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Antimicrobial action of zinc oxide nanoparticles in combination with ciprofloxacin and ceftazidime against multidrug-resistant *Acinetobacter baumannii*

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ABSTRACT

Acinetobacter baumannii is a serious concern amongst hospitalised patients worldwide and its resistance to antibiotics has emerged as a threat to public health in recent years. Metal oxide nanoparticles were found to be effective for overcoming bacterial resistance owing to their antibacterial activities. The aim of this study was to investigate the combined effects of zinc oxide nanoparticles (ZnO-NPs) and the conventional antibiotics ciprofloxacin and ceftazidime as well as their mechanisms of action against resistant *A. baumannii*. ZnO-NPs were prepared by the solvothermal method and were characterised by various methods. Broth microdilution and disk diffusion methods were used to determine the antibacterial activities of ciprofloxacin and ceftazidime antibiotics in the absence and presence of a subinhibitory concentration of ZnO-NPs. The mechanism of action of ZnO-NPs alone and in combination with these antibiotics was assessed by flow cytometry, DNA extraction, fluorescence and scanning electron microscopy. The results showed that the antibacterial activities of both antibiotics increased in the presence of a subinhibitory concentration of ZnO-NPs. Combination of ZnO-NPs with antibiotics increased the uptake of antibiotics and changed the bacterial cells from rod to cocci forms. Bacterial filamentation was also observed and exhibited no DNA fragmentation. In conclusion, the results of this study indicate that ZnO-NPs potentiate the antimicrobial action of ciprofloxacin and ceftazidime. A mechanism is proposed to explain this phenomenon.

1. Introduction

Over the past decades, the prevalence of nosocomial infections due to drug-resistant *Acinetobacter baumannii* has increased and this is a serious concern amongst hospitalised patients worldwide [1]. This Gram-negative bacterium often infects the bloodstream, lungs, urinary tract and skin [2]. Fluoroquinolones (e.g. ciprofloxacin) and β-lactams (e.g. ceftazidime) are some common treatment options for *A. baumannii* infections [3]. However, the incidence of resistance to these antibiotics continues to increase [2]. β-Lactam resistance has been ascribed to enzymatic degradation by β-lactamases as well as to non-enzymatic mechanisms, including changes in the structure and number of outer membrane proteins, overexpression of efflux pumps, and alterations in the affinity and expression of penicillin-binding proteins [4]. The mechanisms underlying resistance to the quinolones are (i) modification of the structure of DNA gyrase through mutations in the quinolone resistance-determining regions of the *gyrA* and *parC* genes and (ii) the activity of multidrug efflux pumps such as AdeABC and AdeM that decrease intracellular drug accumulation [4].

Zinc oxide nanoparticles (ZnO-NPs) have been demonstrated to have strong bactericidal properties and wide ranging applications [5,6]. The antibacterial activity of ZnO-NPs has been found to be due to disruption of the cell membrane, oxidative stress induction, and the generation of reactive oxygen species (ROS) [7]. Previous studies have demonstrated that phagocytic cells are able to reduce ROS damage and host tissue toxicity following internalisation and lysosomal degradation of nanoparticles [8].

This study was carried out in response to the significance of nanoparticles in overcoming the antibiotic resistance in pathogenic

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microbes. The synthesised ZnO-NPs were tested for their antibacterial efficacy individually and in combination with ciprofloxacin and ceftazidime antibiotics against *A. baumannii*. Furthermore, for the first time, the mechanism of action of ZnO-NPs on the efficiency of both antibiotics against resistant *A. baumannii* was investigated.

2. Materials and methods

2.1. Preparation of ZnO nanofluids

Synthesis of ZnO-NPs was carried out by the solvothermal process [9]. To make stable ZnO nanofluids for antimicrobial tests, glycerol was used as the base fluid and ammonium citrate as a dispersant. ZnO-NPs and dispersant were used in a 1:1 ratio and were thoroughly stirred using a magnetic stirrer with glycerol solution at room temperature for ca. 24 h [10].

