9th International Conference on
Nanomaterials: Applications & Properties '2019
Odesa, Ukraine, 15-20 Sept. 2019

Morphological Changes in Gram-Negative Microorganisms Treated with Silver and Copper Nanoparticles

Yevhenia Husak
Medical Institute
Sumy State University
Sumy, Ukraine
e.gusak@med.sumdu.edu.ua

Viktoriia Holubnycha
Medical Institute
Sumy State University
Sumy, Ukraine
v.golubnychaya@med.sumdu.edu.ua

Viktoriia Korniienko
Medical Institute
Sumy State University
Sumy, Ukraine
v.kornienko@med.sumdu.edu.ua

Aziza Yusupova
Medical Institute
Sumy State University
Sumy, Ukraine
ms.aziza.yusupova@gmail.com

Petro Myronov
Medical Institute
Sumy State University
Sumy, Ukraine
p.myronov@med.sumdu.edu.ua

Anton Savchenko
Medical Institute
Sumy State University
Sumy, Ukraine
sava20077@ukr.net

Viktoriia Ivchenko
Sumy National Agrarian University,
Sumy, Ukraine
ivchenkovd@gmail.com

***Abstract* — Bacterial resistance became the crucial problem in current medicine. Due to this searching for new agents with antibacterial properties is a key point. Nanoparticles possess antibac-terial as well as many other unique features that can help to solve this problem. The most promising among them is Cu NPs and Ag NPs.**

The morphological changes in K. pneumonia and E. coli strains after their treatment with Cu NPs and Ag NPs were examined by scanning electron microscopy (SEM).

Silver and cooper nanoparticles cause significant changes in the Gram-negative bacteria structure that are manifested as shortening of the bacteria cells, disruption of the cell wall surface and violation of cell divisions.

Keywords — copper, silver, nanoparticles, antimicrobial activity, bacterial cell wall.

I. Introduction

Nowadays infections caused by bacteria still stay a main reason of the mortality and morbidity worldwide. *Klebsiella pneumoniae* and *Escherichia coli* are the most common Gram-negative bacteria cause sepsis and hospital acquired infections [1]. Crucial role of antibiotics in fight with infection diseases has been destroyed due to appearance and distribution of antibiotic-resistant bacterial strains. Antibiotic resistance is caused by formation of several protective mechanisms in microorganisns [2].

Nanoparticles have a wide application in medicine due to its unique chemical and physical properties. The main particularities of the nanoparticles action are their low toxicity and few antibacterial mechanisms activation at the same time. The substantial rise of their application as potential antibiotic substitute is indicated previously [2, 3]. However, the size, structure, shape, surface properties, particle agglomeration and optical characteristics of NPs influence on its antimicrobial properties.

The precise antibacterial mechanism of the metallic NPs action is poorly understood. There are few common hypothesis of NPs antibacterial activity: 1) their influence on bacterial membrane permeability, 2) oxidative stress induction, and 3) activation of intracellular antibacterial effects [3].

The Cu and Ag NPs are a promising alternative to the drugs with antibacterial properties. Moreover, cooper is essential chemical element that stimulates metabolic processes, enzyme activity, and growth of the organism. Some studies reported the mechanism of Cu-NPs bactericidal activity. Raffi et al and Ruparelia et al. [4] suggested that Cu ions originating from the NPs may interact with phosphorus and sulfur-containing biomolecules such as DNA and protein to distort their structures and thus disrupt intracellular biochemical processes. The mechanisms of AgNPs antibacterial activity is more often discussed issues rather than antibacterial action of other types of nanoparticles [5]. Two antibacterial mechanisms are widely accepted for it, namely contact killing and ion-mediated killing.

Due to all listed above studying of the antibacterial activity and mechanism of Ag NPs and Cu NPs action against Gram-negative bacteria is a topical issue of the present time.

II. Materials and Methods

2.1 Materials

Silver nitrate ($AgNO_3$) (impurity > 99,9%, p.a.), polyvinylpyrrolidone (PVP — K25, MW 24000), ethylene glycol (EG) (impurity > 99,9%), sodium hypophosphite monohydrate ($NaH_2PO_2 \cdot H_2O$), copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), isopropyl alcohol (99% pure, p.a.) were used as raw materials received from Sigma-Aldrich. Distilled water from electric distiller DE 20 was used throughout the experiments. Nutrient broth and nutrient agar were purchased from Hi Media India.

978-1-7281-2830-6/19$31.00 ©2019 IEEE

2.2 Synthesis of Cu NPs and Ag NPs.

Copper and silver nanoparticles were synthesized by the methods described early [8]. After that each type of nanoparticles was treated with low-frequency ultrasound (22±1,65 kHz) for 1 minute.

