Short Comunication

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A Study of the Bactericidal Effect of Copper Oxide Nanoparticles on *Shigella Sonnei* and *Salmonella Typhimurium*

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ABSTRACT

BACKGROUND AND OBJECTIVE: Microbial resistance is one of the most important challenges in dealing with infectious diseases. Therefore, finding or synthesizing new antimicrobial agents is very important. Copper oxide (CuO) is considered for its antibacterial effect against microbial resistance. This study was conducted to investigate the antibacterial effects of copper oxide nanoparticles on *shigella sonnei* and *salmonella typhimurium* bacteria, which have new strains associated with microbial resistance.

METHODS: In this applied fundamental research, copper oxide nanoparticles were synthesized from copper sulfate in sizes of 33 and 56 nm, using a chemical reduction method. Then, the antibacterial effects of copper oxide nanoparticles on the standard strain of *shigella sonnei* (ATCC–9290) and *salmonella typhimurium* (PTCC–1609) were investigated using minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and bacterial death kinetics.

FINDINGS: The MIC obtained in *shigella sonnei and salmonella typhimurium* treatment with a 33 nm nanoparticle were 2500 mg/ml and 5000 mg/ml, respectively, and the value for 56 nm nanoparticle for both bacteria was 5,000 mg/ml. The obtained MBC in the treatment of *shigella sonnei* and *salmonella typhimurium* using 33 nm nanoparticle was 5000≤ IU/ml and 10,000≤ IU/ml, respectively, and the same for 56 nm nanoparticle for both bacteria was equal to 10,000≤ IU/ml.

CONCLUSION: The research proves that copper oxide nanoparticles have a bactericidal effect on *shigella sonnei* and *salmonella typhimurium*, and that the bactericidal effect of smaller nanoparticles is greater than that of bigger nanoparticles, while the antibacterial effects on *shigella sonnei* was more significant.

KEY WORDS: *Copper Oxide Nanoparticle, Shigella Sonnei, Salmonella Typhimurium, MIC, MBC, Bacterial Death Kinetics.*

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Introduction

Gram-negative bacteria are increasingly becoming resistant to most antibiotics. Among the gram-negative bacteria that have become resistant, *shigella sonnei* and *salmonella typhimurium* bacteria can be noted. The *shigella sonnei* bacterium is from *enterobacteriaceae* family and is a potent infectious agent (1). In the treatment process for *shigella sonnei* infection, there is a problem with resistance to antibiotics such as ampicillin, trimethoprim and tetracycline (2).

The *salmonella typhimurium* bacterium also belongs to the *enterobacteriaceae* family (3). Nowadays, the emergence of Multidrug–resistant Salmonella typhi (MDRST) is a global threat (4,5). Therefore, the detection and synthesis of new antimicrobial agents to counteract antibiotic resistance is of great importance. Consequently, much attention is now focused on nanoparticles, which have prominent antimicrobial effects due to their physiochemical properties, which are due to their small size and high specific surface area (6).

Special conditions of bacteria, such as cell walls, metabolic pathways or planktonic physiological conditions, affect the effectiveness of nanoparticles on target bacteria. Nanoparticles generally destroy the target bacteria with a destructive effect on the membrane load cell and its integrity, and the production of free oxygen radicals (ROS). Copper oxide nanoparticles (CuO) have recently been considered as antimicrobial agent. The bactericidal properties of nanoparticles, depending on their size, stability and concentration, are used as antibacterial agents. Highly ionic copper oxide nanoparticles can be synthesized in crystalline morphologies with high specific surface area (8, 9).

Ramyadevi et al. investigated the antimicrobial effects of copper nanoparticles on *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae*, whose results indicated antimicrobial effects of copper nanoparticles (10). Akhavan et al. reported the effects of copper and copper oxide nanoparticles loaded on a thin film of silica, and stated that the inhibitory effects of copper nanoparticles on *E. coli* are stronger than those of copper oxide nanoparticles (11). The aim of the present study is to investigate the antibacterial effects of copper oxide nanoparticle as a new antibacterial agent in two different sizes and depending on different concentrations on two gram-negative bacteria, *shigella sonnei* and *salmonella typhimurium*, considering the increased resistance of gram-negative bacteria and its challenges.

