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The efficacy of combination the amoxicillin and new complex of iodine for lungs infection of mice caused by *Klebsiella pneumonia*

Abstract. In this work, the effectiveness of combination therapy of a new iodine complex (PA) and amoxicillin was investigated. For this, an experimental model of mouse lung infection caused by multi-resistant *Klebsiella pneumonia* ATCC 700603 strain was used. Therapy was administered for 14 days with amoxicillin at a dose of 10 mg/kg and PA at doses of 20 and 200 mg/kg. In addition, the post-therapeutic effect was assessed on day 7. The level of bacteria was studied in the lungs and blood. The concentration of antibiotic and PA in blood was also measured. As a result, it was found that on day 14 the level of bacteria in the lungs and blood significantly decreased with a combination of 10 mg/kg amoxicillin and 200 mg/kg PA. 7 days after the completion of therapy, complete elimination of bacteria occurred in the groups of 20 and 200 mg/kg PA in combination with an antibiotic. With the combined effect of PA and amoxcillin, the level of the antibiotic increases, this may indicate an improvement in bioavailability. These results demonstrate the effectiveness of PA in combination with amoxicillin.

Key words: iodine, amoxicillin, infection, mice, *Klebsiella pneumonia*, combination therapy.

Introduction

Iodine belongs to biophilic elements, but due to the dispersion in nature and extremely low concentrations in the body of terrestrial animals, its role predominantly consists in the formation of thyroid hormones. Iodine is significantly different from other halogens – chlorine and bromine. It forms a variety of compounds related to supramolecular chemistry [1]. These properties make it possible to obtain compounds with various pharmacological activities by complexation with bioorganic ligands, such as amino acids and carbohydrates [2]. For example, iodine adducts with dextrin and lithium cation is effective against gram-positive and gram-negative bacteria. Its pharmacological effect has been examined on rats infected with *Staphylococcus aureus* 209D strain [3]. Polyiodide also forms a variety of complexes with nanomaterials (dendrimers, nanotubes, nanoparticles, ect.) [4]. These complexes have high antimicrobial activity and lack of resistance to them. For example AgNPs with trans-cinnamic acid and povidone-iodine had the antibacterial activity against Gram-positive and Gram-negative bacteria. While the Gram-negative species were more susceptible to TCA-AgNP-NPI than the Gram-positive bacteria [5]. In addition to direct antimicrobial activity against viruses, bacteria, and fungi, certain iodine complexes are also able to affect gene activity, especially oxidative stress [6-8]. Such an action of iodine can affect some mechanisms of antimicrobial resistance, for example, the formation of biofilms or trigges decolonization [9]. Considering the ability of iodine to interact with reactive oxygen species (ROS), and especially with H_2O_2, O_2^-, and OH(•) radical [1], an increase in the stress load on the cell can be suggested. This approach is implemented in antimicrobial photodynamic inactivation under the action of a photosensitizer and iodides [10]. However, when using iodides, their activation is necessary, as in this case with UV light or metal nanoparticles [11].

The cooperative antimicrobial effect is also achieved by the combined action of hydrogen peroxide and molecular iodine solutions against bacteria and yeast without any activation [12]. The source of ROS in a bacterial cell is thereby required that would interact with iodine to form reactive halogen species, iodine in this case [1]. An antibiotic may serve as such a source. It is known that certain antibiotics induce ROS formation inside a bacterial cell, which leads to global changes in metabolism [13].

Therefore, one of the objectives in improving the effectiveness of the antibacterial action of antibiotics,

especially against resistant forms, may be their potentiation due to the formation of reactive iodine species. To this end, it is necessary to maintain the oxidation potential of iodine in the reducing medium, which is achieved by complexation with various bioorganic ligands [2].

In order to achieve this effect, a stable complex of iodine with dextrin and various peptides has been obtained after acid hydrolysis of mammalian albumin (PA). The length of dextrin units does not exceed 15 glucose units. Polyiodide is located inside the dextrin helix [4,14]. Peptides additionally coordinate iodine with their carboxyl groups, protecting it from molecules with more pronounced electron donor properties [2].

