Original Article

Antibacterial and Immunomodulatory Effects of Hexamethylenetetramine (Methenamine) Silver Nitrate

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Abstract

Introduction: Currently, developing new antibacterial drugs as alternative antibiotics is a very active area of research, due to widespread prevalence of resistant strains of microorganisms. This work intends to investigate antibacterial properties and influence on immune blood cells of the silver-based compound hexamethylenetetramine (methenamine) silver nitrate with general formula [Ag(CH₂)₆N₄]NO₃.

Materials and Methods: The antibacterial effect of the silver complex was investigated by agar diffusion and serial dilution methods. Silver complex have been investigated for its impact on the phagocytic activity of neutrophils and on immune cells during the reaction of blast transformation of lymphocytes (RBTL).

Results: Studies have shown that hexamethylenetetramine silver nitrate possesses both bactericidal and bacteriostatic dose-dependent effect on tested bacterial strains, including Staphylococcus aureus, Proteus vulgaris, Pseudomonas aeruginosa, Streptococcus pneumoniae. Escherichia coli were shown to be the most susceptible bacteria. Cytotoxic effect of silver salt on lymphocytes was detected in high dosage in RBTL. No significant immunosuppressive impact on neutrophils phagocytic activity of tested complex was shown.

Conclusion: Agents of nosocomial infections were highly susceptible to the drug. Complex has proved to be promising as a prospective antibacterial drug with wide range of activity.

Introduction

At present, the problem of nosocomial infections is very acute and constitute a serious public health problem. The antibiotics era overshadowed the promise that silver based compounds held, but now silver, once again seems to be prospective antibacterial agent, (Jung et al., 2008). Multidrug resistant microorganisms have become a big challenge for medicine. Antibiotic-resistant strains of microorganisms have boosted interest in discovering alternative antibacterial agents (Silnikov et al., 2015). One of possible mechanism of silver action in vivo is denaturing the deoxyribonucleic acid (DNA) molecule by disrupting the hydrogen bonding between the two anti-parallel strands (Thurman and Gerba, 1989).
Currently, there are many drugs that act on the basis of silver - silver nitrate, protargolum, colloidal silver and others, which could have a significant level toxicity. Some report about lower toxicity levels of silver nanoparticles compared with ionic silver drugs (Kvitek et al., 2011, Sondi and Salopek-Sondi, 2004). However, silver nanoparticles are rather difficult for in vivo application, especially for intravenous administration, and antimicrobial action of nanoparticles depends strongly of size and even its form (Sukdeb et al., 2007). In this regard, it is typical to develop novel silver-based compounds with high antibacterial activity and low toxicity. Study of the immunomodulatory properties of biopharmaceutical compounds is very important for the design of new medicines. In addition, Substances acting on cell-mediated immunity in vitro will provide general and non-specific effect on the entire immune system as a whole because of the high interconnectivity of various components. The purpose of this work was to study antibacterial properties and effects on immune cells of nitrate hexamethylenetetramine of silver - [Ag(CH2)6N2]NO3. It should be noted hexamethylenetetramine salts were mostly used for urinary tract infections. Since it is activated by interacting with the acidic compounds in urine, is suitable for lower urinary tract treatment. Hexamethylenetetramine salts are used in histology as sustainable stains (Dawson and Filipe, 1976) and this silver stain is suitable for electron microscopy (Mase, 1988). In this work we suggest an application of silver salt of hexamethylenetetramine as antibacterial agent of wide spectrum. Previously, the low genotoxic properties of hexamethylenetetramine silver complex was established (Plotnikov et al., 2015). Based on these results, we provide in vitro investigation of silver complex as rapid evaluation method of immune response and antibacterial spectrum. Further in vivo testing is required for complex assessment of biological effects.

Materials and methods

Study of antibacterial activity

The antibacterial effects of the silver complex were investigated by agar disk-diffusion tests and serial dilution method (Reller, 2009). Growth detection of the microorganisms was carried out in test tubes with the meat-peptone and Petri dishes with meat-peptone agar. The complex was prepared as a 2.5 mg/ml aqueous solution for serial dilution test. The study of the bacteriostatic and bactericidal action of the drug was carried out in vitro on microorganisms Streptococcus pneumoniae, Escherichia coli, Staphylococcus aureus, Proteus vulgaris, Pseudomonas aeruginosa. These microorganisms were of different types and allowed to evaluate antibacterial properties against main nosocomial infection agents, both Gram-negative and Gram-positive bacteria. Series of test tubes containing nutrient medium with the drug in the dilutions rates of 1:2 to 1: 256 were prepared for each experiment with microorganisms. Inoculation was accomplished with 1 ml of standardized bacterial suspension 5×10^5 CFU/ml (Colony Forming Unit per ml) into each of tubes. Bacteria were incubated at 37°C for 5 days. Detection of growth was performed every 24 hours. Maximum inhibitory dilution of the drug is determined by taking the mean value of dilutions in the two adjacent tubes (one of which has growth of the microorganism and the next has no growth). Maximum bactericidal dilution was determined as a dilution in last tube with test-cultures, which gave no growth within 5 days of cultivation.

