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IN VITRO ASSESMENT OF 0.2 % ZINC-HYALURONATE ACTIVITY AGAINST MICROORGANISMS ISOLATED FROM PATIENTS WITH DIFFICULT HEALING LEG ULCERS

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ABSTRACT

The development of wound infection and selection of resistant microorganisms is a significant problem in the process of granulation and epithelization of poorly healing leg ulcers. In a number of cases, the attempts for systemic treatment with some of the most potent antimicrobials, according to the susceptibility data, are unsuccessful. It is established that the local application of hyaluronic acid fosters the process of epithelization. In a complex with Zinc ions, hyaluronic acid possesses antiseptic effect. The aim of the study: To assess the in vitro activity of 0.2% Zinc-hyaluronate solution /Hyaluricht ®/ against the most common bacteria isolated from patients with poorly healing leg ulcers. Material and methods: A total of ten bacterial strains /Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, and Serratia marcescens/ isolated from ten patients with long-lasting chronic leg were tested. Antibiotic susceptibility testing was determined by standard disk diffusion method. All Escherichia coli and Serratia marcescens strains were screened for the production of extended-spectrum β-lactamases by using the double-disk test. An inoculum suspension from fresh 18-h culture of the tested strains on blood agar was prepared in 0.2% Zinc-hyaluronate solution. The time kill experiments were performed. Results: Time-kill experiments demonstrated fast bactericidal activity even against highly resistant to antimicrobials strains. In our experiments the time for killing depended on the species affiliation and the bacterial suspension density. As a conclusion, the results confirm the data given in the medical literature for the antimicrobial activity of the preparation Hialuricht® and support the approved position in the clinical practice for its favorable therapeutic effect in the local treatment of infected ulcers.

Introduction

The reasons leading to the formation of poorly healing leg ulcers are chronic diseases of the arterial and venous systems, as well as some connective tissue and autoimmune diseases (3). The process of healing of these ulcers is long lasting and complicated. Their treatment includes systemic vasoprotective, venous tonic and vasodilating remedies, and antibiotics but also preparations for local treatment as enzymes for cleaning and necrosis removal, antimicrobial, epithelizing and protective creams and ointments (2, 4). In spite of the usage of contemporary non-adherent and exudate absorbing polyurethane and hydrocolloid dressings, surgical treatment of the ulcer bottom and edges is often necessary, followed by the application of skin autografts (10).

The development of wound infection and selection of resistant microorganisms

(*Staphylococcus aureus*, *Pseudomonas aeruginosa* and other Gram-negative rods) is a significant problem in the process of granulation and epithelization. In a number of cases, the attempts for systemic treatment according to the susceptibility data with some of the most potent antimicrobials are unsuccessful. Because of that, the treatment of poorly healing leg ulcers caused by resistant microorganisms becomes of problem for clinical practice (5).

The reduced concentration of the natural biopolymer hyaluronic acid in the tissues of patients with poorly healing leg ulcers additionally delays the process of cellular proliferation and regeneration. It is established that the local application of hyaluronic acid fosters the process of epithelization by creating optimal conditions for the activation and migration of the cells participating in the process of tissue regeneration. (9) In a complex with Zinc ions, hyaluronic acid possesses antiseptic effect (1). Hyaluricht® solution is an original preparation of the Hungarian Company Gedeon Richter, containing 0.2 % Zinc-hyaluronate. The aim of this study is to assess by time-kill experiments the *in vitro* activity of Hyaluricht® against the most common bacteria isolated from patients with poorly healing leg ulcers.

Materials and Methods

Clinical bacterial strains. A total of ten bacterial strains isolated from ten patients with long-lasting chronic leg ulcers were tested. Eight patients were women and two were men, aged 51 to 83 years. Seven of them had venous ulcers, two had arterial ulcers and one had atonic leg ulcer. Despite of long-standing systemic and local treatment all patients had recurrent ulcers of the lower limbs.

The species distribution of the tested strains is as follows: *Staphylococcus aureus* (2 strains), *Pseudomonas aeruginosa* (3 strains), *Escherichia coli* (2 strains), and *Serratia marcescens* (2 strains).

