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Investigating the Susceptibility and Death Kinetic of Pseudomonas Aeruginosa Bacterium Standard and Clinical Strains to the Copper Oxide Nanoparticle

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ABSTRACT

Microbial resistance is considered as one of the challenges in coping with infectious diseases. Thus, to find or synthesize new antimicrobial substances is very important. Copper oxide (CuO) has drawn the attention of the researchers due to its antibacterial effect in coping with microbial resistance. In this research, the antibacterial effects of copper oxide nanoparticles on the standard and clinical strains of Pseudomonas aeruginosa, which its new strains have been associated with microbial resistance, were examined and compared. In this experimental-interventional research, a copper oxide nanoparticle was synthesized at a size of 33 nm using copper sulfate chemical reduction method. Then, the antibacterial effects of copper oxide nanoparticle on standard strain (ATCC 27853) and clinical strain of Pseudomonas aeruginosa were examined using minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and bacteria death kinetics. Data derived from investigating the bacteria death kinetics were analyzed using statistical tests at a significant level of p value (<0.05).
Results: The MIC obtained in the standard strain and clinical strain with 33 nm nanoparticle was 5000 mg / ml and 2500 mg / ml, respectively. In addition, the obtained MBC in the bacterial standard strain and clinical strain with 33 nm nanoparticle was ≥ 10,000 IU / ml and ≥5000 IU / ml, respectively. The effect of nanoparticle on each of the strains was at significant level compared to control group. However, the difference between the effects of nanoparticle on strains in comparison to each other was not at the significant level. The current research revealed that the copper oxide nanoparticle had an Impressive Inhibitory effect on the standard and clinical strains of Pseudomonas aeruginosa, depending on nanoparticle's concentration and size. Thus, studies conducted in this area provide appropriate conditions to achieve new antibacterial substances.

Key words: Copper Oxide Nanoparticle, Pseudomonas Aeruginosa, MIC, MBC, Bacterial Death Kinetics

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INTRODUCTION

Gram-negative bacteria are increasingly resisting to most of the antibiotics. Pseudomonas aeruginosa is one of the gram-negative bacteria, which become resistant to the antibiotics. This bacilli bacterium is a gram negative and opportunistic bacterium, which can live in all environments and it is involved in many of human infections, including endocarditis, septicemia, meningitis and chronic lung infections in patients with cystic fibrosis. Pseudomonas aeruginosa is one of the serious pathogens agents in hospital infections and cause of death in people with leukemia and severe burns. This bacterium is directly associated with antibiotic resistance, so

that nearly 30% of hospital infections are related to the antibiotic resistance of this bacterium [1,2 and 3]. Hence, to find and synthesize new antimicrobial substances to cope with antibiotic resistance is very important. Therefore, nanoparticles have drawn the attention of researchers nowadays. Due to their physicochemical properties, resulting from their small size and high specific surface area, they have significant antimicrobial effects [4]. Nanoparticles are able to fill the gaps caused by failure of antibiotics, which includes coping with multidrugs resistant bacterial mutants and bacterial biofilms. Nowadays, metal nanoparticles, metal oxide, and organic nanoparticles are used to achieve this goal. Special conditions of bacterium, such as cell wall, metabolic pathways, or physiological conditions of planktonic, biofilm, or growth rate influence the impacts of nanoparticles on target bacterium. In general, nanoparticles destroy the target bacterium by leaving destructive impact on the bacterium cell membrane, its integrity, and production of reactive oxygen species (ROS). Nanoparticles in fact, act as nanocatalyst [5]. Copper oxide (CuO) has recently been regarded as an antimicrobial substance and copper oxide nanoparticle have been studied to improve its antibacterial properties. Bactericidal properties of nanoparticles depend on their size, stability, and concentration used as an antibacterial substance. A few studies have been carried out so far on the antimicrobial properties of copper oxide and its action. However, reports suggest that copper oxide nanoparticles have the potential to be used as an antibacterial agent. Copper oxide is cheaper than silver compounds and it has appropriate physical and chemical stability. Highly ionic nanoparticles of copper oxide have the potential to be used as antimicrobial agents and they can be synthesized in crystalline and with high special surface morphologies [6,7]. In a research carried out by Ahmad (et al..2014) on the synthesis of copper oxide nanoparticle and its antimicrobial effect, it was revealed that 24_{nm} copper oxide nanoparticles have very good antimicrobial effects on various bacterial strains [8]. Shaffiey (et al..2014) also examined the antibacterial effects of copper oxide nanoparticles on Aeromonas hydrophila bacterium and reported that copper oxide nanoparticles have the potential to be used as an antimicrobial agent against the Aeromonas hydrophilia bacterium [9]. The objective of this research was to investigate and compare the antibacterial effects of copper oxide nanoparticles as a new antibacterial substance based on different concentrations on standard and clinical strains of gram-negative bacterium of Pseudomonas Aeroginosa, given the increasing resistance of gram-negative bacteria and its challenges.

