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Antibacterial action of lysozyme against osteomyelitis agents: S. *aureus* and S. *epidermidis*

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ABSTRACT

Introduction The use of lysozyme as a bactericidal agent against the leading pathogens of chronic osteomyelitis can become an alternative or supplement to existing antibacterial drugs.

Purpose To study the antibacterial effect of lysozyme against clinical strains of Staphylococcus *aureus* and Staphylococcus *epidermidis*

Materials and methods Control strains of Staphylococcus aureus (ATCC 25923), Staphylococcus epidermidis (ATCC 14990) and clinical strains (n = 48), including *MRSA* (n = 6) and *MRSE* (n = 6), isolated from wounds and fistulas of patients with chronic osteomyelitis were used as test cultures. The antibacterial effect of lysozyme was assessed using the disk diffusion method.

Results Lysozyme exhibited bactericidal activity against control strains of S. *aureus* and S. *epidermidis*, the growth inhibition zone of bacteria was 11–12 mm. Among clinical strains of S. *aureus*, 87.5 % were sensitive to lysozyme, the growth inhibition zone diameter was 9–13 mm. No bactericidal effect was observed against three strains of S. *aureus*, including two *MRSAs*, and continuous bacterial growth was observed around the disk. Among strains of S. *epidermidis*, the antibacterial activity of lysozyme was observed against 79.2 % of isolates, the growth inhibition diameter was 8–11 mm. Resistance of three *MRSE* strains to lysozyme was noted. Lysozyme enhanced the effect of vancomycin and cefoxitin against methicillin-sensitive staphylococci and norfloxacin and vancomycin against methicillin-resistant staphylococci.

Discussion Despite the inhibitory effect found, the use of lysozyme alone may be limited due to its possible degradation by proteases, as well as some immunogenicity. There are studies on the synergism of the combined action of lysozyme with various antibiotics on gram-positive and gram-negative bacteria. The data obtained in our experiment showed an increased antibacterial effect by the combined action of antibiotics and lysozyme against the leading causative agents of osteomyelitis.

Conclusion It has been established that lysozyme has an antibacterial effect against clinical strains of S. *aureus*, S. *epidermidis*, including *MRSA* and *MRSE*, isolated from wounds of patients with chronic osteomyelitis. An increased antibacterial effect is observed by a combined action of lysozyme with cefotaxime, norfloxacin and vancomycin.

Keywords: chronic osteomyelitis, lysozyme, resistance, antimicrobial peptides, antibiotics

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INTRODUCTION

Commonly, osteomyelitis is caused by bacteria of the genus Staphylococcus, mainly S. *aureus* and S. *epidermidis* (including methicillin-resistant strains *MRSA* and *MRSE*), that demonstrate a high degree of resistance to conventional antibiotics. This makes the treatment of patients with osteomyelitis difficult and requires the search for new, more effective drugs [1–4].

As an alternative or addition to existing antibacterial drugs, the use of lysozyme as a bactericidal agent against the leading causative agents of chronic osteomyelitis may be a relevant area. Lysozyme is an antimicrobial enzyme found in various biological fluids, such as saliva, tears, and breast milk [5, 6]. Lysozymes are divided into three main families: chicken type (c-type), goose type (g-type), and invertebrate type (i-type). Phage type, bacterial type, and plant type lysozymes are also known. Chicken and human lysozymes are c-type lysozymes. The chicken one consists of 129 amino acid residues (14.3 kDa), the human one of 130 amino acid residues (14.7 kDa). There is 59 % sequence identity between human and chicken lysozymes, but the antibacterial activity of chicken lysozyme is three times lower than that of the human one. However, its use is limited due to insufficient availability [5, 7, 8].

Since lysozyme is a natural component of the body, it is generally well tolerated and has a low risk of toxicity, so it is used for medical purposes. Lysozyme destroys the peptide glycans that make up the wall of bacterial cells, which leads to osmotic destruction and death of bacteria [8]. The combination of lysozyme with antibacterial drugs may enhance their effect [9, 10]. Lysozyme is also able to modulate the body's immune response [7, 8]. Currently, lysozyme is already used as preservation and antiseptic agent [6, 8]. The prospect of using lysozyme as an antibacterial agent against the leading causative agents of osteomyelitis may expand the scope of its application in medicine.

Purpose To study the antibacterial effect of lysozyme against clinical strains of Staphylococcus *aureus* and Staphylococcus *epidermidis*.

MATERIALS AND METHODS

Control strains of Staphylococcus *aureus* (ATCC 25923), Staphylococcus *epidermidis* (ATCC 14990) and their clinical strains (n = 48), including *MRSA* (n = 6) and *MRSE* (n = 6), isolated from wounds and fistulas of patients with chronic osteomyelitis were used as test cultures.