In this study, we evaluated the efficacy of new complex of iodine and amoxicillin in a mouse infection model of *Klebsiella pneumonia* ATCC 700603. In addition, post-treatment effect (PTE) was studied 7 days after therapy. To see one of the mechanisms for enhancing the action of the antibiotic, the effect of PA on the concentration of amoxicillin in the blood of infected mice was studied.

Materials and methods

Test substance. PA is a dark brown powder with a faint odor of iodine (Scientific Center for Anti-Infectious Drugs). Concentration of molecular iodine in PA is 54.1 g/kg. Amoxicillin (amx) (Sigma-Aldrich, USA).

Bacterial strain and media. In the present study we have used of the extended-spectrum β-lactamase reference strain *K. pneumoniae* ATCC 700603 resistant to amoxicillin, cefepime and meropenem. Bacteria were cultured and maintained in Mueller-Hinton broth and agar (Himedia, India) for testing.

Animals. The outbred young male mice CD-1, weighing 10-14 g. The animals were received from the supplier of the Scientific and Practical Center for Sanitary and Epidemiological Expertise and Monitoring, Almaty. Mice were given *ad libitum* access to food and water. The animals were housed in individually ventilated cages (Tecniplast, Italy), in special biosafety level 2 (ABSL-2) rooms under 12/12 lighting mode. The study was approved by the Bioethics Committee of the Scientific Center for Anti-Infectious Drugs (Protocol No. 18, 04.03.20).

Experiment with infected mice. The potentiating activity of PA was examined *in vivo* in murine lung infection model without neutropenia [15]. Mice were infected using an intratracheal injection of 0.05 ml of a bacterial suspension with a tracheal cannula under anesthesia with isoflurane. Once that's happened, the 5 groups were formed, 10 mice in each group. Treatment of animals has started 48 hrs after infection. The mice were euthanized by CO_2 asphyxiation after 14 and 21 days therapy. The blood and lungs were aseptically removed, homogenized, and processed for bacterial CFU determination in tissue. Smears were prepared from the grown colonies, stained with the Gram stain method and examined under a microscope (Leica, Austria). Blood concentrations of amx and PA were also determined after 30, 60 and 120 min. The blood samples were centrifuged at 5000g for 15 min at 4°C. Plasma samples were stored at -80 °C until assay. The design of the experiment is shown in Table 1.

Table 1 – Design of the experiment

Group No.	Conditions of treatment
1	Healthy mice (negative control)
2	Infected and untreated mice (positive control)
3	Amx 10 mg/kg
4	PA 200 mg/kg
5	PA 200 mg/kg and amx 10 mg/kg
6	PA 20 mg/kg and amx 10 mg/kg

The examined doses of PA, 20 and 200 mg/kg, we

re 1/100 and 1/10 of the maximal tolerated dose in mice, which was established in a preliminary experiment. The dose of amx was chosen according to the results of pilot study. PA solutions were administered intragastrically once a day, amx was given intramuscularly once a day, for 14 days.

Amoxicillin and PA assay. Amoxicillin plasma concentrations were measured using an Agilent 1200 HPLC system (Agilent Technologies, USA) with ultraviolet absorbance detection. PA was measured in the form of iodides using the mass spectrometry with inductively coupled plasma method on an Agilent 7500 ICP-MS (Agilent Technologies, USA). For measurement and calibration were used: "Determination of trace elements. Method for determination of the iodine by mass spectrometry with inductively coupled plasma (ICP-MS)", standard DIN EN 15111: 2007 and the validation technique of measuring the mass concentration of iodide ions in a 1% aqueous solution of tetramethylammonium hydroxide (TMAH) by mass spectrometry with inductively – linked plasma (ICP-MS).

Statistical analysis. Statistical data analysis was performed with GraphPad Prism, 6.0 (GraphPad

Software, San Diego, CA, USA). Mean values and standard deviations were calculated for all quantitative indices. The data were subjected to statistical analysis using the Kruskal-Wallis test with a posteriori comparison of each group to the non-parametric Dunn's criterion. The p-value < 0.05 was considered as significant difference.

Results and discussion

For the control group with no antimicrobial treatment of the all mice presented clinical signs of infection. In the Amx and PA groups, the infected mice showed no clinical signs of infection, but showed a small bacterial load in their lungs at 14 days after infection (Fig. 1). The same picture was observed in the blood of control and experimental animals (Fig. 2). Although the level of bacteria in the blood was lower than in the lungs.