2.3 Cu NPs and Ag NPs characterization

Entire characteristic of the nanoparticles was done with use X-ray diffraction, SEM, transmission electron microscope (TEM), UV-VIS spectroscopy, EDS elemental analysis and total results are reflected in our previous works [6, 7]. Concentration of NPs in the aqua solution was determined by the method of inductively-coupled plasma atomic spectrometry (ICP-AES) using an iCAP 6300 Duo spectrometer (Thermo Scientific Corporation, USA).

2.4 Antibacterial assessment

The mechanism of NPs antimicrobial activity was examined against referents strains of *K. pneumonia* and *E. coli.* The minimum inhibitory concentration (MIC) of NPs obtained in early investigation [6, 7] was measured by tube serial dilution method according to the international recommendations provided by the Clinical and Laboratory Standarts Institute (CLSI). The MIC of the Ag NPs and Cu NPs were 10.0 μg/ml and 0.5 μg/ml respectively. Before investigation of the morphological changes isolates were routinely cultivated overnight in nutrient broth at 37 °C. Then the cultures were diluted with cultivation media to the turbidity equivalent to McFarland 0.5 standard ($1.5x10^8$ CFU/ml). It was used as an inoculum. Concentrations of NPs equivalent to MIC were added into glass tubes containing 2 ml nutrient broth with microorganisms. The tubes containing growth medium and microorganisms without NPs were used as controls. After incubation at 37 ºC for 24 h bacterial suspensions were collected by centrifugation. The bacterial cells were fixed in 2,5% glutaraldehyde with 0.1M phosphate buffer pH 7.2 twice during 15 min. Then after 3 washes in buffer the samples were dehydrated with series of 40, 50, 60, 70, 80, 85, 90, 95, and 100% ethanol and stored in 100% ethanol. Finally, 2 μl of each sample was placed on 1mm clean and defatted glass slides. The glass slides were coated with silver and examined under SEM

The cell morphology of the tested microorganisms was examined by scanning electron microscopy (SEM). Cell dimensions were measured directly from the SEM images to calculate the cell volumes by the following equation:

$$V(\mu m^3) = \pi W^2 L/4 + \pi W^2 R/3,$$

When, W and L mean the width and length, respectively, of the central part of the cylindrical cell, and R was the equatorial radius of the spheroid caps at both ends of the cylinder. Average cellular volumes were calculated by using 30 individual bacteria per population [8].

2.4 Statistic

Data were expressed as means ± standard deviation. Student's t-test on unpaired data was used to assess the statistical significance of the difference. Statistical significance was assumed at a confidence level of 95% ($p < 0.05$).

III. RESULTS

Scanning electron micrographs of *Klebsiella pneumonia* and *Escherichia coli* are presented on Figure 1 and 2.

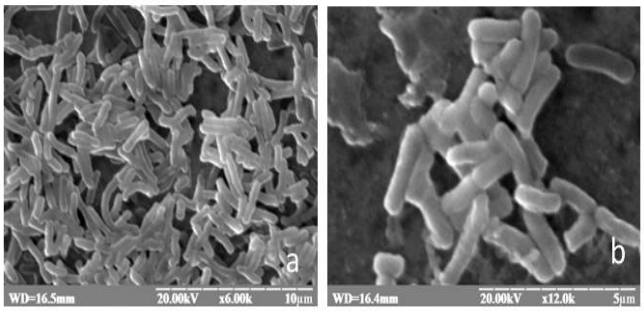

Fig. 1. Scanning electron microscopy images *K. pneumoniae* (a) and *E.coli* (b) in control

As it is shown on Figure 1 the cells in group of control were rod-shaped with smooth and intact cell walls.

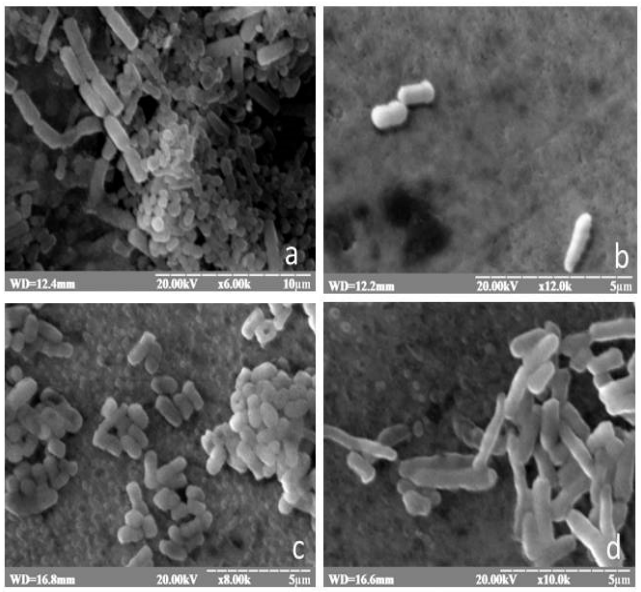

Fig. 2. Scanning electron microscopy images of *K. pneumoniae* in the presence of Cu NPs (a), Ag NPs (b) and *E.coli* in the presence of Cu NPs (c), Ag NPs (d).