2.2. Characterisation of ZnO-NPs

The synthesised ZnO-NPs were characterised using X-ray diffraction (XRD), particle size analyser and scanning electron microscopy (SEM). The XRD pattern of dry nanoparticles was obtained using a Bruker Axs D8 ADVANCE diffractometer (Bruker Corp., Ettlingen, Germany) with CuKα radiation (λ = 1.5406 Å). The average crystalline size of nanoparticles was calculated using the Debye–Scherrer equation:

$$D_{hkl} = \frac{k \times \lambda}{\beta_{hkl} \times \cos\theta_{hkl}} \quad (1)$$

where D_{hkl} is the crystallite size perpendicular to the normal line of (h k l) plane, k is a constant equal to 0.9, λ is the wavelength of the CuKα radiation, β_{hkl} is the full width at half maximum of the (h k l) diffraction peak, and θ_{hkl} is the Bragg angle of (h k l) peak. The particle size distribution of the prepared ZnO-NPs was examined by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS^{TM} instrument (Malvern Instruments Ltd., Malvern, UK). SEM images were obtained with a LEO 1450 VP scanning electron microscope (LEO Elektronenmikroskopie GmbH, Oberkochen, Germany) [9].

2.3. Antibacterial tests

Clinical isolates of *A. baumannii* were obtained from the Microbiology Research Laboratory at Ghaem Hospital (Mashhad, Iran). Species-level identification was confirmed using the biochemical profile by manual and partial 16S rRNA gene sequencing as recommended [11].

A. baumannii was tested for antibiotic sensitivity using the disk diffusion method using the following disks: ampicillin (10 μg); amikacin (30 μg); chloramphenicol (30 μg); tobramycin (10 μg); ciprofloxacin (10 μg); ceftazidime (30 μg); cefalotin (30 μg); gentamicin (10 μg); teicoplanin (30 μg); and cloxacillin (30 μg). According to the antibiotic susceptibility pattern, ceftazidime and ciprofloxacin were considered for the following experiments. Two-fold broth microdilution and disk diffusion methods were performed to analyse the antibacterial activity of ZnO-NPs alone and in combination with ceftazidime and ciprofloxacin antibiotics. Briefly, bacterial cells (10^6 CFU/mL) were treated with ZnO-NPs (0.0625–2 mg/mL), ceftazidime (2–64 μg/mL) and ciprofloxacin (1–16 μg/mL) for 24 h. Bacterial growth was monitored by measuring the optical density at 630 nm (OD_{630}) once every 2 h using an ELx800 Absorbance Microplate Reader (BioTek Instruments Inc., Winooski, VT), and the minimum inhibitory concentration (MIC) was determined [12]. To eliminate the impact of the base fluid and the dispersant on the antibacterial activity of ZnO nanofluids, growth of *A. baumannii* cells was measured in the presence of glycerol and ammonium citrate without ZnO-NPs.

To determine the influence of ZnO-NPs on the antibacterial activity of conventional antibiotics, bacterial cells were treated with different concentrations of ceftazidime (16–64 μg/mL) and ciprofloxacin (4–16 μg/mL) in the presence of a subinhibitory concentration (1/2 × MIC; 0.25 mg/mL) of ZnO-NPs for 24 h. Growth of cells was measured as mentioned above. Inoculated nutrient broth without ZnO-NPs and conventional antibiotics was also used as a positive control. All tests were performed in triplicate and the antibacterial activity was expressed as the MIC and the mean of growth inhibition percentage (GI%) with reference to growth of the positive control. At each concentration, GI% was calculated according to Eq. (2).

$$GI\% = \left(100 - \frac{OD_{630} \text{ in the presence of antibacterial agent (s)}}{OD_{630} \text{ of positive control}} \times 100\right) \quad (2)$$

2.4. DNA fragmentation analysis

Cultures of log-phase *A. baumannii* (10^9 CFU/mL) were treated with 32 μg/mL ceftazidime or 8 μg/mL ciprofloxacin in the absence and presence of a subinhibitory concentration of ZnO-NPs (0.25 mg/mL) for 1 h at 37 °C. Genomic DNA was isolated using a Genomic DNA Isolation Kit (DENAzist Asia, Mashhad, Iran) and DNA fragmentation was evaluated by agarose gel electrophoresis [13].

