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Topics: Antibacterial activity

ZnO nanoparticles enhanced antibacterial activity of ciprofloxacin against *Staphylococcus aureus* and *Escherichia coli*

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Abstract: Nanoparticle metal oxides offer a wide variety of potential applications in medicine due to the unprecedented advances in nanobiotechnology research. In this work, the effect of zinc oxide (ZnO) nanoparticles prepared by mechano–chemical method on the antibacterial activity of different antibiotics was evaluated using disk diffusion method against *Staphylococcus aureus* and *Escherichia coli*. The average size of ZnO nanoparticles was between 20 nm and 45 nm. Although ZnO nanoparticles (500 μg/disk) decreased the antibacterial activity of amoxicillin, penicillin G, and nitrofurantoin in *S. aureus*, the antibacterial activity of ciprofloxacin increased in the presence of ZnO nanoparticles in both test strains. A total of 27% and 22% increase in inhibition zone areas was observed for ciprofloxacin in the presence of ZnO nanoparticles in *S. aureus* and *E. coli*, respectively. The enhancing effect of this nanomaterial on the antibacterial activity of ciprofloxacin was further investigated at three different contents (500, 1000, and 2000 μg/disk) against various clinical isolates of *S. aureus* and *E. coli* The enhancing effect of ZnO nanoparticles on the antibacterial activity of ciprofloxacin was concentration-dependent against all test strains. The most enhancing activities were observed in the contents of the 2000 μg/disk. © 2010 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 93B: 557–561, 2010.

Key Words: bioactive material, nanomedicine, nanotechnology

INTRODUCTION

Despite prodigious efforts, bacterial resistance is still considered as a major drawback in chemotherapy of many infection diseases.[1] Recently, however, certain natural products and synthetic compounds have successfully shown to increase antibacterial activity of antibiotics against different clinically isolated resistant test strains.[2–11] Moreover, there are extensive reports on antibacterial effects of silver[12–15] and copper nanoparticles.[16] Also the combination of silver nanoparticles with antibiotics was investigated in our previous work, where we demonstrated that antibacterial activity of penicillin G, amoxicillin, erythromycin, clindamycin, and vancomycin against *Staphylococcus aureus* and *Escherichia coli* significantly increases in the presence of silver nanoparticles.[17] However, the effect of other nanomaterial such as zinc oxide (ZnO) nanoparticles in combination with antibiotics has not been yet investigated. ZnO is widely used as food additive, food supplement, and pharmaceutical ingredient.[18–21] Among other metal oxide nanomaterials, ZnO nanoparticles are famous for their catalytic efficiency, chemical stability, and strong adsorption ability.[22]

In this study, for the first time, the antibacterial activity of different antibiotics was evaluated against different clinical strains of *S. aureus* and *E. coli* either in presence and absence of sub-inhibitory concentrations of ZnO nanoparticles, using disk diffusion assay. ZnO nanoparticles showed considerable enhancing effects on the antibacterial activity of ciprofloxacin against different clinical strains of *S. aureus* and *E. coli*.

MATERIALS AND METHODS

Synthesis of ZnO nanoparticles

ZnO nanoparticles were freshly prepared by a recently described mechanochemical method using anhydrous $ZnCl_2$, anhydrous Na_2CO_3, and NaCl as starting materials.[23] NaCl was used as dilutive additive to the starting powder. The stoichiometric mixture of the starting powders was milled according to the reaction below:

$$ZnCl_2 + Na_2CO_3 + 8:6\,NaCl \rightarrow ZnCO_3 + 10:6\,NaCl$$

The diameter of balls and their w/w ratio to powder mass were 10 mm and 10:1, respectively. Mechanochemical milling process was then carried out with planetary mill for 9 h at 250 rpm. Powder was calcined in air at 300°C for 30 min.[23]

$$ZnCO_3 \rightarrow ZnO + CO_2(g)$$

Finally, ZnO nanoparticles were obtained when calcinated sample powders were washed with distilled water for thrice and dried. A colloidal stock solution of ZnO nanoparticles in ethanol (10 mg/mL) was prepared. This colloidal