Methods

Materials and strains of studied bacteria: This applied fundamental research was approved by the ethics committee of Kermanshah University of Medical Sciences. In this study, *shigella sonnei* (ATCC 9290) and *salmonella typhimurium* (PTCC 1609) strain and culture media, Muller Hinton Agar, Nutrient Broth, BHI, Tryptone Broth, Peptone Water and brain agar were used. All cultivation media were prepared from Merk Company.

Synthesis and characterization of copper oxide nanoparticles

Synthesis of copper oxide nanoparticles: To prepare an aqueous colloidal copper solution, One-step chemical recovery method was used according to Han et al. (12). In this method, 0.25 gr copper sulfate pentahydrate (CuSO4 5H2O) in 100 ml distilled water and 5 gr Polyvinylpyrrolidone (PVP K-30) (Merck-Germany) were used. In addition, 0.25 gr NaBH4 was used for copper oxide recovery. Different amounts of ascorbic acid were used to prepare copper oxide with different sizes.

Nanoparticle Characterization: To measure the size and morphology of copper oxide nanoparticles, Zeta Sizer (Malvern zeta sizer nano – 25) and scanning electron microscope (SEM) (MIRA3 - TESCAN) were used, respectively.

Antimicrobial susceptibility test for copper oxide nanoparticles

Disc Diffusion Test: For this test, a 24 – hour culture of *shigella sonnei* and *salmonella typhimurium* was prepared in the BHI (Merck – Germany) medium. Then, the turbidity of the bacteria was determined by 0.5 McFarland with naked eye and using a standard spectrophotometer (Bahar Afshan – Iran). The bacterial strains were separately cultured on a sterilized swab (Merck–Germany) and loaded with 20 µl of nanoparticles onto standard sterilized disks (Padtan – Iran). For control, unloaded sterilized disks (Padtan – Iran) were used. After incubation, the diameter of the inhibition zone was measured.

Agar well diffusion test: In this test, the bacterial suspension was prepared at a concentration of 0.5 McFarland. Then the bacterial specimen was cultured on Muller Hinton Agar and a well was created using sterile punch inside the plate. Each well with 10 mg /

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ml of both nanoparticle samples was separately inoculated on separate plates and the wells containing DMSO (Merck-Germany) were prepared as control. After incubation, the diameter of the inhibition zone was measured.

MIC and MBC determination tests: Broth Microdilution MIC Testing was used to determine MIC and MBC. In this method, a 96 – well microplate (SPL-South Korea) was used according to the method by Raeisi et al. (13). Both *shigella sonnei* and *salmonella typhimurium* bacteria were cultured and incubated separately in BHI liquid medium (Merck – Germany). Various concentrations of copper oxide nanoparticles were prepared using the Tryptone Soya Broth (Merck – Germany). A concentration of 0.5 McFarland was obtained from bacterial suspensions. The dilution was carried out using Peptone water (Merck – Germany). Dilutions, 2 to 7 times more than the dilution of the main copper oxide nanoparticle solutions were prepared in BHI containing tubes. Then, 160 µl of BHI, 20 ml of the concentrations of copper oxide nanoparticles and 20 ml of bacterial inoculation were added to each nanoparticle and each bacterium was separately added to the microplate wells. For positive control, 200 µl of the BHI medium and nanoparticles, and for negative control, 180 µl of BHI medium plus 20 µl of bacterial inoculation were added to the wells. After incubation, the first transparent MIC well and the second transparent MBC well were considered.