2.5. Flow cytometry

Assessment of cellular uptake of ZnO-NPs and antibiotics was performed by a method previously described by Kumar et al. [14]. A log-phase *A. baumannii* cell suspension (10^9 cells) was treated with ceftazidime (32 μg/mL) or ciprofloxacin (8 μg/mL) in the absence or presence of 0.25 mg/mL ZnO-NPs. Uptake of ZnO-NPs and conventional antibiotics by the cells was evaluated using a flow cytometer (BD FACSCalibur^{TM}; BD Biosciences, San Jose, CA). The data were analysed using Flowing Software 2.4.1 (http://www.flowingsoftware.com).

2.6. Fluorescence and scanning electron microscopy

A log-phase *A. baumannii* cell suspension (10^5 cells) was treated with ceftazidime (32 μg/mL) or ciprofloxacin (8 μg/mL) in the absence or presence of 0.25 mg/mL ZnO-NPs for 1 h at 37 °C and was then centrifuged at 10,000 rpm for 5 min. Morphological changes were assessed by fluorescence microscopy (BX51; Olympus, Tokyo, Japan) and SEM (LEO 1450 VP).

3. Results

3.1. Characterisation of ZnO nanofluids

The crystalline nature of the ZnO-NPs was confirmed by XRD analysis (Fig. 1A). A number of strong Bragg reflections can be seen, which correspond to the (1 0 0), (0 0 2), (1 0 1), (1 0 2), (1 1 0), (1 0 3), (2 0 0), (1 1 2) and (2 0 1) reflections of wurtzite hexagonal phase of ZnO-NPs. The diffraction peaks match well with those in the JCPDS card (Joint Committee on Powder Diffraction Standards, Card No. 89-1397). The crystallite size of the ZnO-NPs was estimated using the Debye–Scherrer formula to be 17.08 nm. The mean particle size determined by DLS was 78 ± 7 nm (Fig. 1B). Clearly, the mean particle size obtained by DLS is greater than that of the actual particle size, since the hydrodynamic radius is probed with

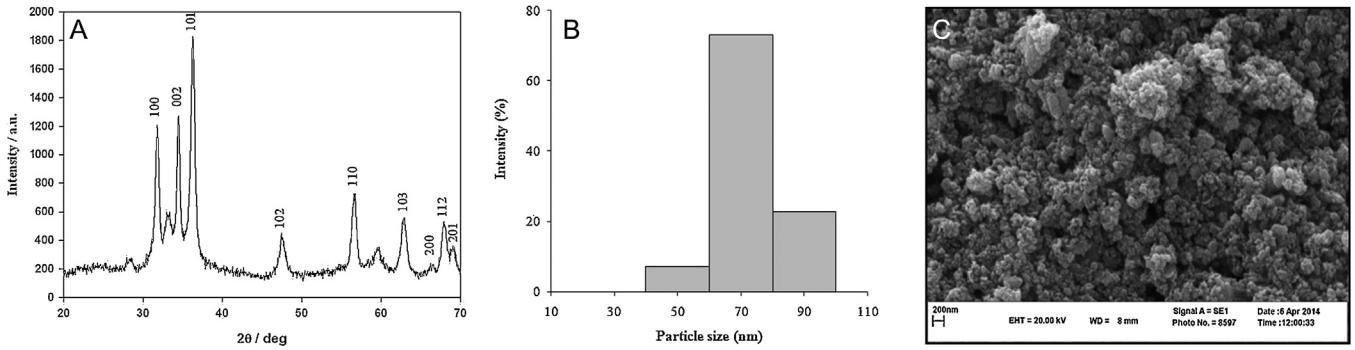

Fig. 1. (A) X-ray diffraction (XRD) pattern, (B) particle size distribution and (C) scanning electron microscopy (SEM) image of zinc oxide nanoparticles (ZnO-NPs).

DLS. The SEM image of the synthesised ZnO-NPs showed that the shape of ZnO-NPs is approximately spherical (Fig. 1C).