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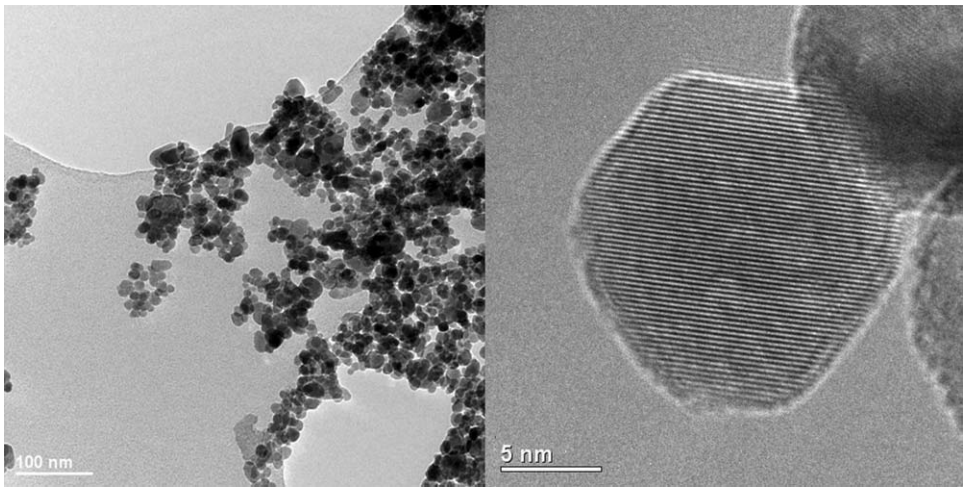

FIGURE 1. Transmission electron micrographs of prepared zinc oxide nanoparticles using a mechanochemical method.

stock solution was sonicated (ultrasound Elma T 780/H) for 15 min and used for further biological experiments and sample characterization by transmission electron microscopy (Philips CM200).

Disk diffusion assay

The disk diffusion susceptibility test was carried out on Müeller-Hinton agar (Difco, Germany) plates in order to examine the antibacterial activity of candidate antibiotics against test strains. Standard antibiotics disks listed in Table I were purchased from Mast Co., UK. To determine the combined effects, each standard paper disk was further impregnated with ZnO nanoparticles at a sub-inhibitory amount of 500 μg/disk. A single colony of test strains was grown overnight in Müeller-Hinton liquid medium (Difco) on a rotary shaker (200 rpm) at 35°C. The inocula were prepared by diluting the overnight cultures with 0.9% NaCl to a 0.5 McFarland standard and applied to the plates along with standard and test disks containing ZnO nanoparticles. Different clinical test strains of *S. aureus* and *E. coli* were isolated at Ghods Polyclinic Laboratory (Tehran, Iran) and used as test strains throughout this investigation. The confirmation of all bacterial isolates was carried out by conventional microbiological identification methods.[24] ZnO nanoparticles at the content of 500 μg/disk was tested as control. After incubation at 35°C for 18 h, the inhibition zones were measured. Mean surface area of the inhibition zone (mm^2) was calculated from mean diameter of each tested antibiotic. The percent of increase in the inhibition zone areas for different antibiotics against *S. aureus* were calculated as $(b^2 - a^2)/a^2 \times 100$, where a and b are the inhibition zones for A and B, respectively. In the same way, $(d^2 - c^2)/c^2$ was used for antibiotics against *E. coli*. All experiments were performed in triplicate.

RESULTS

Figure 1 shows representative transmission electron micrograph images recorded from the ZnO nanoparticle drop-coated film that was synthesized by mechano–chemical method. According to transmission electron micrograph data, particles vary in size from 20 to 45 nm. The combination effect of ZnO nanoparticles (500 μg) with different antibiotics was primarily investigated against two clinical isolates of *S. aureus* and *E. coli* by the disk diffusion method (Fig. 2). The diameters of inhibition zones (mm) in antibiotic disks either in presence or lack of ZnO nanoparticles are outlined in Table I. Although the ZnO nanoparticles decreased the antibacterial activity of amoxicillin, penicillin G, and nitrofurantoin against *S. aureus*, the antibacterial activity of ciprofloxacin increased in the presence of ZnO nanoparticles against both test strains. In detail, the surface areas of inhibition zones of ciprofloxacin in both *S. aureus* and *E. coli* plates increased in presence of ZnO nanoparticles by 27% and 22%, respectively. No enhancing effect on the antibacterial activity other antibiotics was observed against mentioned test strains at the concentration tested (500 μg/disk).