MATERIALS AND METHODS

2-1-Materials and strains of the bacteria investigated

This experimental-interventional research was conducted and under the supervision of the Ethics Committee of Kermanshah University of Medical Sciences. In this research, standard strain (ATCC: 27853) and clinical strain of Pseudomonas aeruginosa and culture media, Mueller Hinton Agar, nutrient broth, BHI, Tryptic Soy Broth, Peptone Water and brain agar were used. All culture media were prepared from **Merck** Company (Merck-Germany).

2-2-Synthesis and investigation of copper oxide nanoparticle properties

2-2-1-Synthesis of copper oxide nanoparticle: To prepare aqueous solution of copper colloid, a one-stage reduction chemical method was used by Han (et al.. 2011) [10]. For this purpose, 0.25_{gr} of copper sulfate ($CuSO_4.5H_2O$) was dissolved in 100_{ml} distilled water. Then, 5_{gr} of Polyvinylpyrrolidone (PVP-K30) (Merck-Germany) was dissolved in the prepared aqueous solution. $NaBH_4$ was used to reduce the copper oxide. Accordingly, 0.25_{gr} of this substance was added to the mentioned aqueous solution under stirring conditions and at the environment temperature and atmosphere. At the beginning of the process, the copper sulfate solution changed to a green color, and finally the color of solution got brown. After keeping the mixture for 30 minutes, ascorbic acid at the required amount was added to the mixture and kept for 30 minutes at 60°C. After completing the copper oxide reduction process, the solution was rinsed with ethanol and centrifuged at 4000_{rpm} to collect the copper colloid. Different values of ascorbic acid were used to control the size of copper oxide nanoparticle.

2-2-2-Investigating the nanoparticle properties: To investigate the size and morphology of copper oxide nanoparticles, Zeta Sizer device (Malvern zeta sizer nano-25) and scanning electron microscope (SEM) (MIRA3-TESCAN) were used.

2-3-Tests to determine the antimicrobial susceptibility

2-3-1-Disk diffusion test: To perform this test, 24-hour culture of standard strains and clinical strains of the bacterium of Pseudomonas aeruginosa was performed at BHI medium (Merck-Germany) at 37°C. Then, the turbidity of the culture of bacterial strains was regulated by using 0.5 McFarland standard method (Bahar Afshan-Iran) and by using a spectrophotometer (Jenway 6305) at a 600_{nm} wavelength so that 1.5×10^8 CFU/ml to be obtained from each bacterial strain. Then, turbidity obtained from the bacteria strain was cultured separately by using sterile swab on Mueller Hinton agar (Merck-Germany). In the next stage, $20_{\mu l}$ synthesized nanoparticle was loaded on the 6_{mm} sterile standard disks (Padtan-Iran) in 8_{cm} plates containing Mueller Hinton agar culture medium. Unloaded disks (Padtan-Iran) were also used for control. Then, the discs were placed on a culture medium of Mueller Hinton agar, which bacteria had been cultured on it, and then, they were incubated for 37°C for 24 hours. After 24 hours, the diameter of the inhibition zone formed around the disks loaded with the nanoparticles was measured using caliper and reported in Mm unit.