Susceptibility testing and antibiotics. Antibiotic susceptibility testing was determined by standard disk diffusion method using 10U penicillin, 1μg oxacillin, 10μg ampicillin, 20/10 μg amoxicillin/clavulanic acid, 75 μg azlocillin, 100 μg piperacillin, 100/10 μg piperacillin/tazobactam, 30 μg cephalothin, 30 μg cefuroxime, 30 μg cefotaxime, 30 μg ceftriaxone, 30 μg ceftazidime, 30 μg cefepime, 10 μg imipenem, 10 μg meropenem, 30 μg vancomycin, 30 μg teicoplanin, 10 μg gentamicin, 30 μg amikacin, 15 μg erythromycin, 2 μg clindamycin, and 5 μg ciprofloxacin. Disks for the agar diffusion procedures were obtained from Becton Dickinson Microbiology Systems (Cockeysville, Md.). Breakpoints recommended by the NCCLS were used to determine susceptibility or resistance (8).

Test for production of extended-spectrum β-lactamases (ESBLs). All *E. coli* and *S. marcescens* strains were screened for the production of ESBLs by using the double-disk test (6). A potentiation of the zones of cefotaxime, ceftriaxone, ceftazidime, or aztreonam by clavulanic acid represented a positive test result and was indicative of the possible presence of an ESBL.

Time-kill experiments. An inoculum suspension from fresh 18-h culture of the tested strains on blood agar was prepared in 0.2% hyaluronate solution. The turbidity is adjusted to obtain a turbidity optically comparable to that of the 0.5 McFarland standard. The inoculum suspension was incubated at 37 ºC for 24 h. 0.1 ml of the undiluted suspension and 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} dilutions were inoculated on Mueller-Hinton agar, Becton Dickinson Microbiology Systems (Cockeysville, Md.), poured in Petri dishes with thickness of the agar 5 mm for colony counting. The inoculum was performed at the moment of preparation of the suspension (0h) and at 1st hour, 2nd hour, 3rd hour, 4th hour, 5th

TABLE 1

Susceptibility patterns of bacterial strains isolated from poorly healing leg ulcers

Strain No	Species	Susceptibility patterns
1.	*Staphylococcus aureus* (Lab. № 206)	Pen^S Oxa^S Gen^S Ak^S Ery^S $Clin^S$ Cip^S Van^S Tei^S
2.	*Staphylococcus aureus* (Lab. № 306)	Pen^R Oxa^S Gen^S Ak^S Ery^S $Clin^S$ Cip^S Van^S Tei^S
3.	*Staphylococcus aureus* (Lab. № 606)	Pen^S Oxa^S Gen^S Ak^S Ery^S $Clin^S$ Cip^S Va^S Tei^S
4.	*Pseudomonas aeruginosa* (Lab. № 207)	Azl^S Pip^S Taz^S Caz^R Imp^S Mer^S Gen^S Ak^S Cip^R
5.	*Pseudomonas aeruginosa* (Lab. № 307)	Azl^R Pip^R Taz^S Caz^R Imp^S Mer^S Gen^I Ak^R Cip^R
6.	*Pseudomonas aeruginosa* (Lab. № 604)	Azl^R Pip^R Taz^I Caz^R Imp^I Mer^I Gen^R Ak^R Cip^R
7.	*Escherichia coli* (Lab. № 158)	Ap^R Azl^R Pip^R Amc^R Cf^R Cxm^R Ctx^R Cro^R Caz^R Fep^R Imp^S Mer^S Gen^R Ak^R Cip^R ($ESBL^+$)
8.	*Escherichia coli* (Lab. № 7546)	Ap^R Azl^R Pip^R Amc^R Cf^R Cxm^R Ctx^R Cro^R Caz^R Fep^R Imp^S Mer^S Gen^R Ak^I Cip^R ($ESBL^+$)
9.	*Serratia marcescens* (Lab. № 493)	Ap^R Azl^S Amc^R Cf^R Cxm^R Ctx^S Cro^S Caz^S Fep^S Imp^S Mer^S Gen^I Ak^S Cip^S
10.	*Serratia marcescens* (Lab. № 605)	Ap^R Azl^S Amc^R Cf^R Cxm^R Ctx^S Cro^S Caz^S Fep^S Imp^S Mer^S Gen^S Ak^S Cip^S