In the second round of the test—in order to investigate the effect of ZnO nanoparticles on antibacterial activity of ciprofloxacin—the experiment was repeated; this time with 5 μg/disk concentration of ciprofloxacin supplemented either with or without three sub-inhibitory levels of ZnO nanoparticles (500, 1000, and 2000 μg/disk), using four clinical isolates of *S. aureus* and three clinical isolates of *E. coli*. (Table II). As clear from the Table II, ZnO nanoparticles improved the antibacterial activity of ciprofloxacin in a concentration-dependent manner. This pattern was true for all clinical isolates and the most enhancing activity was observed in concentration of 2000 μg/disk. ZnO nanoparticles caused 39% to 63% increase in inhibition zone areas of ciprofloxacin in different isolates of *S. aureus*. However, for *E. coli* isolates, the increase in inhibition zones varied from 17% up to the considerable amount of 93% in presence of ZnO nanomaterials.

DISCUSSION

In this study, the antibacterial activity of ZnO nanoparticles alone was tested at the concentrations of 500, 1000, and

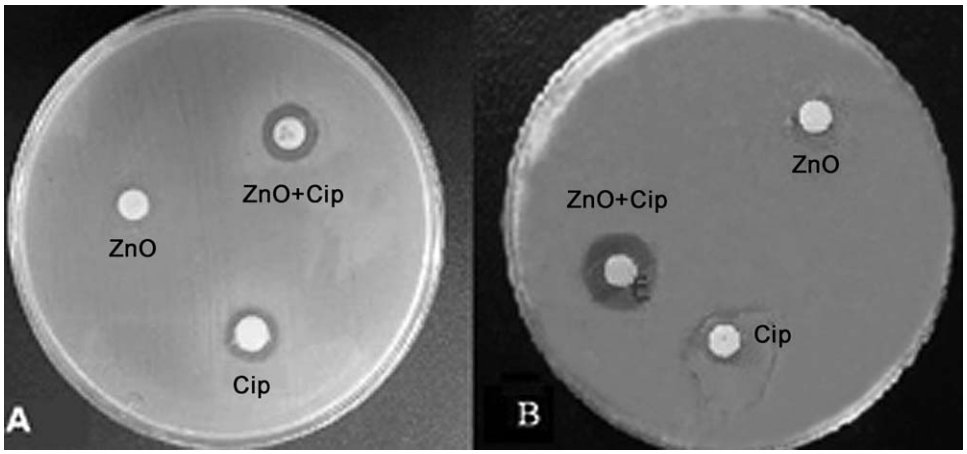

FIGURE 2. Agar disk diffusion assay testing the effect of ciprofloxacin against *Escherichia coli* (A) and *Staphylococcus aureus* (B) both in presence and absence of zinc oxide (ZnO) nanoparticles (500 μg/disk). Test strains were spread onto Müeller-Hinton Agar plates. A ciprofloxacin standard disk was further loaded with ZnO nanoparticles (500 μg/disk) (Cip + ZnO) and placed onto the left side of the inoculated agar plates. Ciprofloxacin standard disks (Cip) and ZnO nanoparticles containing disks (500 μg/disk) were also applied as controls. In both test strains, inhibition zones around ZnO nanoparticle containing ciprofloxacin disks were broader than nanoparticle-free disks.

2000 μg/disk. The ZnO nanoparticle levels of 500, 1000, and 2000 μg/disks were chosen to guarantee that the effect produced was due to the combination effect of ZnO nanoparticle–ciprofloxacin and not to the effect of the ZnO nanoparticles themselves.

Candidate antibiotics were carefully chosen because they represent major classes of antibiotics (penicillins, cephallosporins, macrolides, aminoglicosides, tetracyclines, fluoroquinolones, lincomycin derivatives, nitrofurans, and glycopeptides). However, different antibiotics showed different activities in presence of ZnO nanoparticles. In detail, antibacterial activity of amoxicillin, penicillin G, and nitrofurantoin against *S. aureus* decreased, whereas that of ciprofloxacin increased in both test strains. The remaining antibiotics were almost indifferent to the presence of ZnO nanoparticles.