Agar disk-diffusion tests was performed according to the following procedure. On solid nutrient media meat-peptone agar (MPA) in Petri dishes, added 1 ml of suspension of microorganisms prepared for optical turbidity standard 1×10^6 CFU/mL, and evenly distributed. After that, different paper disks with a wide range of concentrations (0.05 to 5.0 mg/ml of silver complex) were placed on the inoculated agar surface. Test plates were incubated for 24 h at 35°C. The zones of growth inhibition around tested disks were measured to the nearest millimeter.

Study of influence on immune blood cells

Influence of complex in reaction of blast transformation lymphocytes

The impact of the complex on immune cells of human blood was observed in the reaction of blast transformation of lymphocytes (RBTL). The method is based on an assessment of the lymphocytes transformation and proliferation when exposed to various antigens and mitogen phytohemagglutinin.
Methenamine Silver Properties

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(PHA). All blood samples were obtained from healthy donors. Lymphocytes were isolated by gradient centrifugation and resuspended with standard media RPMI 1640, containing 20% fetal bovine serum, L-glutamine, streptomycin. Aliquots of 0.1 ml (2×10⁶ cells/ml) of the cell mixture were placed in microculture plates. Complex was added to plate in concentrations 0.1 mg/ml - 0.001 mg/ml with or without PHA. Control group contained no substances. Cultural plates were sealed and incubated for 72 hours at 37°C. Then all samples were centrifuged and smears were made. Smear samples were stained by the Giemsa method. Five hundreds cells were counted in every smear for transformed lymphocytes determination. Lymphocyte transformation was detected by method (Novikov and Novikova, 1996).

Influence of complex on phagocytic activity of leucocytes

The phagocytic activity of the neutrophils was studied using phagocytosis method (Novikov and Novikova, 1996). The Gram-positive bacteria, *Staphylococcus aureus* - H209, used as the phagocytic substrate. The Bacteria were added to the leukocyte suspension. The investigated complex was added to the microculture plates in different concentration (0.1-0.001 mg/ml). The samples were placed for 30 minutes in an incubator and shaken every 10 minutes. After incubation, cells were fixed with formalin. Then centrifuged to make a smear for determining phagocytosis. Smears stained with Romanovsky-Giemsa and counted. The phagocytic index was calculated as the number of neutrophils positive for *S. aureus* ingestion per 100 neutrophils. The avidity index was calculated as the total number of *S. aureus* cells engulfed per 100 positive neutrophils and divided by 100. The index of phagocytosis completeness was calculated as the number of *S. aureus* killed in phagocytes divided by the total number of the microbes engulfed by phagocytes per hundred cells.

**Results**

The studies revealed a strong antibacterial effect in vitro on all the studied cultures of microorganisms, as shown in Tables 1, 2. Table 1 shows silver-based complex revealed both bactericidal and bacteriostatic activity for all tested bacteria cultures. The most pronounced antibacterial effect was revealed against *Escherichia coli*, where bactericidal effect is provided in concentration 0.02 mg/ml and bacteriostatic (growth inhibition) - in a dilution of 1:192. The next in row of microorganisms based on sensitivity to the drug are *Proteus vulgaris* and *streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*. Antibacterial activity of the complex revealed by the agar diffusion method in general correlated with the results obtained by serial dilutions method, as could be seen in Fig. 1. The Maximum growth inhibition (23±2 mm) was

### Table 1: Antibacterial properties of tested substance.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Maximum inhibitory dilutions</th>
<th>Minimal bactericidal concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>1:96</td>
<td>0.04 mg/ml</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em></td>
<td>1:96</td>
<td>0.03 mg/ml</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>1:96</td>
<td>0.03 mg/ml</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>1:96</td>
<td>0.03 mg/ml</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>1:192</td>
<td>0.02 mg/ml</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Control</th>
<th>0,1 mg/ml</th>
<th>0,01 mg/ml</th>
<th>0,001 mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferation without PHA, %</td>
<td>6±0.3</td>
<td>5±0.5</td>
<td>7±0.6</td>
<td>7±0.4</td>
</tr>
<tr>
<td>PHA-induced proliferation, %</td>
<td>89±1.5</td>
<td>48±2.1*</td>
<td>67±2.8</td>
<td>89±2.3</td>
</tr>
</tbody>
</table>

(*significance (p<0.05), compared to control)
observed for bacteria Escherichia coli; Followed by, Streptococcus pneumoniae – 20±1 mm; Proteus vulgaris – 18±1 mm and Pseudomonas aeruginosa - 17±1 mm. The results of the silver complex influence on reaction of lymphocyte blast transformation are shown in Table 2.

Materials from Table 3 shows that the drug has a certain inhibiting effect on blast cells only at high concentration of 0.1 mg / ml (52.5% inhibition), followed by normalization in the rest of the concentration spectrum, that indicating low immunosuppressive influence. The results of studies of the drug impact on phagocytic activity are presented in Table 3.