At the same time, the morphology of the microorganisms treated with NPs (Figure. 2) was changed dramatically and there were a lot of coccobacilus bacteria after treatment with Cu NPs.

As it seen on the picture 2 the cell surface became rough with thickening on the cell ends after cultivation of the microorganisms with Ag NPs. The following results (tabl.1) provide further evidence of the bacterial cell damage with nanometals.

TABLE I. THE MEAN CELL SIZES OF THE *K. PNEUMONIAE* POPULATIONS. MEAN VALUE ± STD. ERROR.

	Width (μm)	**Length (μm)**
Contr	0.4413±0.0571	1.9994±0.6219
NPs Cu	0.3272±0.0939	0.8028±0.1705
NPs Ag	0.5073±0.0737	1.2652±0.2665
	Radius (μm)	**Volume (μm^3)**
Contr	0.2479±0.0352	0.3561±0.001712
NPs Cu	0.3272±0.0341	0.1041±0.001495
NPs Ag	0.2423±0.0407	0.3208±0.001368

TABLE II. THE MEAN CELL SIZES OF THE E. COLI POPULATIONS. MEAN VALUE ± STD. ERROR.

	Width (µm)	Length (µm)
Contr	0,4916±0.0708	1,9350±0,3904
NPs Cu	0,5947±0,1119	1,0665±0,3665
NPs Ag	0,5060±0,0881	1,8103±0,6829
	Radius (µm)	**Volume (µm^3)**
Contr	0,2770±0,0539	0,4371±0,0018
NPs Cu	0,3292±0,0388	0,4179±0,0041
NPs Ag	0,2786±0,0299	0,4385±0,0210

As it is reflected in the tables 1, 2 the untreated cells of *K. pneumoniae* and *E. coli* were corresponded normal size of these microorganisms. It was about 1.9994±0.6219 µm and 0.8028±0.1705µm respectively.

Dramatic shortening of the *K. pneumoniae* cell after Cu NPs and Ag NPs treatment was detected. Length of microorganisms was respectively 0.8028±0.1705m and 1.2652 ± 0.2665 µm. Moreover, after *K. pneumoniae* incubation with Ag NPs significant decreasing of the of bacteria amount in the slide was found.

Similar changes were revealed after *E. coli* incubation with Cu NPs. The bacteria became shorter than in control group (1.0665 ± 0.3665 µm length). Incubation with Ag NPs did not cause significant influences on size of the cells but surface of the bacteria became more irregular and rough.

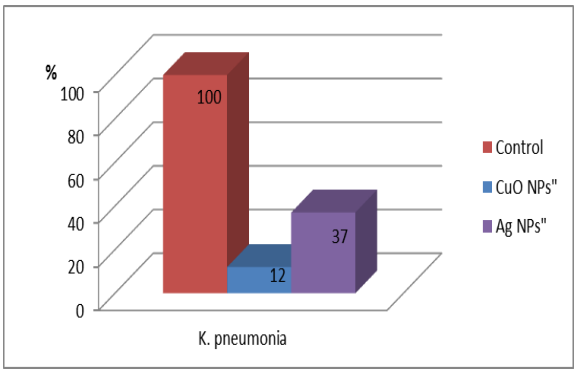

Fig. 3. Changes in volume of *K. pneumonia* experimental group compared to control strain, %.

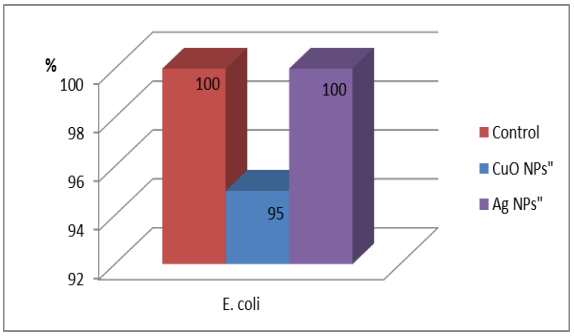

Fig. 4. Changes in volume of *E. coli* experimental group compared to control strains, %.

