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TO THE MECHANISM OF ANTIBACTERIAL ACTION OF COLLOIDAL SILVER: CHANGES IN MEMBRANE-ASSOCIATED PROTON ATPASE ACTIVITY IN *ESCHERICHIA COLI* AND *ENTEROCOCCUS FAECALIS*

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It has been shown that colloidal silver (Ag nanoparticles, Ag NPs) significantly inhibited membrane-associated ATPase activity of *Escherichia coli* ATCC25922 and *Enterococcus faecalis* ATCC 29212, which was enhanced in the presence of *N,N'*-dicyclohexylcarbodiimide, inhibitor of the proton F_0F_1-ATPase. The effects depended on concentration of Ag NPs. It is suggested that the F_0F_1-ATPase, a key enzyme of bacterial membranes, could be a target for Ag NPs, and alterations of its functions might be a mechanism for antibacterial action of these NPs.

Silver nanoparticles – antibacterial action – bacteria – proton ATPase – N,N'-dicyclohexylcarbodiimide

Ցույց է տրվել, որ կոլոիդային արծաթը (Ag նանոմասնիկները) զգալիորեն արգելակում է *Escherichia coli* ATCC25922-ի և *Enterococcus faecalis* ATCC 29212-ի թաղանթներին կապակցված ԱԵՖ-ազի ակտիվությունը, որը ուժեղացվել է պրոտոնային F_0F_1-ԱԵՖազի ապգելակիչ *N,N'*-երկցիկլոհեքսիլկարբոերկիմիդի առկայությամբ: Արդյունքները կախված էին Ag նանոմասնիկների կոնցենտրացիայից: Ենթադրվում է, որ բակտերիային թաղանթների հիմնական ֆերմենտ F_0F_1-ԱԵՖազը կարող է լինել Ag նանոմասնիկների թիրախ, և դրա գործառույթների խանգարումը կարող է լինել այդ նանոմասնիկների հակաբակտերիային ներգործողության մեխանիզմը:

Արծաթի նանոմասնիկներ – հակաբակտերիային ներգործողություն – բակտերիաներ – պրոտոնային ԱԵՖազ – N,N'-երկցիկլոհեքսիլկարբոերկիմիդ

Было показано, что коллоидное серебро (наночастицы (НЧ Ag) значительно ингибирует мембраносвязанную АТФ-азную активность *Escherichia coli* ATCC25922 и *Enterococcus faecalis* ATCC 29212, которая усиливается в присутствии дициклогексилкарбодиимида ингибитора протонной F_0F_1-АТФазы. Эффекты зависели от концентрации НЧ Ag. Предполагается, что F_0F_1-АТФаза, ключевой фермент бактериальных мембран, может быть мишенью для НЧ Ag, а подавление ее функций – механизмом антибактериального действия этих НЧ.

Наночастицы серебра – антибактериальное действие – бактерии – протонная АТФаза – N,N'-дициклогексилкарбодиимид

The growing resistance of pathogens to antibiotics requires the development of alternative approaches. It is necessary to search for compounds with high biological activity, which could successfully compete with antibiotics and chemotherapeutic agents for their antibacterial properties.

Currently, materials of small (nano) size are considered promising, among which much attention is paid to nanoparticles (NPs) of transition metals and, in particular, silver (Ag) [14, 17]. The effects of these nanoparticles (NPs) on various microorganisms attract much attention, since NPs can be used as an alternative to antibiotics [14, 17]. The antibacterial effect of colloidal Ag on various Gram-negative and Gram-positive bacteria, including *Escherichia coli* and *Enterococcus faecalis*, has been shown [8, 11, 14]. Here numerous factors such as structure, shape, size of the NPs, synthesis reaction, type of stabilizer, etc., are important, which can lead to different effects [14, 17]. Moreover, these NPs can suppress antibiotic-resistant strains of *E. coli* [4]. It is interesting that Ag NPs can be used for protection of dairy products [1] and cleaning drinking water [8], as shown.

The mechanisms of inhibitory action of Ag NPs on bacteria, however, remain unclear. It is assumed that Ag NPs can adsorb on the surface of bacteria, penetrate cell walls and plasma membranes into the cell, alter their structure and functions and bind to proteins and nucleic acids [4, 14, 16]. Some changes in membrane transport and membrane-associated enzymatic activity, especially proton ATPase activity, the key enzyme of bioenergetic value [2, 3, 9, 13], are suggested. These ways can be dramatic ones leading to cells damaged and died.

Therefore, the aim of the present study was to investigate the activity of the membrane-associated proton ATPase under the influence of Ag NPs on *E. coli* and *E. faecalis*. This would lead to revealing some mechanisms of antibiotic action of Ag NPs.

