THE in vitro ANTIMICROBIAL ACTIVITY OF HIGHLY DISPERSED SILICA AND POLYHEXAMETHYLENE GUANIDINE HYDROCHLORIDE COMPOSITE FOR TREATING LOCAL INFECTIONS

A. I. Doroshenko, O. B. Balko*, Ye. P. Voronin**, A. M. Doroshenko, G. V. Zaychenko

Bogomolets National Medical University
Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine*
Chuiko Institute of Surface Chemistry of the National Academy of Sciences of Ukraine**

Antimicrobial drug resistance (ADR) is an urgent global problem for all countries; it has a negative effect on the treatment outcome of patients. The problem can be solved by creating and introducing new antimicrobial compounds and complex drugs. Development of the combined antimicrobial agent which would show the expressed antimicrobial action and sorption properties remains relevant. Polyhexamethylene guanidine hydrochloride (PHMG-HC), being a high-molecular cationic surfactant of the guanidine group, was chosen as an antimicrobial component.

Aim. To determine the antimicrobial activity of highly dispersed silica (HDS), a composite (code name CMU-211) of HDS and PHMG-HC, and PHMG-HC polymer solution.

Materials and methods. 5% suspension of HDS modified by PHMG-HC polymer; 5% suspension of HDS and 20% aqueous solution of polyhexamethylene guanidine hydrochloride were used in the work. The antimicrobial activity of substances was studied using test-strains of such microorganisms as Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella enterica, Klebsiella pneumoniae, and Candida albicans.

Results. The composite HDS/PHMG-HC has been shown to have a high activity against C. albicans and S. aureus with the minimum inhibitory concentration (MIC) of ~10 μg/mL (calculated with reference to HDS/PHMG-HC), as well as the marked effect against E. coli (MIC of ~20 μg/mL), S. enterica (MIC of MIC ~40 μg/mL) and P. aeruginosa (MIC ~40 μg/mL). The relatively low activity of CMU-211 was reported against K. pneumoniae (MIC ~80 μg/mL). The activity of the composite HDS/PHMG-HC was similar to that of PHMG-HC.

Conclusions. The composite HDS/PHMG-HC developed exhibits the marked antibacterial activity against gram-positive, gram-negative microorganisms, as well as C. albicans.

Key words: antibacterial activity; antibiotic resistance; antifungal action; polyhexamethylene guanidine hydrochloride; silica nanoparticles

A. I. Дорошенко, О. Б. Балко*, Є. П. Воронін**, А. М. Дорошенко, Г. В. Зайченко
Національний медичний університет імені О. О. Богомольця
Інститут мікробіології і вірусології імені Д. К. Заболотного НАН України*
Інститут хімії поверхні імені О. О. Чуйка НАН України**

Antимікробна активність in vitro нанодисперсного кремнезему і композиту полігексаметиленгіанідину гідрохлориду для лікування місцевих інфекцій

Резистентність до антимікробних препаратів є актуальною проблемою для всіх країн світу, що негативно впливає на результати лікування хворих. Вирішити поставлений проблему можна шляхом створення та впровадження нових антимікробних сполук та комплексних лікарських засобів. Актуальним залишається розробка комбінованого антимікробного засобу, який би проявляв виражену антимікробну дію та сорбційні властивості.

Мета. Визначити антимікробну активність суспензії нанодисперсного кремнезему (НДК), композиту НДК/ПГМГ-ГХ і розчину полімера ПГМГ-ГХ для лікування місцевих інфекцій.

Матеріали та методи. В роботі була використана 5% суспензія НДК, модифікована полімером ПГМГ-ГХ; 5% суспензія НДК та 20% водний розчин полігексаметиленгіанідину гідрохлориду. Дослідження антимікробної активності речовин проводили на мікроорганізмах: Escherichia coli; Staphylococcus aureus; Pseudomonas aeruginosa; Salmonella enterica; Klebsiella pneumoniae; Candida albicans.