2-3-2-Agar well diffusion test: Broth Nutrient Medium (Merck-Germany) was used to prepare bacterial suspension with concentration of 0.5 McFarland. Then, the bacterial strains were cultured on Mueller Hinton Agar medium (Merck-Germany), and a well was created by using a sterile punch inside the plate. Each well with 10 $_{mg/ml}$ of nanoparticle was inoculated on separate plates and the wells containing Dimethyl Sulfoxide (DMSO) (Merck-Germany) were prepared as control. Plates were incubated at 37°C for 24 hours. After 24 hours, the diameter of the inhibition zone formed around the wells containing the nanoparticle was measured using caliper and reported in Mm unit.

2-4-Test to determine minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Broth Microdilution MIC Testing method was used to determine MIC and MBC. In this method, 96-wells micro-plates (SPL-South Korea) were used based on the model proposed by **Raeisi** (et al..2016) [11]. Each of the two strains of the bacterium of Pseudomonas aeruginosa was separately cultured 18 hours before testing in the liquid BHI medium (Merck-Germany) and incubated at 37°C. Then, BHI and agar culture media (Merck-Germany) were prepared. Different concentrations of copper oxide nanoparticles were prepared by using the Tryptic Soy Broth medium (Merck-Germany). To regulate the level of bacterial inoculum, the number of 1.5×10^{8} $_{CFU/ml}$ was obtained from the studied bacteria by using the 0.5 McFarland method. Then, to confirm at the specified time, OD of the cultured bacteria was read using a spectrophotometer. To achieve 10^{6} $_{CFU/ml}$ bacterial inoculation, dilution through Peptone water medium (Merck-Germany) was used. In the next stage, dilutions 2 to 7 times more than the dilution of the main solutions of copper oxide nanoparticle were prepared in tubes containing BHI (78 to 10000 $_{mg/ml}$) to determine MIC and MBC. First, $160_{\mu l}$ BHI, $20_{\mu l}$ of prepared concentrations of copper oxide nanoparticle, and $20_{\mu l}$ of bacterial inoculation were added for each bacterial strain separately for micro-plate wells. Accordingly, the volume of contents in the wells was $200_{\mu l}$ and considering the creation of dilution of 0.1 in the wells, the concentration of substances in the well was diluted by10 times, and the approximate number of bacteria in the wells reached to $10^{5}_{CFU/ml}$. For positive control, $200_{\mu l}$ of BHI medium and nanoparticle and for negative control, $180_{\mu l}$ of BHI medium along with $20_{\mu l}$ of bacterial inoculation were added to wells. The micro-plates were shaken for 30 seconds at 300 $_{rpm}$ in a plate thermo-shaker device and incubated for 37 hours at 37°C. After the incubation, the wells were examined in terms of creation- or non-creation of turbidity and the first transparent well was considered to be MIC and the second one was considered to be MBC well.

2-5-Investigating the bacterial death kinetics in broth medium:

In order to investigate the lethal effects and lethal rate of nano-emulsions, 8_{ml} of BHI broth medium was poured in four tubes, and 1_{ml} of 18-hour culture of the bacterial strain was inoculated into the BHI medium. Then, 1_{ml} of copper oxide nanoparticle solution with dilutions of 0.1, 0.01 and 0.001 was added to three tubes separately. Physiology serum was added to the fourth tube as control. Then, media were incubated at 37°C with about 150_{rpm} and each of the considered concentrations was diluted at intervals of 15, 30, 45 and 60 minutes and cultured on plates containing BHI agar medium. Then, the cultured plates were incubated for 24 hours at 37°C. After incubation, the number of survived bacteria was counted using the counter colony and the bacterium death time curve was plotted. This test was performed in three replications for each bacterial strain separately and standard deviations of values were calculated (p value <0.05).

2-6-Statistical tests: Independent sample T test and one way ANOVA tests were used to analyze the results, and in the case of a significant (p value <0.05), Scheffe test was used. For each of the samples, the culture and counting were performed 3 times and the mean logarithm of the number of bacteria was obtained separately and reported as mean ± SD at each of the sampling times.

RESULTS

3-1-Investigating the size and morphology of copper oxide nanoparticles: in investigating the size of copper oxide nanoparticles synthesized by using Zeta Sizer device, as shown in (Figure-1), showed that using a specified value of ascorbic acid, copper oxide nanoparticle was synthesized with approximate size of 33_{nm}. Scanning Electron Microscope (SEM) investigations also suggest the particle and appropriate morphology of this nanoparticle (Figure-2).

