18.1±0.6	low	0	non	17.3±0.9	low	16.3±1.5	low
2	*E. coli*	21.8±1.8	sufficient	0	non	20.3±2.2	sufficient	18.3±1.1	low
3	*P. aeruginosa*	18.5±1.8	low	0	non	16.0±0.8	low	13.8±2.2	low
4	*C. albicans*	16.7±1.6	low	0	non	15.5±2.1	low	15.1±1.4	low

strains of *S. aureus, P. aeruginosa* was detected after 48 hours of cultivation only. Cryolyophilized xenograft flaps saturated with silver demonstrated antimicrobial properties that were not inferior to the degree of effectiveness of modern dressings, which were used as a positive control (Table 2). Mepilex Transfer Ag dres-

Table 2. Determination of antimicrobial properties of cryolyophilized xenograft with silver nanoparticles in liquid nutrient medium

No.	Micro-organism	Time	Growth of test-strain in the presence of				
			xenograft flap saturated with silver nanoparticles	control			
				xenograft flap without silver nanoparticles	flap of Mepilex Transfer Ag dressing	flap of Atrauman Ag dressing	test-strain in MPB
1	2	3	4	5	6	7	8
1	*S. aureus*	1 hr	+	+	+	+	+++
		24 hrs	-	++	-	-	++++
		48 hrs	+	+++	+	+	++++
		72 hrs	+	++++	+	++	++++
2	*E. coli*	1 hr	-	++	-	-	+++
		24 hrs	-	+++	-	-	++++
		48 hrs	-	++++	-	+	++++
		72 hrs	+	++++	+	+++	++++
3	*P. aeruginosa*	1 hr	-	++	-	-	+++
		24 hrs	-	+++	+	+	++++
		48 hrs	+	++++	++	++	++++
		72 hrs.	++	++++	+++	++++	++++

Continuation of Table 2

1	2	3	4	5	6	7	8
4	*C. albicans*	1 hr	+	++	+	+	+++
		24 hrs	-	+++	-	-	++++
		48 hrs	-	++++	+	+	++++
		72 hrs	+	++++	++	++	++++

Notes: + – very weak microbial growth (growth of single colonies – up to 10 on a medium in a Petri dish), which is less than 10^3 colonyforming units (CFU)/ml;

++ – weak growth (10–25 colonies), which is 10^3 – $5x10^3$ CFU/ml;

+++ – moderate growth (from 50 to 100 colonies), which is 10^4 – 10^6 CFU/ml;

++++ – massive growth (impossible to count the number of colonies), amounting to 10^9 CFU/ml

sings showed the same antimicrobial activity, while Atrauman Ag dressings showed slightly lower antimicrobial activity in comparation with xenografts with silver particles. Moreover, it was noted that xenograft has had better adsorption properties of compared to the control. This phenomena was visible due to more intense green color of the MPB as a result of the release of pigment by *P. aeruginosa* in test tubes with samples of dressings.

The following test-strains were used in studies on the effect of nanocrystals on adhesive properties: gram-positive cocci *S. aureus* ATCC 6538, gram-negative *E. coli* ATCC 25922, *P. aeruginosa* ATCC 9027 and yeast *C. albican* 558SS. Microorganisms were cultivated in MPB in a presence of flaps. IMA The data obtained in this experiment are presented in Table 3.

The IAM of S. aureus was determined as high adhesive. However, a cultivation of these test-strains in the presence of silver nanoparticles led to the staphylococcal adhesive activity reduction to the average level (IAM=2.56±0.24). The adhesiveness of gram-negative bacteria *E. coli* and *P. aeruginosa* was decreased under the influence of silver nanoparticles from medium-adhesive to low-adhesive (IAM became (1.86±0.63) and (2.21±0.59), respectively). The *C. albicans* test-strains demonstrated a medium adhesive potential. The presence of silver nanoparticles during yeast cultivation caused a decrease of the index of microbial adhesiveness to low level. Obtained data has shown the indexes of the adhesiveness of microorganisms under the influence of silver nanoparticles were changed significantly, as the difference between the indexes of adhesiveness of microorganisms in the experiment and control was more than 20 %.