Legend: **S** – sensitive, **R** – resistant, **I** – intermediate; **Pen** – penicillin; **Oxa** – oxacillin; **Ap** – ampicillin; **Amc** – amoxicillin/clavilanic acid; **Azl** – azlocillin; **Pip** – piperacillin; **Taz** – piperacillin/tazobactam; **Cf** – cephalothin; **Cfx** – cefuroxime; **Ctx** – cefotaxime; **Cro** – ceftriaxone; **Caz** – ceftazidime; **Fep** – cefepime; **Imp** – imipenem; **Mer** – meropenem, **Van** – vancomycin; **Tei** – teicoplanin; **Gen** – gentamicin; **Ak** – amikacin; **Ery** – erythromycin; **Clin** – clindamycin; **Cip** – ciprofloxacin; **$ESBL^+$** – producer of β-lactamase with extended spectrum

hour, 6th hour, 8th hour, and 24th hour during the incubation period. The inoculated Petri dishes were incubated at 37 ºC for 24 h. The number of colony forming units (CFU) per 1 ml was determined according to the formula:

CFU/ml = the number of colonies x 10 x reciprocal value of the dilution.

All time kill experiments were performed in duplicate. Time-kill curves were constructed by plotting mean colony counts (log_{10} CFU/ml) versus time (7).

Results and Discussion

Table 1 shows susceptibility patterns of the tested strains. Two of the three *S. aureus* strains (No 1 and 3) were susceptible to all tested antimicrobials and one (No 2) was resistant only to penicillin. Both *E. coli* isolates were ESBL-producers and resistant to aminopenicillins, ureidopenicillins, cephalosporins from 1st – 4th generations, aminoglycosides, and fluoroquinolones and susceptible only to carbapenems. One strain of *P. aeruginosa* (No 6) was highly resistant. It exhibits resistance to ureidopenicillins, aminoglycosides, and fluoroquinolones and intermediate susceptibility to carbapenems and piperacillin/tazobactam. The other two *P. aeruginosa* strains (No 4 and 5) and both *S. marcescens* strains were moderate resistant.

Killing curves of the tested strains are shown on **Figures 1 – 4**. As it seen from the curves, the type of action of 0.2% Zn-hyaluronate is bactericidal. This action is very fast, from 2 to 7 hours, even for strains with high resistance to antimicrobials (strains No 6, 7, and 8).

The data for the time for killing and the suspension density are presented in **Table 2**.

Differences in the time for killing of the separate strains were found. These differences are determined to some extent by the

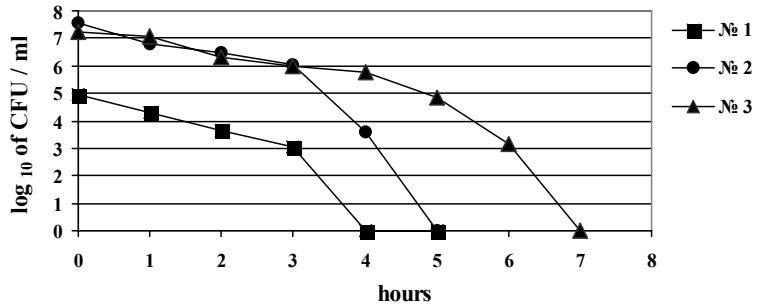

Fig. 1. Time-kill curves of *S. aureus* strains treated with 0.2% Zn-hyaluronate.

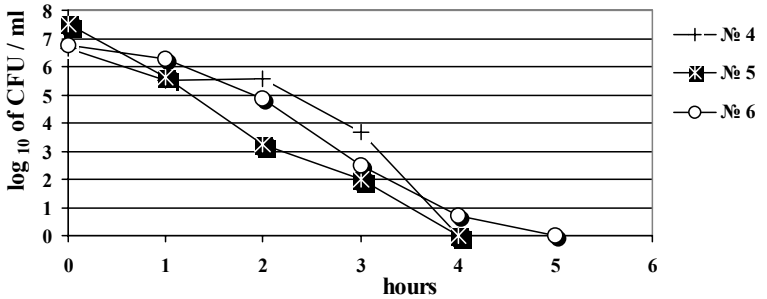

Fig. 2. Time-kill curves of killing of *P. aeruginosa* strains treated with 0.2% Zn-hyaluronate.