Bacteria were identified using a BactoScreen bacteriological analyzer (LLC NPF Litekh). The sensitivity of microorganisms to antibacterial drugs was determined with the disk diffusion method. The results were assessed using the EUCAST criteria (2017–2022). Detection of methicillin-resistant staphylococcal genes in the biological material was carried out with a reagent kit for detection and quantitative determination of *MSSA* and *MRSA*, *MSSE* and *MRSE* DNAs by polymerase chain reaction (PCR) with hybridization-fluorescence detection AmpliSens MRSA-screen-titer-FL.

The antibacterial effect of lysozyme was assessed using the disk diffusion method. Discs made of technical filter cardboard (GOST 6722–75) and discs with antibiotics impregnated with lysozyme (CAS–No. 9001-63-2, 20000 U/mg, AppliChem) at a concentration of 30 μg/ml were placed on the surface of a dense nutrient medium (Müller-Hinton agar) seeded with a daily culture of S. *aureus* or S. *epidermidis*. Petri dishes with the seeded cultures were incubated in a thermostat at a temperature of 37 °C. After 24 hours, the results were recorded by measuring the growth inhibition zone around the disc. The action of lysozyme on the control strains was repeated six times.

Bacterial resistance profiles of S. *aureus*, S. *epidermidis* were analyzed to four antimicrobial drugs (AMD): cefoxitin (FOX), gentamicin (GEN), norfloxacin (NOR), vancomycin (VAN).

For statistical processing of the obtained data, the Gnumeric 1.12.17 software and LibreOffice spreadsheets (version: 5.4.1.2) were used. The samples were tested for compliance with a certain distribution law using the Anderson-Darling criterion. Considering that the data in the samples were subject to normal distribution, the Student criterion was used to test the hypothesis of equality of the mean values. The digital data are presented as the arithmetic mean (M) and standard deviation (SD). Differences were considered significant at $p < 0.05$.

Microbiological studies were conducted at the microbiology laboratory of the Ilizarov National Medical Research Center of Traumatology and Orthopaedics.

RESULTS

The control strains of S. *aureus* и S. *epidermidis* were sensitive to the action of lysozyme (Fig. 1). Among the clinical isolates of bacteria, there were strains sensitive and insensitive to lysozyme.