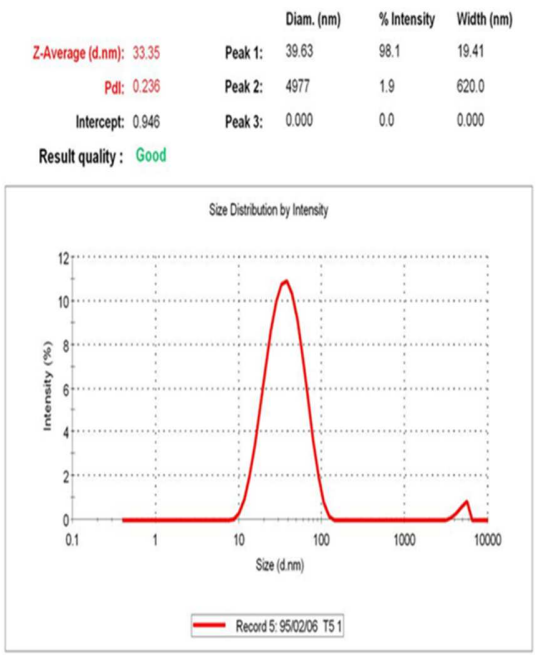

Figure 1. Determining the size of 33nm nanoparticle by a Zeta Sizer device

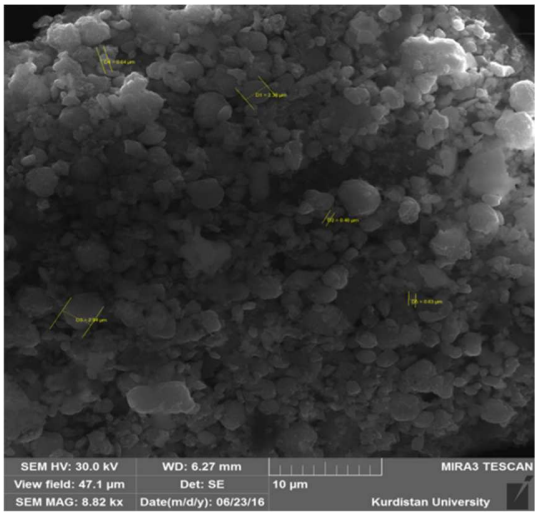

Figure 2. Morphology of copper oxide 33 mm nanoparticles by SEM microscope

3-2-Disk diffusion test and well diffusion test results: The mean diameter of inhibition zone resulting from treatment of bacterial strains with 33_{nm} nanoparticle in the disk diffusion test for standard and clinical strains was $8\pm1_{mm}$ and $9.5\pm1_{mm}$, respectively, and these values in the well diffusion test for standard and clinical strains were $13\pm1.5_{mm}$ and $19\pm1_{mm}$, respectively.

3-3-MIC and MBC test results: The data obtained in determining the MIC and MBC show that copper oxide nanoparticle has a significant antibacterial effects on the clinical strain compared to standard strain. Table-1 shows this difference.

Table1. MIC and MBC test results on standard and clinical strains of Pseudomonas aeruginosa

	Standard strain	Clinical strain
MIC	5000 mg/ml	2500 mg/ml
MBC	≥10000 I.U/ml	≥5000 I.U/ml

3-4-Bacterial death kinetics test results: This test results revealed that growth and survival of the groups treated with bacterial strains of Pseudomonas aeruginosa with 33_{nm} nanoparticle decreased significantly compared to control group. With regard to standard strain, the nanoparticle in dilution of 0.1 has a more significant antibacterial effect compared to other concentrations. In dilution 0.1, the highest inhibitory effect (bacterial N.Log: 6.80) of nanoparticle was seen, and in dilution 0.001, the lowest inhibitory effect of the nanoparticle was seen (Chart-1). In bacterial clinical strain treatment with nm33 nanoparticle, the inhibitory effect of copper oxide nanoparticle was also more significant compared to control

group and the highest inhibitory effect belonged to dilution 0.1 of nanoparticle (bacterial N.Log: 6.77) and this inhibitory effect has lasted continuously until the minute 60. Results also revealed that the lowest inhibitory effect belonged to 0.001 dilution of copper oxide nanoparticle (Chart-2).