Результати. Композит НДК/ПГМГ-ГХ має високу активність проти C. albicans і S. aureus з МІК ~10 мкг/мл (у перерахунку на ПГМГ-ГХ) i виражену активність проти E. coli (МІК ~20 мкг/мл), S. enterica (МІК ~40 мкг/мл) і P. aeruginosa (МІК ~40 мкг/мл). Відносно низьку активність композит НДК/ПГМГ-ГХ проявив щодо K. pneumoniae (МІК ~80 мкг/мл). Активність композиту НДК/ПГМГ-ГХ була подібна до такої ж у ПГМГ-ГХ.

Висновки. Розроблений композит НДК/ПГМГ-ГХ проявляє виражену антибактеріальну дію щодо грампозитивних, грамнегативних мікроорганізмів, а також C. albicans.

Ключові слова: антимікробна активність; антибіотикорезистентність; протигрибкова активність; полігексаметиленгіанідину гідрохлорид; наночастинки кремнезему

UDC (547.304.2:546.284):615.281.9
ISSN 1562-725X (Print)ISSN 2518-1572 (Online)
Antimicrobial drug resistance (ADR) is an urgent global problem for all countries; it has a negative effect on the treatment outcome of patients. Currently, ADR is going beyond purely medical issues and becoming of great social and economic importance [1].

*Staphylococcus aureus* plays a leading role in development of nosocomial *pyo-inflammatory* infections and, recently, there is an increase in its resistance to great part of antimicrobial drugs (AMD) used in clinical practice [2]. Furthermore, the prevalence of resistance has significant variations in different countries [3].

There is also an increase in AMD resistance of *Escherichia coli* strains [4], which are the leading cause of nosocomial *pyo-inflammatory* infections [3,5]. Other problematic drug-resistant pathogens encountered today include *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Candida albicans* [6].

The problem can be solved through development and implementation of novel antimicrobial compounds and combination drugs.

Cationic amphiphilic polymers, including polyhexamethylene guanidine hydrochloride (PHMG-HC), are among the promising antimicrobial agents for topical use [7]. Representatives of the polymeric guanidine family have a broad spectrum of activity against gram-positive, gram-negative bacteria, fungi, and yeast. PHMG-HC has received increasing attention in recent years since this compound can be developed as a highly effective disinfectant in combination with other substances and it can be bound to the substrate material to create covalently bound, nonleaching antimicrobial surfaces [8].

The bactericidal effect of PHMG-HC is based on binding of positively charged disinfectant molecules to the cytosolic membrane and lipopolysaccharides or murein of the cell wall. Bacterial cell death caused by critical changes in the areas being in contact with a disinfectant is followed by the cell wall destruction and cell lysis [9, 10].

As for a sorbent, over the past 15 years the research on the properties of highly dispersed silica (HDS) has been conducted at the Pharmacology Department of Bogomolets National Medical University (Kyiv, Ukraine) together with Chuiko Institute of Surface Chemistry of the National Academy of Sciences (NAS) of Ukraine. The HDS suspension has been found to possess pronounced absorption properties and reduce the toxicity of xenobiotics with different mechanisms of toxic action and chemical structure [11].

The development of a combined antimicrobial agent with the marked antimicrobial activity and absorption properties represents the continued scientific cooperation between the Pharmacology Department of Bogomolets National Medical University and Chuiko Institute of Surface Chemistry of the NAS of Ukraine. As a result of this cooperation, the composite of HDS+PHMG-HC have been developed. The aim of the work was to determine the *in vitro* antimicrobial activity of highly dispersed silica (HDS, code name CMU-212), a composite (code...
Materials and methods

Substances. The following substances were tested in the study:
- CMU-211, which is 5 % HDS suspension modified with PHMG-HC in the ratio of 200 mg of the polymer per 1 g of silica;
- CMU-212, which is 5 % HDS suspension;
- 1 % aqueous solution of PHMG-HC.

CMU-211 and CMU-212 were obtained at Chuiko Institute of Surface Chemistry of the NAS of Ukraine. CMU-212 is 5 % stabilized suspension of highly dispersed silica (i.e., 5 g of SiO\(_2\) in 100 g of the suspension). CMU-211 is also the same 5 % suspension of highly dispersed silica, which additionally contains PHMG-HC in the ratio of 5:1 (i.e. 5 g of SiO\(_2\) and 1 g of PHMG-HC per 100 g of the suspension). In the ratio of 200 mg of PHMG-HC per 1 g of silica a monolayer of adsorbed polymer is formed.