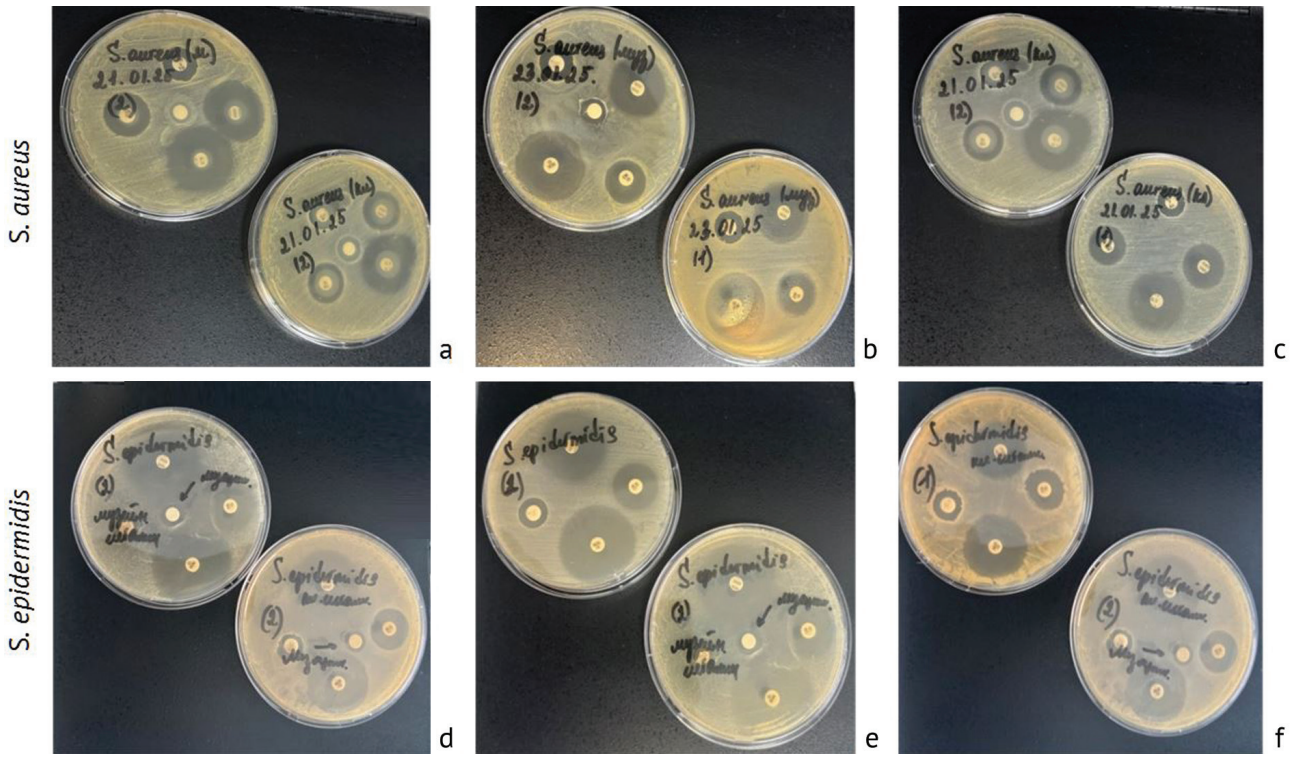

Fig. 1 Antibacterial effect of lysozyme together with and without antibiotics on the control and clinical bacterial strains of S. *aureus*, S. *Epidermidis*: (a, d) — control and clinical stains; (b, e) — control strains; (c, f) — clinical strains

No significant differences in the diameter of growth retardation were observed between the control and clinical strains (Table 1).

Table 1

Growth inhibition of the bacteria S. *aureus* and S. *epidermidis* after action of lysozyme (30 mcg/ml)

Miscroorganism	Diameter of growth inhibition zone, mm
S. *aureus* ATCC 25923 (*n* = 6)	11.3 ± 0. 47
S. *aureus* (*n* = 24)	11.2 ± 1.10
S. *epidermidis* ATCC 12228 (*n* = 6)	10.0± 0.43
S. *epidermidis* (*n* = 24)	10.5 ± 1.05

Lysozyme alone at a concentration of 30 μg/ml showed a bactericidal effect on the control strains of S. *aureus* and S. *epidermidis*, the zone of bacterial growth inhibition was 11–12 mm. Among the clinical strains of S. *aureus*, sensitivity to lysozyme was detected in 87.5 %, the diameter

of the growth inhibition zone was 9–13 mm. In relation with three strains of S. *aureus*, including two *MRSA*, no bactericidal effect was noted, continuous bacterial growth was observed around the disk.

Among S. *epidermidis* strains, antibacterial action of lysozyme was noted against 79.2 % of isolates, growth inhibition diameter was 8–11 mm. Three *MRSE* strains were found to be resistant to lysozyme.

S. *aureus* (*n* = 18) and S. *epidermidis* (*n* = 18) strains were sensitive to the action of the antibacterial drugs tested. Lysozyme enhanced the action of antibiotics, which was expressed in an increased zone of bacterial growth inhibition around the disks. Significant differences were observed for vancomycin and cefoxitin (Table 2).

Lysozyme did not enhance the effect of cefoxitin and gentamicin against methicillin-resistant staphylococci. Significant differences were observed only for norfloxacin and vancomycin (Table 3).

Table 2

Zone of growth inhibition of methicillin-sensitive staphylococci isolated from wounds of patients with chronic osteomyelitis under lysozyme action

Drug (mcg)	Diameter of growth inhibition zone, mm			
	MSSA, (*n* = 18)		*MSSE*, (*n* = 18)	
	–	+ lysozyme (30)	–	+ lysozyme (30)
Cefoxitin (30)	23.3± 0.47	26.5 ± 0.81* *p* = 0.02476	33.3 ± 2.10	35.7 ± 1.90
Norfloxacin (10)	29.0 ± 0.82	30.0 ± 0.79	31.3 ± 0.83	32.0 ± 1.40
Gentamicin (10)	19.0 ± 1.40	19.7 ±1.24	20.8 ± 6.60	22.3 ± 1.80
Vancomycin (5)	13.7 ± 0.94	15.8 ± 0.47* *p* = 0.0404	15.0 ± 0.51	17.3 ± 1.10* *p* = 0.0216

Note: * — level of significance of differences between groups, $p < 0.05$

Table 3

Zone of growth inhibition of methicillin-resistant staphylococci isolated from wounds of patients with chronic osteomyelitis under lysozyme action

Drug (mcg)	Diameter of growth inhibition zone, mm			
	MSSA, (*n* = 18)		*MSSE*, (*n* = 18)	
	–	+ lysozyme (30)	–	+ lysozyme (30)
Cefoxitin (30)	20.3 ± 0.84	20.0 ± 0.82	19.5 ± 1.10	20.0 ±0.80
Norfloxacin (10)	27.3 ± 1.25	30.2 ± 0.61* *p* = 0.0248	29.8 ± 1.03	32.2 ±0.62* *p* = 0.0242
Gentamicin (10)	18.8 ± 0.24	18.5 ± 0.20	10.7 ±0.94	12.0 ± 0.41
Vancomycin (5)	14.3 ± 0.47	14.2 ± 0.62	14.7 ±0.47	17.5 ± 0.72* *p* = 0.025

Note: * — level of significance of differences between groups, $p < 0.05$

DISCUSSION

S. *aureus* is considered a clinically significant pathogen in chronic osteomyelitis, which, interacting with the body's cells through the small colony variant (SCV), biofilm formation and toxin secretion, induces an inflammatory response, causing cell death by apoptosis and necrosis [1]. S. *epidermidis* bacteria also play an important role in the development of infections in chronic osteomyelitis [3, 11]. In the last decade, there has been an increase in the number of bacteria with multidrug resistance, which leads to the ineffectiveness of traditional approaches to the treatment of patients with chronic osteomyelitis and determines the need to search for new drugs [2, 4].