Kinetic study of bacterial death in broth medium: To perform this test in four experimental tubes, 8 ml of BHI liquid medium was poured into the medium and 1 ml of the 18 – hour culture of the bacteria was inoculated into the BHI medium. Then, by adding 1 ml solution of copper oxide nanoparticles (each of the sizes separately) to the tubes, 0.1, 0.01 and 0.001 dilutions were prepared. Physiology serum was added to the fourth tube as control.

The media were then incubated at 37 °C at a speed of 150 rpm and diluted at intervals of zero, 30, 45 and 60 minutes from each of the concentrations at specified times, and cultured on plates containing BHI Agar medium. The plates were incubated and after incubation, the number of bacteria that survived was counted using a colony counter.

Statistical tests: For statistical analysis of the results, one-way and two-way ANOVA and scheffe supplement test were used and $p<0.05$ was considered significant.

Results

Study of the size and morphology of copper oxide nanoparticles: In the study of the size of synthesized copper oxide nanoparticles by a Zeta Sizer, copper oxide nanoparticles were synthesized with two sizes of approximately 33 nm and 56 nm (Fig 1). SEM microscopic examination revealed the particle morphology of these nanoparticles (Fig 1A, 1B).

The results of disk diffusion test and well diffusion test: The obtained data indicate that the smaller size nanoparticles (33 nm) have more inhibitory effects on both bacteria than nanoparticles with a larger size (56 nm). In addition, antibacterial effects of copper oxide nanoparticles on *shigella sonnei* were more than the effect on *salmonella typhimurium* (table 1).

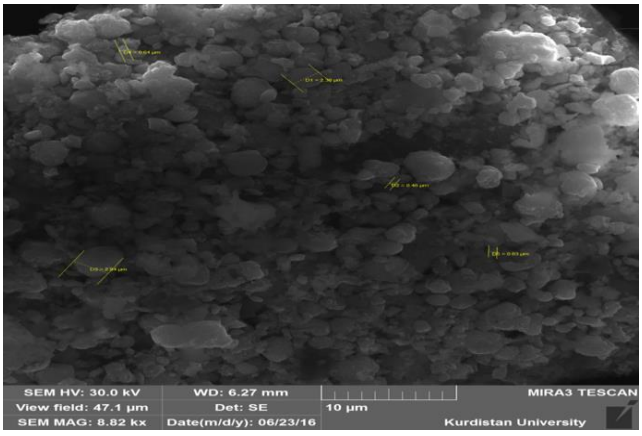

Figure 1A. 33 nm copper oxide nanoparticle morphology with SEM microscope

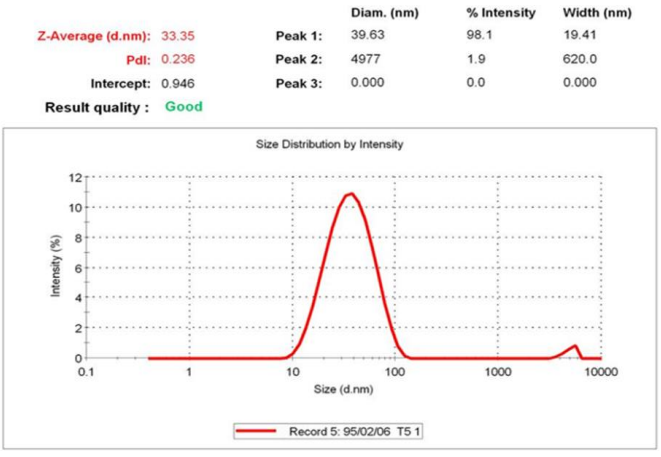

Figure 1B. Determining the size of 33 nm nanoparticle with Zeta Sizer

MIC and MBC test results for copper oxide nanoparticles: Data obtained in determining MIC and MBC indicate that copper oxide nanoparticles have significant antibacterial effects on *shigella sonnei* and *salmonella typhimurium* bacteria. The results of the

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test show that the inhibitory effect of 33 nm nanoparticle on *shigella sonnei* is greater than *salmonella typhimurium* (table 2).