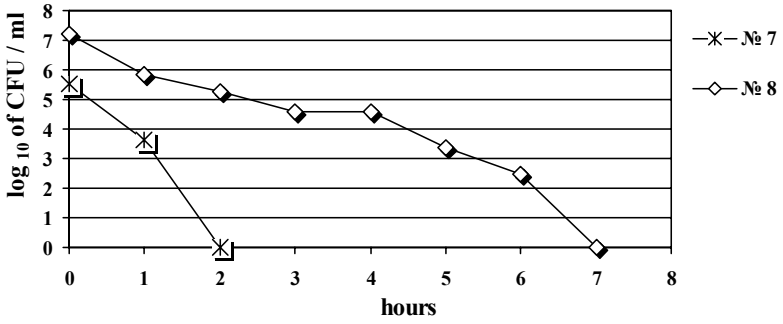

Fig. 3. Time-kill curves of *E.coli* strains treated with 0.2% Zn-hyaluronate.

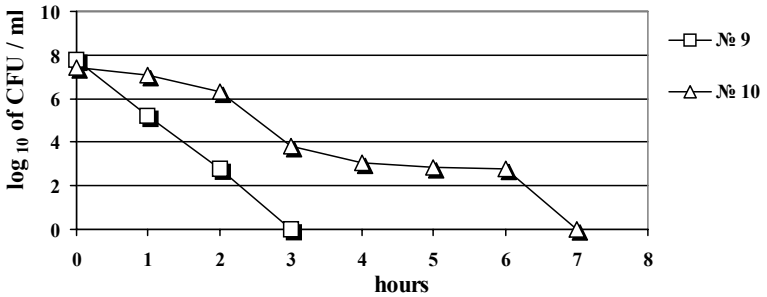

Fig. 4. Time-kill curves of *S. marcescens* strains treated with 0.2% Zn-hyaluronate.

TABLE 2

Comparative data on the time for killing and the suspension density of microorganisms

Microbial species	Suspension density (CFU/ml)	Time for killing the microorganisms
Strain № 1 *Staphylococcus aureus*	10^5	4 hours
Strain № 7 *Escherichia coli*	10^5	2 hours
Strain № 4 *Pseudomonas aeruginosa*	10^6	4 hours
Strain № 6 *Pseudomonas aeruginosa*	10^6	5 hours
Strain № 2 *Staphylococcus aureus*	10^7	5 hours
Strain № 3 *Staphylococcus aureus*	10^7	7 hours
Strain №5 *Pseudomonas aeruginosa*	10^7	4 hours
Strain № 8 *Escherichia coli*	10^7	7 hours
Strain № 9 *Serratia marcescens*	10^7	3 hours
Strain № 10 *Serratia marcescens*	10^7	7 hours

initial density of the bacterial suspension. With suspension density 10^5 CFU/ml, the time for killing varies from 2 h to 4 h, with density 10^6 CFU/ml – from 4 h to 5 h and with density 10^7 CFU/ml – from 3 h to >7 h. The individual peculiarities of the tested strains influence the time for killing as well. Thus two strains *P. aeruginosa* with density 10^6 CFU/ml were killed for 2 h and 4 h, respectively, two strains *E. coli* with density 10^7 CFU/ml – for 5 h and 7 h, respectively and two strains *S. marcescens* with density 10^7 CFU/ml for 3 h and 7 h, respectively.

In vitro studies of Hyaluricht® antibacterial effect upon the most frequently isolated pathogenic microorganisms from patients with leg ulcers were carried out (1). The results show the most significant effect of the preparation against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Enterococcus faecalis*.

In comparison with previous reports, the present study found well expressed bactericidal activity of 0.2% Zn-hyaluronate against *Staphylococcus aureus*, *Pseudomonas aeruginosa Escherichia coli*, and *Serratia marcescens*. Time-kill experiments demonstrated fast bactericidal activity even against highly resistant to antimicrobials strains. In our experiments the time for killing depended on the species affiliation and the bacterial suspension density. This may reflects the species specific biological characteristics rather than a carriage of genes for resistance to antimicrobials.

Conclusions

As a conclusion, the results from the *in vitro* study of 0.2% Zn-hyaluronate solution efficiency against the most frequently isolated microorganisms from patients with *Ulcus cruris* confirm the data given in the medical literature for the antimicrobial activity of the preparation Hialuricht® and support the approved position in the clinical practice for its favorable therapeutic effect in the local treatment of infected ulcers (1, 9).

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