An explanation for the increased activity in the presence of ZnO nanoparticles in *S. aureus* would be based on the assumption that ZnO nanoparticles may interfere with the pumping activity of NorA protein of *S. aureus*. The NorA protein mediates the active efflux of hydrophilic fluoroquinolones from the cell, conferring resistance upon the organism.[25–28] Because recent reports suggest that nano-sized metal oxides such as zinc oxide and titanium dioxide possess the ability to induce faster electron transfer kinetics in the active site of the enzymes,[29,30] it seems likely that ZnO

TABLE I. Inhibition Zones (mm^2) of Candidate Antibiotics Against *Staphylococcus aureus* and *Escherichia coli* (Either in Presence or Absence of ZnO Nanoparticles at Content of 500 μg/disk)[a]

Antibiotics (μg/disk)	*Staphylococcus aureus*		Increase/ Decrease in Area (%)[b]	*Escherichia coli*		Increase in Area (%)[b]
	Antibiotic only (A)	Antibiotic plus ZnO Nanoparticles (B)		Antibiotic only (C)	Antibiotic Plus ZnO Nanoparticles (D)	
Penicillin G 10	18.5 ± 0.5	17 ± 0.0	−15.0	–	–	–
Amoxicillin 10	15 ± 1.0	12 ± 0.5	−36.0	–	–	–
Carbenicillin 100	25 ± 0.5	24 ± 0.5	−7.80	–	–	–
Cephalexin 30	–	–	–	15 ± 0.5	15 ± 0.5	0.0
Cefixime 5	–	–	–	13 ± 1.0	13 ± 0.5	0.0
Erythromycin 5	–	–	–	15 ± 1.0	14 ± 0.0	−12.9
Gentamicin 10	8 ± 0.5	8 ± 0.5	–	–	–	–
Amikacin 30	–	–	–	15 ± 0.5	15 ± 0.5	0.0
Tetracycline 30	12 ± 0.5	12 ± 0.5	0.0	11 ± 0.0	11 ± 0.5	0.0
Ciprofloxacin 5	20 ± 0.5	22.5 ± 1.0	27.0	19 ± 0.0	21 ± 1.0	22.0
Clindamycin 2	12 ± 1.0	12 ± 1.0	0.0	17 ± 0.5	17 ± 0.5	0
Nitrofurantoin 300	27 ± 1.0	25 ± 0.5	−14.0	17 ± 0.5	17 ± 0.5	0
Nalidixic acid 30	–	–	–	–	–	–
Vancomycin 30	19 ± 0.5	18 ± 1.0	−10.0	14 ± 0.5	13± 0.5	−0.13776

ZnO, zinc oxide.

[a] All experiments were done in triplicate.

[b] Mean surface area of the inhibition zone (mm^2) was calculated from the mean diameter of each tested antibiotic. The percent of increase in the inhibition zone areas for different antibiotics against *S. aureus* were calculated as $(b^2 - a^2)/a^2 \times 100$, where a and b are the inhibition zones for A and B, respectively. In the same way, $(d^2 - c^2)/c^2$ was used for antibiotics against *E. coli*.

TABLE II. The Combination Effect of ZnO Nanoparticles in Three Different Contents (500, 1000, and 2000 μg/disk) with Ciprofloxacin Against Seven Clinical Isolates of *Staphylococcus aureus* and *Escherichia coli*[a]

	Zone of inhibition (mm)						
		Antibiotic Plus ZnO Nanoparticles (B)			Increase in Area (%)[b]		
Isolates	Antibiotic only (A)	500 μg	1000 μg	2000 μg	500 μg	1000 μg	2000 μg
S. aureus (isolate 1)	21 ± 0.5	22.5 ± 0.5	24 ± 1.0	26.5 ± 0.5	15	30	59
S. aureus (isolate 2)	18 ± 0.5	20 ± 1.0	22.5 ± 0.0	24.5 ± 0.5	23	56	85
S. aureus (isolate 3)	14 ± 0.0	15.5 ± 0.5	17.5 ± 1.0	20 ± 0.0	23	56	104
S. aureus (isolate 4)	27 ± 1	28 ± 0.5	30.5± 1.0	32 ± 1.0	8	27	40
E. coli (isolate 1)	9 ± 0.5	10 ± 0.5	11 ± 1.0	12.5 ± 1.0	23	49	93
E. coli (isolate 2)	40 ± 1	41.5 ± 0.5	43 ± 1.0	45 ± 1.5	7.6	16	27
E. coli (isolate 3)	36 ± 0.0	36 ± 0.5	37.5± 0.5	39 ± 0.5	0	8	17

ZnO, zinc oxide.