The cell volumes decreased in both groups with Cu NPs treatment (Fig. 3, 4). Moreover, substational drop of the *K. pneumonia* cell volume was revealed.

IV. DISCUSSION

Nowadays increasing number of nanoparticles using as a new alternative to antibiotics [9]. The numbers of studies on the use of metal nanoparticles with antimicrobial properties rises permanently [10].

The study revealed noticeable changes in the microbial cell morphology after treatment with silver and cooper nanoparticles. These changes included alterations in size and shape. Composition of cell wall determines the cell shape. NPs actively influence on bacteria and damage biological structure (lipids, proteins, nucleic acids) [11]. Copper inhibits and disrupts protein and nucleic acid synthesis [12]. Consequently, the most suitable reason of the morphological changes in *K. pneumoniae* and *E. coli* cell after treatment with NPs is chemical modification of the bacterial cell wall structure.

There was revealed a little number of *K. pneumoniae* in the slide after their incubation with Ag NPs and the chains formation in the slide with *K. pneumonia* treated with Cu NPs. Obviously, there can be different reasons. On the one hand used concentration of the silver caused whole destruction of the microorganisms and decreased their amount sharply. This assumption corresponds with other scientists data [9, 10] who indicated the violation of the metabolic properties in bacteria cell membranes, affection of the energy processes in the membrane and its barrier function after NPs treatment. On the other hand some authors indicate silver nanoparticles and Cu ions penetrate into the cell and cause further damage by possibly interacting with sulfur- and phosphorus-containing compounds DNA. Due to this we observed the formation of the cell chain.

V. CONCLUSION

Silver and cooper nanoparticles cause significant changes in the Gram-negative bacteria structure that are manifested as shortening of the bacteria cells, disruption of the cell wall surface and violation of cell divisions.

ACKNOWLEDGMENT

This research supported by H2020 Marie Skłodowska-Curie Actions Grant NanoSurf 777926 and Ukraine MES Grant "Effectiveness of nanocomposite (chitosan-nanometal) antimicrobial action against multiresistant clinical strains" No 62.20.02-01.18/20.).

REFERENCES

[1] C. Elliott, A. Justiz-Vaillant, Int. Biol. Biomed. J., "Nosocomial Infections: A 360-degree Review," vol. 4, No 2, pp. 72-81, 2018.

[2] A. B. Michael Naafs, "Copper Nanoparticles and Antioxidant Stress: Problem Makers or Solvers?," Nutri. Food Sci., vol. 3, No 2, pp. 243-245, 2019.

[3] A. Sirelkhatim, et al., "Review on Zinc Oxide Nanoparticles: Antibacterial Activity and Toxicity Mechanism," Nano-Micro, Lett. vol. 7(3), pp. 219-242, 2015.

[4] N. Karikalan, "Synthesis and charaterization of copper nanoparticles and evaluation of antibacterial activity," Rasayan J. Chem., vol. 11(4), pp. 1451-1457, 2018.

[5] A. Kumar Chatterjee, "Mechanism of antibacterial activity of copper nanoparticles," Nanotechnology, vol. 25(13), p. 135101, 2014.

[6] V. Holubnycha, et al., "Effect of ultrasound treatment on chitosan-silver nanoparticles antimicrobial activity," NAP-2018, IEEE 8th International Conference on Nanomaterials: Applications & Properties, 2018, p. 04NNLS09(4), 2018.

[7] V. Holubnycha, et al., "Antibacterial activity of the new copper nanoparticles and Cu NPs/chitosan solution," NAP-2017, IEEE 7th International Conference on Nanomaterials: Applications & Properties, pp. 04NB10(4), 2017.

[8] Bihong Shi, Xuhua Xia, "Morphological Changes of Pseudomonas pseudoalcaligenes in Response to Temperature Selection," Curr. Microbiol. vol. 46(2), pp. 120-23, 2003.

[9] Linlin Wang, Chen Hu, and Longquan Shao, "The antimicrobial activity of nanoparticles: present situation and prospects for the future," Int. J. Nanomedicine, vol. 12, pp. 1227-1249, 2017.

[10] Yun'an Qing, et al. Int. J. Nanomedicine, "Potential antibacterial mechanism of silver nanoparticles and the optimization of orthopedic implants by advanced modification technologies," vol. 13, pp. 3311-3327, 2018.

[11] Jose Ruben Morones-Ramirez, et al., "The Bactericidal Effect of Silver Nanoparticles," Nanotechnology 16(10), pp. 2346-2353, 2005.

[12] Qing Ling Feng, "A mechanistic study of the antibacterial effect of silver ions on Escherichia coli and Staphylococcus aureus," J. Biomed. Mat. Res., vol. 52(4), pp.662-668, 2000.