Table 1. The results of disk diffusion test and well diffusion tests in the treatment of *shigella sonnei* and *salmonella typhimurium* bacteria with two different sizes of copper oxide nanoparticles

Size of nanoparticle Bacterium		Disc (mm)	Agar well (mm)	Negative control in both tests
Shigella sonnei	33nm	25±2	31.5±1.5	0
	56nm	17±2.5	23±1.5	0
Salmonella typhimurium	33nm	18±2	26±2	0
	56nm	15±2	22±2	0

Table 2. Results of MIC and MBC in the treatment of *shigella sonnei* and *salmonella typhimurium* bacteria with two different sizes of copper oxide nanoparticles

Group	33 nm	56 nm
Shigella sonnei	2500 mg/ml	5000 mg/ml
	5000≥I.U/ml	10000≥I.U/ml
Salmonella typhimurium	5000 mg/ml	5000 mg/ml
	10000≥I.U/ml	10000≥I.U/ml

The results of kinetics of bacterial death: In the treatment of *shigella sonnei* bacterium, the highest inhibitory effect was observed in the dilution of 0.1 and the lowest inhibitory effect of 33 nm nanoparticle was observed in 0.001 (Fig 2A). In treatment with 56 nm nanoparticle, highest inhibitory effect was observed in 0.1 dilution, and the lowest inhibitory effect was observed in 0.001 (Fig 2B).

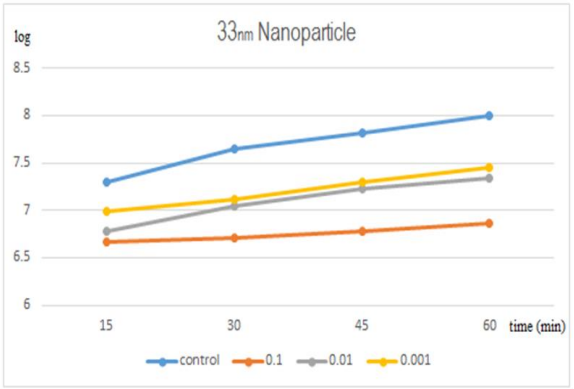

Figure 2A. *Shigella sonnei* death kinetics diagram for treatment with 33 nm nanoparticles based on time

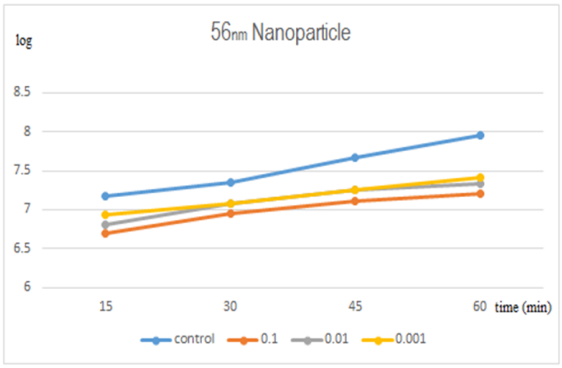

Figure 2B. *Shigella sonnei* death kinetics diagram for treatment with 56 nm nanoparticles based on time

In the treatment of *salmonella typhimurium* bacterium with 33 nm nanoparticles, the highest inhibitory effect was observed in 0.1 dilution and the lowest inhibitory effect was observed in 0.001 dilution (Fig 3A). In treatment with 56 nm nanoparticles, it was fond that the inhibitory effect of growth in 0.1 dilution of the nanoparticle has more significant anti-bacterial effect than other treatments (Fig 3B).