Microbial strains used. To assess the antimicrobial activity of the substances tested the following microbial strains were used: Escherichia coli UCM B-906, Staphylococcus aureus UCM B-918, Pseudomonas aeruginosa UCM B-900, Salmonella enterica UCM B-921, Klebsiella pneumonia UCM B-920, and Candida albicans UCM Y-1918. These strains were obtained from the Ukrainian Collection of Microorganisms (UCM) of D. K. Zabolotny Institute of Microbiology and Virology of the NAS of Ukraine. These microorganisms are the test strains to determine the antimicrobial activity of medicines [12].

Nutrient media. The LB liquid medium (Luria-Bertani broth, Merck, Germany) was used in preparation of the initial and working suspensions of microorganisms and test substances, as well as determination of the minimum inhibitory concentrations (MIC) of the test substances. The LB solid nutrient medium (Luria-Bertani medium, Merck, Germany) in Petri dishes was used to obtain twenty-four-hour cultures of microorganisms and determine the minimum bactericidal/fungicidal concentrations (MBC/MFC) when it was inoculated with aliquots of test and control suspensions.

The study of the antimicrobial activity of the test substances. For each species of microorganisms a line of 12 test-tubes was prepared. All test-tubes were filled with 0.5 ml of the LB medium. The first test-tube of each line was filled with 0.5 ml of the working solution of the corresponding substance (CMU-211, CMU-212 or PHMG-HC), and double serial dilutions were then prepared.

Twenty-four-hour cultures of microorganisms were obtained via cultivation on a slant solid LB medium at 37 °C for 18–24 h. Initial microbial suspensions with turbidity corresponding to 0.5 McFarland standard (1.5×10⁸ CFU/ml) were prepared using 24-hour cultures. Working suspensions of microorganisms were obtained after dilution of the initial suspensions in the ratio of 1:5 (v/v).

After that 0.5 ml portions of each working suspension were transferred into test-tubes containing prepared double dilution of the corresponding test substance (CMU-211, CMU-212 or PHMG-HC). Therefore, the final volume of the solution in the experimental test-tubes was up to 1 mL. The titers of S. aureus, E. coli, P. aeruginosa, S. enterica, and K. pneumoniae were 10° CFU/ml, whereas the titer of C. albicans was 10⁶ CFU/ml, which corresponded to the experimental procedure requirements.

The effect of CMU-211 on microorganisms was studied using 15 mg/mL as the initial concentration which corresponded to 12.5 mg of HDS and 2.5 mg of PHMG-HC per 1 mL of the suspension. For CMU-212 the initial concentration was 12.5 mg/mL of A-300 HDS suspension. Ultimately, as the starting concentration of PHMG-HC 2.5 mg/mL was used.

As for CMU-211, the final concentrations of HDS and PHMG-HC in the last (12th) test-tube were 6.1 μg/mL and 1.22 μg/mL, respectively. For CMU-212 the final concentration of HDS was 6.1 μg/mL, whereas the final concentration of 1.22 μg/mL was used in the PHMG-HC series.

The experimental samples were compared with the negative controls of the microbial growth using the adjustments for suspension turbidity according to the negative controls of the substance purity. For each experimental series of test-tubes, the first concentration with no visible growth of microorganisms was determined. This concentration was denoted as the minimum inhibitory (bacteriostatic) concentration (MIC) of the corresponding substance with respect to a particular species of microorganisms.

The next step was to determine the minimum bactericidal concentration (MBC) of the substances. In this regard, 200 μL portions of the suspension taken from all experimental samples with no visible growth and from all control test-tubes were inoculated on Petri dishes with the solid LB medium. After uniform distribution of each suspension on the surface of agar and its drying plates were incubated at 37 °C for 24 h in a thermostat. Then in each area of the sample application colonies formed were counted, they indicated the number of viable microorganisms in the corresponding bacterial suspensions. This parameter was expressed as colony forming units (CFU). The minimum bactericidal (fungicidal) concentration of the corresponding substance in relation to the species of microorganisms studied was determined by the first concentration, in which the growth in the microbial suspension aliquots applied on the solid medium was less than 200 CFU. The above mentioned concentration for
**Results and discussion**

The data obtained suggest that CMU-211, CMU-212 and PHMG-HC solution had different influence on the microorganisms tested (see Tab.).