[a] All experiments were done in triplicate.

[b] Mean surface area of the inhibition zone (mm^2) was calculated for each tested antibiotic from the mean diameter. The percent of increases of inhibition zone area for different antibiotics against *S. aureus* were calculated as $(b^2 - a^2)/a^2 \times 100$, where a and b are the inhibition zones for A and B, respectively. In the same way, $(d^2 - c^2)/c^2$ was used for antibiotics against *E. coli*.

nanoparticles may interfere with the pumping activity of the this protein. Efflux transporters were identified in other bacteria[31] and ZnO nanoparticles may interfere with these efflux pump systems as well. Another explanation would be that ZnO nanoparticles may enhance the absorption of antibiotics into *S. aureus* cells, which is mainly mediated by membrane Omf protein,[32] thereby improving their performances. Omf protein is considered to be responsible for the permeation of quinolones to the cell membrane.[33–35]

Ciprofloxacin is a well-known member of Fluoroquinolones. The mechanism of action of quinolone agents is based on their ability to penetrate into bacterial cells and to inhibit DNA gyrase,[36,37] an enzyme which is responsible for superhelical twists in bacterial DNA.[38] Therefore, considering the nitrogen atoms of quinolone ring in ciprofloxacin, and the hydroxylated surface[39] of ZnO nanoparticles, it is possible that ciprofloxacin–ZnO nanoparticle system may be stabilized through a network of ionic interactions between protonated nitrogen atoms of quinolone and hydroxylated surface of ZnO nanoparticles.

The interactions of complex-forming metal ions such as Co (II), Ni (II), and Cu (II) with ciprofloxacin is previously characterized by Spectroscopy and X-ray analysis, which reveals that ciprofloxacin is capable of forming complexes with metal ions.[40,41] In this context, it is probable that the electron-donor fluore atom in ciprofloxacin may interact with the chelating Zn atom, thereby stabilizing the ciprofloxacin-ZnO nanoparticle combination. This explanation is in good agreement with the study of Patel et al., which showed the improved interaction of ciprofloxacin with DNA in the presence of the chelating cobalt II elements and the ability of ciprofloxacin to form complexes with chelating agents.[42] Another explanation relies on the presence of the carboxyl group in ciprofloxacin as an obvious target for chelation by metal ions, as recognized in previous studies.[43] The chelation of ring carbonyl group oxygen atom by Mg^{2+} metal ion is demonstrated earlier.[44] It seems likely that the carbonyl group and the carboxylic oxygen's may form complexes with Zn atom to increase the stability of ciprofloxacin–ZnO nanoparticles.

These unique features of ciprofloxacin (three amino groups together with the electron-donor fluore group) may have enabled it to form a fairly strong interaction with ZnO nanoparticles. In this context, the antibacterial activity of other antibiotics tested, either decreased or remained the same, perhaps due to the probability that they form weak hydrogen bonds with hydroxylated ZnO nanoparticles or lack sufficient targets to form complexes with Zn atom. More studies remain to be done, to describe in detail, the mechanism underlying the enhanced activity of ciprofloxacin in combination with ZnO nanoparticles.

CONCLUSIONS

Many efforts have been made to overcome the emerging problem of antibiotic resistance among a variety of disease causing bacteria and advances in the field of nanobiotechnology may offer a great opportunity of research in this field. Therefore, studies on combination of antibiotic agents and synthetic and natural organic or inorganic nanomaterials are of great importance. The potential advantages of using organic or inorganic nanoparticles as drug carriers are well reviewed in the literature.[45–47] Here, for the first time, we report that antibacterial activity of ciprofloxacin against two clinical test strains: *S. aureus* and *E. coli* improves in presence of ZnO nanoparticles. Because of its potential synergistic effect with ciprofloxacin, ZnO nanoparticles may be considered as a valuable adjuvant in combination therapy of ciprofloxacin. However, the antibacterial activity of antibiotics such as amoxicillin against *S. aureus* was decreased considerably in the presence of ZnO nanoparticles; therefore, the combination of ZnO nanoparticles with these antibiotics cannot be recommended for possible combination therapy.

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