On the 14th day after the treatment of animals with Amx and PA, the CFU level in the lungs and blood significantly decreased compared to the group treatment with the antibiotic alone. The most effect was observed in the antibiotic and PA group at a dose 200 mg/kg. The decrease of CFU in the lungs of animals was better than when treatment only the antibiotic or PA (p=0.0091). Similar results were obtained in blood tests (p=0.0005). The resistance to infection of *K. pneumoniae* ATCC 700603 is due to the unsatisfactory activity of neutrophils and, in particular, their oxidative activity [16]. In addition, this strain carries genes for resistance to amoxicillin [17]. However, it is known that iodides can be enhancing phagocytosis. This mechanism is based on the formation of reactive oxygen species against microorganisms generated by haloperoxidases in the innate immune system. The myeloperoxidase system of neutrophils is active in the presence of chlorides, which are ubiquitous ions. Although the affinity of this enzyme for iodides is higher due to low concentrations in the blood, its role in protection is insignificant. However, if the concentration of iodides in the blood is increased, this effect will noticeably increase [18]. The reaction proceeds according to the scheme: ($H_2O_2 + I^- \rightarrow OI^- + H_2O$). This process becomes most effective at sufficiently high iodide concentrations [3].

Therefore, to study the long-term effect of the immune system, the lungs and blood of animals were studied on the 7th day after therapy. Figures 3 and 4 shows the results on day 7 after therapy.

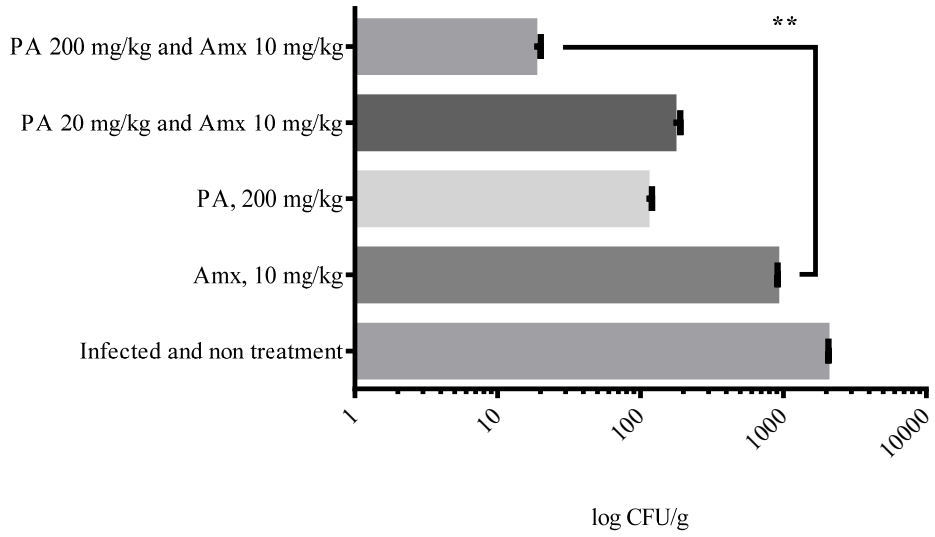

Figure 1 – Effectiveness of *K. pneumoniae* eradication from mouse lungs on day 14 of treatment with PA and Amx. Conditions of treatment are presented on the y-axis, the number of bacteria converted to $\log_{10}$ CFU unit per gram of lungs on the x-axis.
Note: **Statistical significance compared with Amx monotherapy at a level of p <0.01