As shown in Table 3, new silver complex has no considerable impacts on the phagocytic activity in all dosages, but index of phagocytosis completeness within low concentration ranges slightly increases. These Findings indicate absence of toxic effects on immune blood cells.

**Discussion**

Obtained results indicate significant antibacterial properties of hexamethylenetetramine silver complex (Table 1). Bacteria *E.coli* was revealed as relatively more susceptible strain. That is rather predictable for silver salts. However, silver complex revealed similar high level of antibacterial activity against both Gram-positive and negative bacteria. Produced inhibition zones were within the maximum range of 39% for different strains (Figure 1). Reportedly, silver causes higher antibacterial effect against Gram-negative isolates (Cavassin et al., 2015). The mechanism of activity of silver drugs resides in ionic silver at a concentration of $10^{-6}$ to $10^{-8}$ mol/L, while Ag$^0$ is inactive, its antimicrobial activity results from combination with, and alteration of, microbial proteins, with eventual structural and metabolic disruption (Maillard and Hartemann, 2012). Silver ions activity can be enhanced through combination with other antimicrobial agents.

Reportedly, microbial population size can be a limiting factor for inhibition activity by silver ions, with the increase of bacterial population size, the ratio of silver ions to each cell is decreased (Zhao and Stevens, 1998). Consequently, minimal bactericidal concentration (MBC) of silver ions varied from 20 to

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**Table 3: Influence of silver-based complex on neutrophils phagocytic activity, M±SEM (*significance (p≤0.05), compared to control)**

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Control</th>
<th>0,1 mg/ml</th>
<th>0,01 mg/ml</th>
<th>0,001 mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active phagocytosis percentage</td>
<td>31±0.5</td>
<td>24±1.5*</td>
<td>27±1.2</td>
<td>32±0.6</td>
</tr>
<tr>
<td>Avidity index (bacteria/phagocyte)</td>
<td>7±0.2</td>
<td>4±0.2*</td>
<td>4±0.5</td>
<td>6±0.3</td>
</tr>
<tr>
<td>Percentage of phagocytosis</td>
<td>53±0.9</td>
<td>51±1.4</td>
<td>58±1.7</td>
<td>62±1.3*</td>
</tr>
<tr>
<td>completeness</td>
<td></td>
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</tbody>
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**Fig. 1. Zones of growth inhibition of test-cultures of bacteria under the influence of the complex.**
70 µM, depending on the starting concentration (population density) of E. coli. That correlates to results in Table 1 (recalculated to silver ions), where MBC for E.coli was 80 µM. MBC for all tested bacterial strains varied within a range of 80-160 µM of silver ions. Thus, bactericidal effect mainly depends on silver ion concentration in tested substance and less on hexamethylenetetramine component, but the level of toxicity of this complex strictly depend on hexamethylenetetramine. Different anionic component caused different level of genotoxicity (Plotnikov et al., 2015).

The possible mechanism of such effect can be twofold. Initially, reversible, metabolically independent surface binding; then, a metabolically dependent irreversible, intracellular accumulation (Slawson et al., 1990). Silver has been shown to have similar effect against multidrug resistant and susceptible bacteria, which serves as an important advantage in medical application (Cavassin et al., 2015). These results (Table 2) also revealed a notable, antiproliferative impact on lymphocytes. Influence on lymphocytes is significantly dose-dependent and is highly expressed in high dosage. Cytotoxic action against mitogen-stimulated lymphocyte proliferation under influence of tested complex correlate to action of silver ions, as described (Zapata-Sirvent and Hansbrough, 1993). However, no modulation of T-cell proliferation was observed in the presence of Ag-nanoparticles (Greulich et al., 2011). Stimulating influence on index of phagocytosis completeness was unexpected; this parameter was slightly increased in low concentrations of tested substance.

Study (Haase et al., 2014) revealed no significant impact on phagocytosis. So results (Table 3) are mostly linked to methenamine influence. Comparison of the effects of Ag nanoparticles and ionic silver (Ag⁺) on cells of the innate immune system, in particular on neutrophil granulocytes and macrophages revealed elevation of intracellular levels of reactive oxygen species and reduced protein phosphatases. That was concluded as non-specific component responsible for immunomodulatory activity of complex.

**Conclusion**

Thus, tested silver-based complex has a dose-dependent bactericidal and bacteriostatic effect against all studied strains of microorganisms, including those causing nosocomial infections. The experiment showed no suppressive effect of the drug on untreated donor blood cells in the test RBTL and phagocytic activity test. A Noticeable inhibitory effect of the drug was observed only in the high concentration in PHA-stimulated RBTL. Based on this result, Thus, tested silver complex could be considered as a potential candidates for an antibacterial drug with low toxicity.

**Acknowledgment**

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**Conflict of Interest**

Authors declare no conflict of interests.

**References**


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