Silver nanoparticles can be easily incorporated in dressings and have significantly decreased wound-healing time and increased bacterial clearance from infected wounds [16]. Our studies have shown that cryolyophilized xenografts saturated with silver nanoparticles can be effectively used to prevent the

Table 3. The adhesiveness of test-strains under the action of silver nanoparticles

Microorganism	IAM				
	xenograft flap saturated with silver nanoparticles	flap of Mepilex Transfer Ag dressing	flap of Atrauman Ag dressing	xenograft flap without silver nanoparticles	without flap
S. aureus	2.56 ± 0.24*	2.81 ± 0.23*	2.94 ± 0.41*	4.61 ± 0.27	5.52 ± 0.41
E. coli	1.86 ± 0.63*	1.89 ± 0.52*	2.25 ± 0.48*	3.9 ± 0.73	3.93 ± 0.28
P. aeruginosa	2.21 ± 0.59*	1.94 ± 0.34	2.47 ± 0.80	3.84 ± 0.36	3.88 ± 0.81
C. albicans	1.78 ± 0.32	2.16 ± 0.37	2.35 ± 0.54	2.70 ± 0.93	2.85 ± 0.43

Notes: * – the presence of reliability at a significance level of $p < 0.05$ relative to control (test-strain in the MPB);

– transition of strain to a category with lower adhesiveness.

development of purulent-inflammatory complications in the treatment of burns, as this metal exhibits antimicrobial properties. It is proved that there is a difference in the efficiency of silver nanoparticles against gram-positive and gram-negative flora. The difference in the degree of a gram-negative and gram-positive microflora sensitivity to silver nanoparticles, apparently, is due to the peculiarities of the structure of the cell membrane, which is confirmed by other studies [3, 17, 18].

The results showed that silver nanoparticles which were saturated xenografts and dressings, caused the transition of test-strains of *S. aureus* from the category of high-adhesive to the category of medium-adhesive, and test-strains of *E. coli, P. aeruginosa, C. albicans* – from the category of medium-adhesive to the category of low-adhesive. The decrease in the adhesiveness of gram-positive microorganisms is probably due to the blockade of silver nanoparticles of the surface structures of microbial cells required for binding to erythrocyte fibronectin. The decrease in the adhesive activity of gram-negative bacteria is due to the destructive action of metal nanoparticles against the fimbrial structures of bacteria that provide adhesion. Reducing the adhesive potential of test-microorganisms is a pathogenetically approach to prevent purulent-inflammatory complications of burn wounds.

Conclusions. The obtained data allows considering the possibility of using xenografts saturated with silver nanoparticles for the local treatment of burns in order to prevent purulent-inflammatory complications that may occur. It shows promising results in healing of contaminated wounds too.

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Електронна адреса для листування: pokryshko@tdmu.edu.ua

Н. В. ТУЗЮК, О. В. ПОКРИШКО

Тернопільський національний медичний університет імені І. Я. Горбачевського МОЗ України

МІКРОБІОЛОГІЧНЕ ОБҐРУНТУВАННЯ ВИКОРИСТАННЯ КСЕНОТРАНСПЛАНТАНТІВ, НАСИЧЕНИХ НАНОКРИСТАЛАМИ СРІБЛА, ДЛЯ ЛІКУВАННЯ ОПІКОВИХ РАН

Мета роботи: вивчити антимікробну ефективність насичених нанокристалами срібла ксенотрансплантантів, які використовуватимуться у лікуванні опікових ран.

Матеріали і методи. Протимікробну ефективність ксенотрансплантантів, насичених нанокристалами срібла, досліджували *in vitro* методом дифузії в агар і в рідкому поживному середовищі, а також вивчаючи вплив нанокристалів срібла на адгезивну активність тест-культур: *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027 та *Candida albicans* ATCC 885-653.

Результати досліджень та їх обговорення. Протимікробні властивості срібла, якими були насичені клапті кріоліофілізованої ксеношкіри, не поступалися за ступенем ефективності сучасним перев'язувальним матеріалам, які використовували як позитивний контроль (абсорбуючі стерильні пов'язки Mepilex Transfer Ag (Mölnlycke, Sweden) та Atrauman Ag (Heidenheim, Germany)) у дослідженнях. Наносрібло виконує роль антимікробного бар'єру в рані та знижує показники адгезивного потенціалу мікроорганізмів, що важливо для запобігання контамінації опікових ран.

Отримані результати дають змогу розглядати можливість використання ксенотрансплантантів, насичених нанокристалами срібла, для місцевого лікування опікових ран з метою профілактики гнійно-запальних ускладнень, що можуть виникати.

Ключові слова: опікові рани; ксенотрансплантант; нанокристали срібла; антимікробні властивості.