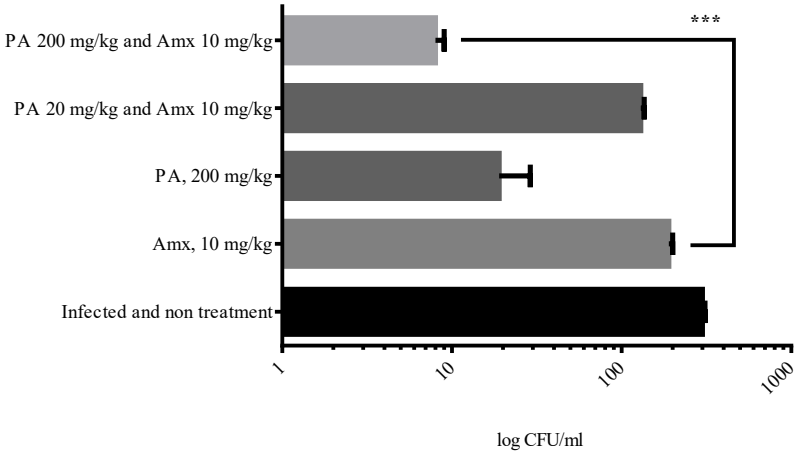

Figure 2 – Effectiveness of K. pneumoniae eradication from mouse blood on day 14 of treatment with PA and Amx. The treatment conditions are presented on the y-axis, the number of bacteria converted to log10 CFU unit per ml of blood on the x-axis.
Note: ***Statistical significance compared with Amx monotherapy at a level of p <0.001

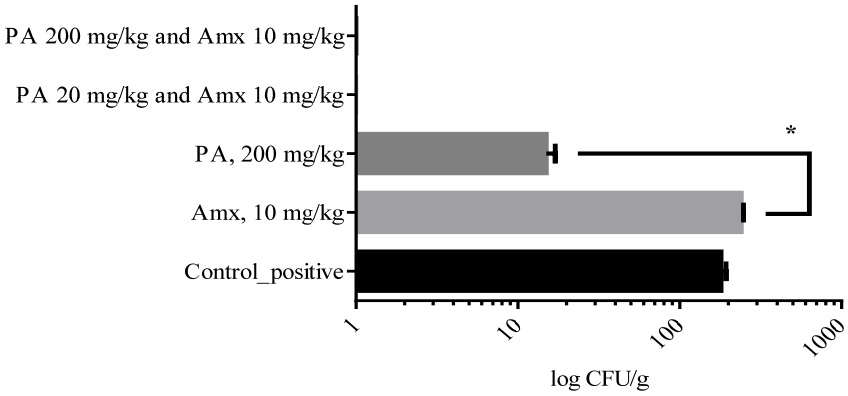

Figure 3 – Effectiveness of *K. pneumoniae* eradication from mouse lungs on day 7 after PA and Amx treatment. The treatment conditions are presented on the y-axis, the number of bacteria converted to $\log_{10}$ CFU unit per ml of blood on the x-axis.
Note: *Statistical significance compared with Amx monotherapy at a level of p<0.05

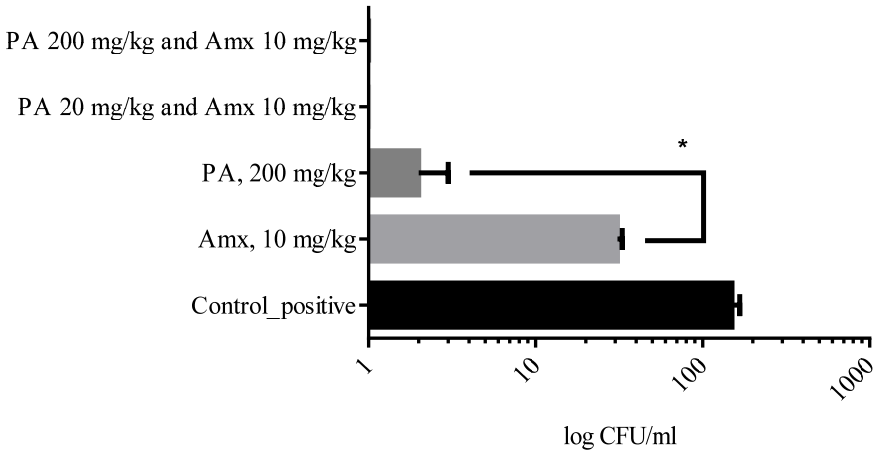

Figure 4 – Effectiveness of *K. pneumoniae* eradication from mouse blood on day 7 after PA and Amx treatment. The treatment conditions are presented on the y-axis, the number of bacteria converted to $\log_{10}$ CFU unit per ml of blood on the x-axis.
Note: *Statistical significance compared with Amx monotherapy at a level of p<0.05

On 7th day after therapy, the *K. pneumoniae* was not detected in the lungs and blood of experimental animals from the PA and Amx treatment groups. The post-treatment effect is probably associated with prolonged elimination of iodine from the body. Even after a single dose of 70 mg NaI, iodide levels did not achieve baseline even on day 3. At the same time, the effectiveness of the action of iodides was against *Mannheimia haemolytica* and *Bibersteinia trehalosi* insignificant at concentrations of 500 μM and below [19]. Only when combined with an antibiotic is a significant effect achieved. This can be explained by the effect of molecular iodine on bacterial resistance, as was shown earlier *in vitro* [20]. Also this effect can be explained because an increase in the concentrations of both iodides and antibiotics.