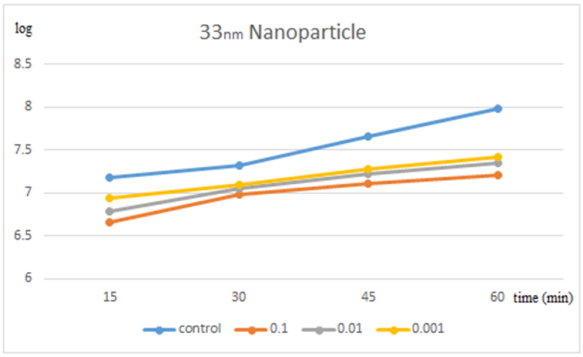

Figure 3A. *Salmonella typhimurium* death kinetics diagram for treatment with 33 nm nanoparticles based on time

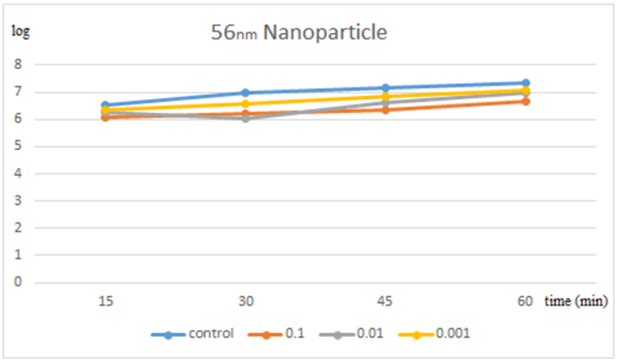

Figure 3B. *Salmonella typhimurium* death kinetics diagram for treatment with 56 nm nanoparticles based on time

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Statistical analysis of the effects of copper oxide nanoparticles on *shigella sonnei* and *salmonella typhimurium* bacteria showed that there was a significant difference between the logarithms of the number of bacteria at different times and also between nanoparticles of different sizes and the control group. In addition, there was a significant difference between the effect of 33 nm and 56 nm nanoparticles (p=0.000).

Discussion

The results of this study showed that copper oxide nanoparticles can be used to overcome the *shigella sonnei* and *salmonella typhimurium* gram-negative bacteria. The antimicrobial effects of these nanoparticles are dependent on their physical, chemical and functional properties (14). It seems that the key factors in the effect of copper oxide nanoparticles on bacteria include size, solubility, treatment time and structure of nanoparticles, and its destructive effect mechanisms include oxidative stresses, coordination effects, non-homeostasis effects and genotoxicity effects (15).

Similar to other studies that showed the effect of copper oxide nanoparticles on bacteria, the present study also showed that copper oxide nanoparticles have a significant antibacterial effect (16). Since nanoparticles exhibit stronger antibacterial properties at smaller sizes (6), in this study, we tried to show the difference in the antibacterial effects of two different sizes of copper oxide nanoparticles. The results showed that the smaller nanoparticle (33 nm) had stronger antibacterial effects than the larger nanoparticle (56 nm) at specific concentration and time. One of the reasons for this is that the smaller nanoparticles have edges and corners that are more exposed to the release of their ions, which, in turn, produce more lethal effects. In addition, the antimicrobial effects of the nanoparticle on the *shigella sonnei* bacterium are more pronounced, and the results also prove that, in addition to the size of copper oxide nanoparticles, the concentration used along with the studied bacteria is a golden factor in the bactericidal effect of these nanoparticles. On the other hand, the release of Cu^{2+} ions in a nutrient medium can easily occur (8). Regarding the antibacterial effects of copper oxide nanoparticles, the release of Cu^{2+} in nutrient medium can have an effect on its bactericidal effects. An important advantage in using copper nanoparticles in overcoming bacterial resistance is that increased bacterial resistance to this type of material is very low. Since the antibacterial mechanism of the nanoparticles is multi-functional, it simultaneously targets the cell wall, the cellular respiratory system, the genomic mechanism, and the proteinization process.

In fact, the bacteria will not have the opportunity, and the potential for repair, and the possibility of finding a way to survive. Due to low cost of synthesis and significant antibacterial effects of copper oxide nanoparticles, the development and use of these nanoparticles in smaller sizes or loaded onto surfaces as antimicrobial agents in counteracting bacterial resistant strains in the disinfection of therapeutic or military sites can be an appropriate goal.

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