**The antimicrobial activity of CMU-211 (HDS suspension modified with PHMG-HC), as well as their components alone in relation to the test strains of microorganisms**

<table>
<thead>
<tr>
<th>Species of microorganisms</th>
<th>HDS and PHMG-HC (CMU-211)</th>
<th>PHMG-HC (CMU-212)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC, μg/mL</td>
<td>MBC, μg/mL</td>
</tr>
<tr>
<td>Substance In relation to PHMG-HC</td>
<td>Substance In relation to PHMG-HC</td>
<td>Substance</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong> UCM B-906</td>
<td>117.19</td>
<td>19.53</td>
</tr>
<tr>
<td><strong>Staphylococcus aureus</strong> UCM B-918</td>
<td>58.6</td>
<td>9.77</td>
</tr>
<tr>
<td><strong>Pseudomonas aeruginosa</strong> UCM B-900</td>
<td>234.37</td>
<td>39.06</td>
</tr>
<tr>
<td><strong>Salmonella enterica</strong> UCM B-921</td>
<td>234.37</td>
<td>39.06</td>
</tr>
<tr>
<td><strong>Klebsiella pneumoniae</strong> UCM B-920</td>
<td>468.76</td>
<td>78.13</td>
</tr>
<tr>
<td><strong>Candida albicans</strong> UCM Y-1918</td>
<td>58.6</td>
<td>9.77</td>
</tr>
</tbody>
</table>

* The minimum fungicidal concentration (MFC) is denoted. HDS = highly dispersed silica; MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration; PHMG-HC = polyhexamethylene guanidine hydrochloride.

Note. * The minimum fungicidal concentration (MFC) is denoted. HDS = highly dispersed silica; MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration; PHMG-HC = polyhexamethylene guanidine hydrochloride.

S. aureus, E. coli, P. aeruginosa, S. enterica, and K. pneumoniae were denoted as MBC. For C. albicans, the appropriate term 'minimum fungicidal concentration (MFC)' was used. The cultures from the positive and negative controls of growth were assessed on the presence of the confluent growth, and the cultures from the negative controls of the medium and purity of substances were assessed on the absence of the microbial growth. If these requirements for control samples were met, the experiment was considered to be conducted appropriately.

**Results and discussion**

The antimicrobial activity of CMU-211 (HDS suspension modified with PHMG-HC), MICs of CMU-211 against S. aureus and C. albicans were the lowest and were equal to 48.83/9.77 μg/mL. However, to achieve MIC for S. enterica the concentration of CMU-211 needs to be two times higher (195.31/39.06 μg/mL). K. pneumoniae appeared to be the most resistant, and it was required at least 390.63/78.13 μg/mL of CMU-211 to inhibit its multiplication. P. aeruginosa was also characterized by high resistance to the substance since its MIC was the same as that of S. enterica and was equal to 195.31/39.06 μg/mL. Thus, CMU-211 had the most prominent inhibitory effect against S. aureus and C. albicans, while the weakest effect was on K. pneumoniae (see Tab.).

The assessment of MBCs showed that bacteriostatic concentration of CMU-211 against S. enterica and K. pneumoniae was also proved to be bactericidal. However, the concentrations of the substance had to be twice as high as MICs to ensure the bactericidal (fungicidal) effect on other microorganisms studied. Therefore, for CMU-211, the lowest MBCs/MFCs of 97.66/19.53 μg/mL were proven to be against S. aureus and C. albicans, while the weakest effect was on K. pneumoniae (see Tab.).