Materials and methods. *Colloidal silver, bacteria and nutrient media, membrane vesicles.* The studies used co3loidal Ag, known under the trade name “Silverton” (“Tonus-Les” Lab, Armenia), obtained by electrochemical synthesis [5]. The concentration of colloidal Ag was determined by the flame method (acetylene - air) using an atomic absorption spectrophotometer (Shimadzu series AA-7000 / AAS, Japan).

Bacteria *E. coli* ATCC 25922 and *E. faecalis* ATCC 29212, wild types, from the American Type Culture Collection (ATCC) were used. Bacteria were grown in peptone (Roth, Germany) or triptone (Roth, Germany) medium, glucose (20 mM) was added, under anaerobic conditions at pH 7.3 and 7.0 and 37 ^{0}C to the stationary phase, as described earlier [3, 4, 9, 13-15].

Membrane vesicles were obtained by lysing cell using lysozyme (Roth, Germany) with ethylenediaminotetraacetic acid (10 mM) and subsequent osmotic shock according to the method of Konings and Kabak [6] The amount of protein in membrane vesicles was determined by the method of Lowry [7] using serum bovine albumin (Sigma, USA) as a standard.

Determination of ATP-ase activity of bacterial membranes. Membrane-associated ATPase activity was determined by the amount of inorganic phosphate (P_{inorg}) that was released after membrane reaction of membrane vesicles (50-90 μg protein/ml) with ATP (Tris-salt, Sigma, USA) (3 mM) by Tausky and Shorr's colorimetric method [12] using a spectrophotometer (GENESIS 100, Thermo Scientific, Germany) [3, 13, 15]. The assay mixture was 50 mM Tris-Cl buffer (pH 7.5), containing 1 mM $MgSO_4$ and 100 mM KCl. The corrections were made for blanks without ATP or membrane vesicles. ATPase activity was expressed in nmol P_{inorg} /min per μg protein.

To determine the ATPase activity of the proton F_0F_1-ATPase, *N,N'*-dicyclohexylcarbodiimide (DCCD) (Sigma, USA), which is an inhibitor of this ATPase in the bacteria studied [2, 3, 9, 13], was used. The membrane vesicles were pre-incubated with DCCD (0.1 mM) for 10 min. DCCD was dissolved in ethanol, however small amount of this solvent (final concentration of <1 %) had no effect on ATPase activity.

It should be noted that the membrane vesicles obtained by the above method [6] are right-side-out and not largely accessible to ATP, but they serve as a convenient model for determining membrane-associated ATPase activity [3, 15], as it was shown that they determined up to 80 % of the total and F_0F_1-ATPase activity of inverted or in-site-out vesicles [3, 13].

Data processing. The results were processed statistically with the help of the Microsoft Excel 2013 program, while the reliable ones accepted the difference, when the Student's criteria of accuracy (P) was <0.05.

Results and Discussion. To study the effect of Ag NPs on ATPase activity of membrane vesicles, before introducing ATP, membrane vesicles were pre-incubated with different concentrations of colloidal Ag for 10 min at 37 °C, so that the final concentration of Ag NPs in solution was 10 mg/l or 20 mg/l.

The results obtained have shown that Ag NPs have a suppressive effect on the ATPase activity of *E. coli* membrane vesicles (fig. 1). With increasing concentration of NPs, an increase in the effect was observed (fig. 1). The inhibiting effect of Ag NPs was greatly enhanced after the addition of DCCD. Thus, the inhibitory effect of 10 mg/l Ag NPs in the presence of DCCD increased ~1.5-fold (fig. 1). The same was observed when DCCD was added to 20 mg/l Ag NPs; in this case membrane ATPase activity decreased by ~1.7-fold (fig. 1).

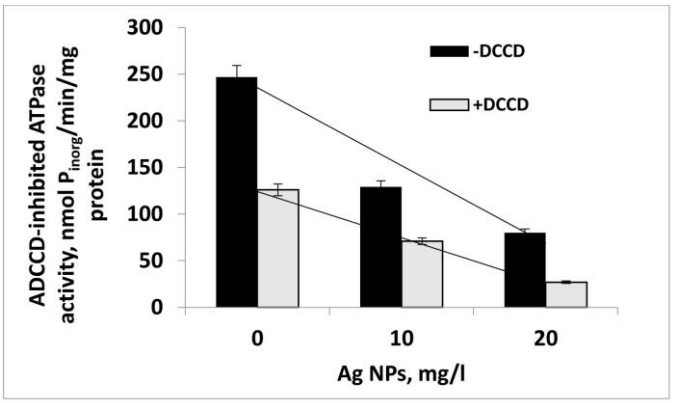

Fig. 1. ATPase activity of *E. coli* ATCC 25922 membrane vesicles treated with different concentrations of colloidal Ag, in the absence and presence of DCCD. For details, see Materials and methods.