The results of measuring the concentration of amoxicillin and PA. The concentration of antibiotic and PA in iodides form (Table 2) was measured after 30, 60 and 120 min on the last day of administration of the test items.

Table 2 – Concentrations of amoxicillin and iodide in infected mice

Groups	Concentration of Amx, mg/L			Concentration of I, mg/L		
	30 min	60 min	120 min	30 min	60 min	120 min
Amx 10 mg/kg	3.88±0.34	1.34±0.01	0.33±0.01	-	-	-
PA 20 mg/kg and amx 10 mg/kg	7.92±0.51*	1.69±0.03	1.37±0.09*	9.61±3.99	14.16±4.05	10.01±7.05
PA 200 mg/kg and amx 10 mg/kg	9.43±0.72*	1.73±0.20	1.06±0.06	56.09±6.27	103.36±33.31	70.59±9.28

Note: Significant difference from the amx group ($p < 0.05$).

As shown in Table 2, the concentration of antibiotic increases with increasing dose of PA. Moreover, the concentration of the antibiotic increased twofold or more. These results suggest the possibility that the antibiotic may work by increasing bioavailability when exposed to PA. In this case concentration of iodides in the blood was in the range from 74.5 to 801.2 μM (56.09 – 103.36 mg/L). This can explain the effectiveness of PA therapy in combination with antibiotic. The same therapeutic effect is obtained when using green tea extracts or mucoactive drugs [21, 22]. It should be noted that iodide concentrations above 1 μM have a protective effect on cells in severe inflammation, when a huge amount of HClO is generated [23]. The administered iodine dose is high enough, but does not exceed LOAEL – 250 mg/kg (the lowest concentration of a chemical used in a toxicity test that has a significant adverse effect on the exposed population of test organisms compared with the controls) [24].

Conclusion

One of the approaches in the treatment of antibiotic-resistant infections is a combination of drugs. It can be both antibiotics and substances that do not have a direct antimicrobial effect. For example, beta-lactamase inhibitors, which break down beta-lactam antibiotics but do not inhibit bacterial growth. Another approach is based on combination of antibiotics with substances that affect pharmacokinetics. This results in an increased bioavailability of the antibiotic. The third way would be to change the susceptibility of resistant bacteria to antibiotics. Also, an increase in the effectiveness of anti-infective therapy can be achieved by influencing the components of the immune system, for example, by increasing phagocytosis. Among such drugs that affect both the sensitivity of bacteria to antibiotics and those that enhance phagocytosis are various iodine complexes. In this work, we presented the results of a study of the combination of a new iodine complex and amoxicillin in mouse lung infection caused by the multi-resistant *K. pneumonia* ATCC 700603 strain. Amoxicillin at a dose of 10 mg/kg was injected intramuscularly, and PA at a dose of 20 and 200 mg/kg was injected intragastrically. The maximum efficacy was found with a combination of 200 mg/kg PA and 10 mg/kg amoxicillin. A decrease in the level of bacterial load occurs in the lungs and blood. At the same time, there is also an increase in the concentration of the antibiotic in the

distribution phase (30 min), which may indicate an increase in bioavailability. In addition, 7 days after completion of therapy, animals in the groups receiving combination therapy experienced complete elimination of bacteria. Also, in the PA 200 mg/kg group, the level of bacteria in the lungs and blood was significantly lower than in the Amx 10 mg/kg group. That is, *K. pneumonia* ATCC 700603 retained its antibiotic resistance at all times. We believe that the effectiveness is achieved not only through an increase in susceptibility of bacteria, as shown earlier, but also through an increase in bioavailability of antibiotic. Thus, it can be concluded that the new complex compound of iodine has a potentiating effect on the antibiotic in lung infections caused by the multi-resistant *K. pneumonia* ATCC 700603 strain.

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