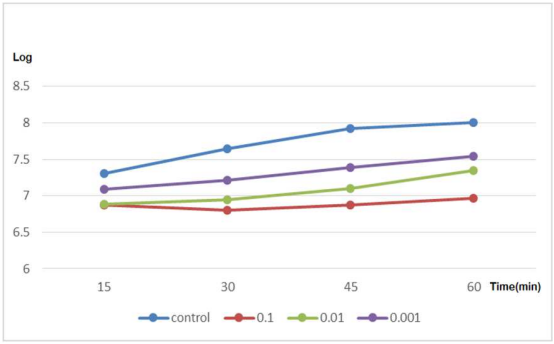

Chart1. standard strain death kinetics of Pseudomonas aeruginosa to treatment with 33nm nanoparticle in terms of time

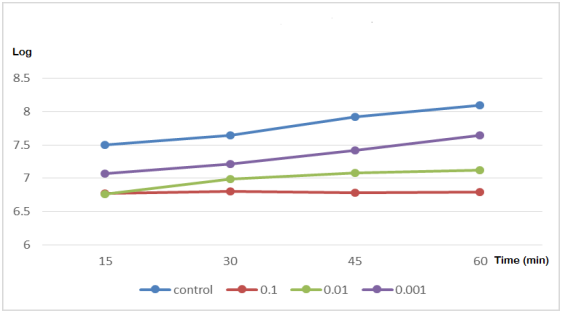

Chart 2. clinical strain death kinetics of Pseudomonas aeruginosa to treatment with 33nm nanoparticle in terms of time

3-5-Results of statistical analysis: results of Independent sample T test with regard to inhibitory effect of copper oxide nanoparticle on Pseudomonas aeruginosa bacteria strains revealed that the difference between the logarithms of the number of bacteria at different times with the control group is significant for each strain (0.05 p value <). Moreover, one-way ANOVA test results and Scheffe complementary test revealed that logarithm difference between standard strain and clinical strain bacteria was not significant.

DISCUSSION AND CONCLUSION

The current research results revealed that copper oxide nanoparticle had inhibitory effects on Pseudomonas aeruginosa bacterium and this inhibitory effect is not significantly different in comparing the standard strain with clinical strain. The antimicrobial effects of these nanoparticles depend on their physical, chemical and functional properties. It seems that the key factors involved in the effect of copper oxide nanoparticles on bacteria to be size, solubility, treatment time, and nanoparticle structure, and its destructive mechanisms often include production of reactive oxygen species (ROS), cation releasing, destruction of biomolecules, reduced ATP, and membrane interactions [12]. In the current research, similar to other previous research, which suggests the effect of copper oxide nanoparticles on bacteria [13], results showed that copper oxide nanoparticles have a significant antibacterial effect. In addition, studies have shown that nanoparticles show stronger antibacterial effects at smaller sizes [14,15]. Some of the reasons involved in this regard include smaller nanoparticles, edges and corners more likely release their ions, and nanoparticles at higher concentrations have more ability in releasing the ion, which these ions would cause more lethal effects [16, 17 and 18]. Our research also indicated that the concentration and size of used copper oxide nanoparticles in vicinity of Pseudomonas aeruginosa strains are considered as key factors in lethal effect of this nanoparticle. It should be noted that Cu^{2+} ions are released easily in a nutrient medium [7]. With regard to antibacterial effects of copper oxide nanoparticles, as current research, the facilitated release of Cu^{2+} in a nutrient medium can be effective in its bactericidal effects. An important advantage of using copper nanoparticles in overcoming drug-resistant bacteria is that the possibility of bacterial resistance to this type of substance is very low, since the antibacterial mechanism of the nanoparticles is multi-functional, and it targets the cell wall, the cell respiratory system, the genomic mechanism, and the proteinization process simultaneously. The bacterium in fact will not have the opportunity to restore potential and the possibility to achieve an approach for survival. Given the low-cost synthesis and significant antibacterial effects of copper oxide nanoparticles, it seems that the development and use of these nanoparticles at smaller sizes or loaded on surfaces, as an antimicrobial agent in coping with bacterial-resistant strains in disinfecting the therapeutic or military places can be an appropriate goal.

Conflict of interest

The authors of this research declared no conflict of interest.

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