In the case of *E. faecalis* ATCC 29212, the same effects have been observed as in *E. coli*, with the only difference being that in this case the effect of both Ag NP and the combined effect of Ag NP and DCCD were more pronounced (fig. 1). An increase of the inhibitory effect was observed in both cases: with elevated concentration of Ag NPs and in combination with DCCD (fig. 2). With *E. faecalis*, the suppressive effect of Ag NPs after the addition of DCCD was also enhanced. Thus, the suppressive effect of 10 mg/l Ag NPs in the presence of DCCD increased by ~1.9-fold (fig. 2), which was almost twice as much as in the case of *E. coli* with the same concentrations of Ag NP. The same was observed with a combination of 20 mg/l Ag NPs with DCCD; in this case, the ATPase activity of the membrane vesicles decreased by ~1.9-fold.

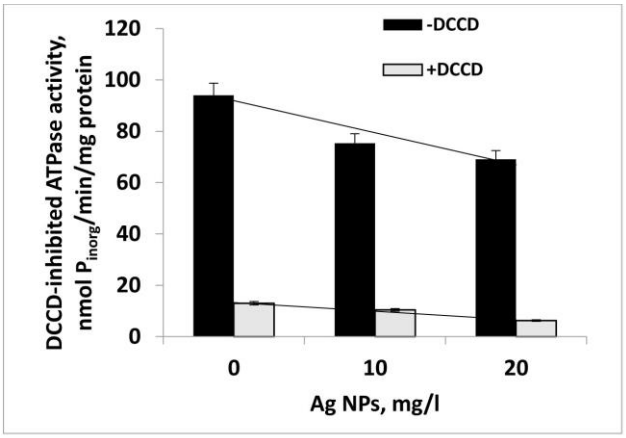

Fig. 2. ATPase activity of *E. faecalis* ATCC 29212 membrane vesicles treated with different concentrations of colloidal Ag (Ag NPs), in the absence and presence of DCCD. For details, see Materials and methods.

From the obtained results, the inhibition of the F_0F_1-ATPase activity of the membrane vesicles of both bacteria was calculated, caused by exposure to only DCCD (fig. 3). To determine the DCCD-sensitive ATPase activity of the membrane vesicles, calculated the difference between the ATPase activity values, caused by the exposure of Ag NPs, and the ATPase activity value, due to the combined exposure of Ag NPs and DCCD were calculated. From these results it can be seen that DCCD had an inhibitory effect on the F_0F_1-ATPase activity of both bacteria. Moreover, when processing membranes only by DCCD, the DCCD-sensitive ATPase activity was much greater than when processing membranes pre-treated with Ag NPs (fig. 3). It turned out that Ag NPs led to a decrease in DCCD-sensitive ATPase activity, but in combination with DCCD they caused a stronger inhibition of ATPase activity than each of these agents solely (comp. figs. 1-3). By increasing the concentration of colloidal Ag, the combined inhibitory effect on ATPase activity can be enhanced, and in the enhancement of the combined inhibitory effect, the proportion arriving at DCCD, on the contrary, decreased, which indicated that the gain was provided by increasing the concentration of Ag NPs. Besides, the concentration of 10 mg/l Ag NPs was optimal to significantly inhibit ATPase activity (fig. 3).

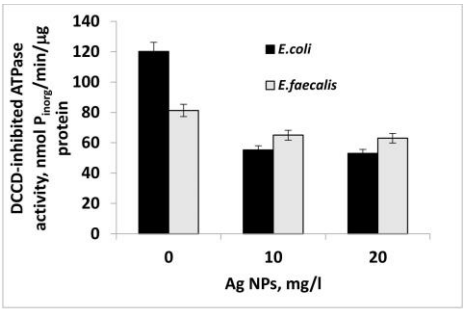

Fig. 3. DCCD-inhibited ATPase activity of *E. coli* ATCC 25921 and *E. faecalis* ATCC 29212 membrane vesicles treated with different concentrations of colloidal Ag (ag NPs). For details, see Materials and methods.

From this, it can be assumed that the mechanism of the antibacterial activity of Ag NPs may involve the same functional subunits of the F_0F_1-ATPase complex, or the same molecular mechanisms as those affected by DCCD were targeted. It is possible that Ag NPs, as well as DCCD, which interact with the "c" subunit of F_0 complex [2, 9, 13, 15], led to conformational changes, causing a decrease in membrane-associated ATPase activity.

It should be noted that in our work the idea was put forward that the memrane-associated proton F_0F_1-ATPase serves as an important target of the cell for many physical and chemical factors, such as the electromagnetic field of extremely high frequencies and antibiotics [10], and the alteration of its structure and activity can cause antibacterial action.

Thus, it can be concluded that Ag NPs significantly inhibit membrane-associated ATPase activity, including the F_OF_1-ATPase, which can lead to impaired membrane functions and death of bacterial cells.

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