A promising direction is considered to be the use of antimicrobial peptides of the innate immune system [12]. It is known that peptides obtained as a result of lysozyme cleavage exhibit antimicrobial activity, primarily against gram-positive bacteria [13]. They can act directly (lytic effect) or indirectly (modulate the immune system). The antibacterial mechanism of lysozyme is due to its muramidase activity, which hydrolyzes the β-l.4-glycosidic bond of peptide glycans, the ability to bind to nucleic acids of microorganisms and cause mutation or decay of bacterial genetic material [5, 14, 15].

Due to differences in the mechanisms of bacterial resistance to antibiotics and antimicrobial peptides, clinical use of lysozyme has a lower risk of developing resistance in microorganisms. It is believed that resistance to peptide-degrading enzymes in bacteria develops rarely, resulting from horizontal transfer of resistance determinants rather than de novo mutation [16].

The available literature reports on an inhibitory effect of lysozyme obtained from egg white on drug-resistant bacteria, including *MRSA* [17]. In our study, 87.5 % of *MSSA*, 79.2 % of *MSSE*, and 50 % of *MRSA* and *MRSE* strains were sensitive to the action of lysozyme.

The use of lysozyme alone may be limited by the possibility of its degradation by proteases present in body fluids, as well as some immunogenicity, which may cause immune reactions when used repeatedly [18, 19]. The issue of lysozyme as an allergen remains controversial. Some researchers believe that lysozyme, being a component of the human immune system, does not cause an allergic reaction [20]. Other studies have shown that it acts as a weak allergen [21, 22]. Moreover, being applied directly on the wound surface, lysozyme can be easily washed off by exudate. In this regard, new methods for delivering lysozyme to the site of infection are being developed to increase the effectiveness of its action and reduce side effects [23]. Those include hydrogels, nanofilms, fibrous membranes and composite systems with modified lysozyme, which could improve the stability of lysozyme and reduce its immunogenicity [24, 25].

One of the alternative uses of peptides is their combination with traditional antibiotics to treat patients with osteomyelitis [26]. The data obtained in our experiment showed an increased effect of antibiotics on all sensitive microorganisms, but significant differences were found for the combination of lysozyme with vancomycin and cefoxitin. In relation to methicillin-resistant staphylococci, an increase in antimicrobial activity was noted only for the combination of lysozyme with vancomycin and norfloxacin.

The study of the combined action of lysozyme obtained from egg white with various antibiotics (gentamicin, ofloxacin, oxacillin, rifampicin, polymyxin B, vancomycin, ciprofloxacin and tetracycline) on gram-positive and gram-negative bacteria, including drug-sensitive and drug-resistant strains, a synergistic mechanism of action was established that reduces the resistance of microbes [9, 26, 27]. Antibacterial peptides change the permeability of the cell membrane, allowing more antibiotic to penetrate the cell and bind to intracellular targets, enhancing its action and reducing the side effects of high concentrations [9, 10].

Researchers have shown that the bactericidal effect of the combined action of lysozyme and antibiotics against planktonic cells and biofilms obtained *in vitro* is more pronounced compared to the use of the drugs separately [28].

At the same time, it is necessary to consider the fact that all bacteria have both general and specific mechanisms of protection against innate immunity factors. The resistance mechanisms used by gram-positive bacteria, including S. *aureus*, include changes in the charge and composition of the cell wall. The sensitivity of gram-positive bacteria to antimicrobial peptides depends on: the content of negatively charged teichoic acids in the cell wall which bind lysozymes and reduce

their enzymatic activity [8, 19]; inactivation of peptides due to their binding to surface or secreted proteins and polysaccharides; cleavage of antimicrobial peptides by bacterial proteases; adaptation of bacteria to the effects of antimicrobial peptides; displacement of antimicrobial peptides by efflux pumps and transport systems [16, 29, 30].

CONCLUSION

It has been established that lysozyme has an antibacterial effect against clinical strains of S. *aureus*, S. *epidermidis*, including *MRSA* and *MRSE*, isolated from wounds of patients with chronic osteomyelitis. An increased antibacterial effect is observed by a combined action of lysozyme with cefotaxime, norfloxacin and vancomycin.

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