The antimicrobial activity of CMU-212 (HDS suspension). The antimicrobial properties of CMU-212, a suspension of HDS A-300 and one of the components of CMU-211, were also studied. It was found that CMU-212 failed to show the antimicrobial effect on
all microorganisms tested. Application of this substance in the maximum concentration of 12.500 μg/mL did not cause growth retardation in the test strains of microorganisms in the liquid medium. Plating of microorganisms incubated with the whole range of the test concentrations in test-tubes on the solid medium proved their viability since they demonstrated a confluent growth on the surface. Hence, it was concluded that CMU-212 failed to show the antimicrobial activity against the test strains (see Tab.).

The antimicrobial activity of PHMG-HC. The data obtained showed that PHMG-HC solution had a high level of the antimicrobial activity against the test strains studied. The indicators of MIC for this substance appeared to be the lowest for S. aureus and C. albicans (4.88 μg/mL). E. coli and S. enterica were less sensitive to PHMG-HC and demonstrated no growth in the presence of 19.53 μg PHMG-HC per 1 mL. Among the representatives of the Enterobacteriaceae family used in the study K. pneumoniae showed the highest resistance since the bacteriostatic effect was obvious only when the concentration of PHMG-HC was 39.06 μg/mL. P. aeruginosa appeared to have the similar level of resistance. Therefore, PHMG-HC was the most active in inhibiting the growth of E. coli and S. enterica, but it was 8 times less effective against K. pneumoniae and P. aeruginosa (see Tab.).

Summarizing the results of the antimicrobial activity assessment it should be noted that CMU-211 has a high activity against C. albicans and S. aureus and a marked effect on E. coli and S. enterica. The relatively low activity of CMU-211 was reported against K. pneumoniae and P. aeruginosa, however, the effect on P. aeruginosa was consistent with the activity of PHMG-HC applied alone.

According to the multiplicity of difference between MICs and MBCs/MFC for individual species of microorganisms the activity of the HDS + PHMG-HC composite (i.e. CMU-211) is similar to that of PHMG-HC (see Tab.). Thus, a twofold increase in the concentration of both substances converts the bacteriostatic/fungistatic effect against P. aeruginosa and C. albicans into a bactericidal and fungicidal effect, respectively. PHMG-HC demonstrated more prominent growth-inhibiting effect against S. aureus (MIC of PHMG-HC was 4.88 μg/mL vs 9.77 μg/mL for CMU-211), but the MBC values were the same for both substances (19.53 μg/mL). The antimicrobial activity against E. coli was higher with PHMG-HC since the MBC/MIC ratio was equal to 1; however, the HDS+PHMG-HC composite gave the MBC/MIC ratio of 2. For other microorganisms, the MBC/MIC ratios were the same for both substances and were equal to 1.

As far as we know, no similar studies assessing the antimicrobial activity of the HDS + PHMG-HC composite were found in the available literature. Oulé et al. [9] studied the antimicrobial activity of PHMG-HC and showed that complete death of E. coli could be achieved when this substance was applied in the concentration of 0.005 %. According to Gregirchak et al. [13] MIC of PHMG-HC for E. coli and S. aureus was 19 μg/mL and 9 μg/mL, respectively, it was close to the estimates obtained in our study.

CONCLUSIONS

1. The 5 % suspension of HDS modified with PHMG-HC polymer added in the ratio of 5:1 (i.e. 200 mg of the polymer per 1 g of the suspension) developed at the Chuiko Institute of Surface Chemistry of the NAS of Ukraine, exhibits the marked antibacterial activity against gram-positive, gram-negative microorganisms, as well as C. albicans.

2. This HDS+PHMG-HC composite (CMU-211) has showed the highest activity against S. aureus and C. albicans. MIC and MBC/MFC are 9.77 μg/mL and 19.53 μg/mL, respectively, calculated with reference to PHMG-HC.

3. The composite has been found to have different activities against gram-positive and gram-negative bacteria. Gram-negative microorganisms, such as E. coli, P. aeruginosa, S. enterica and K. pneumonia, have been found to have slightly lower sensitivity to the composite compared to the gram-positive S. aureus. It is assumed that this difference may be associated with the peculiarities of the bacterial wall structure. However, these hypothesis needs further testing.

Conflict of interests: authors have no conflict of interests to declare.

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