3.2. Antibacterial activity

A. baumannii was found to be resistant to ciprofloxacin, ceftazidime, ampicillin, amikacin, cefalotin and cloxacillin. Growth of *A. baumannii* cells in the absence and presence of ZnO-NPs, ceftazidime and ciprofloxacin was determined. Ceftazidime and ciprofloxacin did not show any remarkable growth inhibition, whereas ZnO-NPs showed a significant growth inhibition compared with the control. The MIC of ZnO-NPs was 0.5 mg/mL and its growth inhibitory effect was concentration-dependent. The means of GI% at 0.0625, 0.125 and 0.25 mg/mL concentrations of ZnO-NPs were 9.03 ± 0.4, 13.9 ± 0.81 and 22.6 ± 1.81, respectively. Although the base fluid and dispersant had no effect on the growth of *A. baumannii* at a subinhibitory concentration of ZnO-NPs, they were excluded from the antibacterial activity of ZnO nanofluids to determine only the antibacterial influence of ZnO-NPs on the growth of bacterial cells.

The combination of ZnO-NPs with ceftazidime and/or ciprofloxacin antibiotics against *A. baumannii* was investigated using the microdilution and disk diffusion methods. The means of GI% of antibiotics in the absence and presence of a subinhibitory concentration of ZnO-NPs are shown in Table 1. The antibacterial activity of both antibiotics increased in the presence of 0.25 mg/mL ZnO-NPs and the maximum growth inhibition was observed at 8 μg/mL ciprofloxacin and 32 μg/mL ceftazidime.

The diameters of the inhibition zone around the ciprofloxacin and ceftazidime disks are shown in Table 2. *A. baumannii* exhibits resistance against both antibiotics, but in combination with ZnO-NPs the zone of inhibition was increased.

3.3. DNA ladder assay

The amount of DNA from untreated and treated *A. baumannii* cells was evaluated by agarose gel electrophoresis. The intensity of the genomic DNA band was quantified using ImageJ software (US National Institutes of Health, Bethesda, MD). The DNA of bacterial cells treated with ZnO nanofluids and antibiotics alone and in combination did not show any changes in DNA quantity. Also, the DNA electropherograms exhibited no fragmentations or degradations in all tested genomic DNA samples.

3.4. Detection of antibiotic uptake by flow cytometry

After 60 min of treatment, the *A. baumannii* cells exhibited a pronounced increase in the uptake of ciprofloxacin and ceftazidime antibiotics in the presence of ZnO-NPs, as indicated by the 37.1% and 26.2% increase in the intensity of side scatter (SSC), respectively (Fig. 2B and C) compared with the absence of ZnO-NPs. As shown in Fig. 2A, ZnO-NP treatment alone caused a small increase in granularity as evident by an 8.6% increase in SSC compared with the control. Any increase in the intensity of SSC can be attributed to increased internalisation of antibacterial agents.

3.5. Morphological changes of bacterial cells

Morphological changes in bacterial cells treated with conventional antibiotics in combination with ZnO-NPs were observed by fluorescence microscopy and SEM. The ZnO-NPs-treated cells revealed the highest fluorescence intensity, and the combination of ZnO-NPs with antibiotics caused the formation of filaments (Fig. 3). SEM analyses showed that *A. baumannii* cells treated with a combination of ZnO-NPs and each antibiotic resulted in transformation of the shape of some cells from rod to cocci, or filamentous and multiple dents also appeared on the surface of the cells (Fig. 4).

4. Discussion

The scope of this study is based upon exploring the potential of ZnO-NPs to improve the efficacy of antibiotics against multidrug-resistant *A. baumannii* along with their effect on membrane permeability. To the best of our knowledge, this is the first report on the mechanism of action of ZnO-NPs on the efficiency of ciprofloxacin and ceftazidime antibiotics against resistant *A. baumannii*. ZnO-NPs alone and in combination with both antibiotics proved to be effective in the control of *A. baumannii*. In addition, the increased antibacterial activity of ceftazidime and ciprofloxacin in the presence of ZnO-NPs may be due to membrane damage and accumulation of antibacterial agents in the cells.

Table 1
Growth inhibition percentage (GI%) of *Acinetobacter baumannii* cells after 24 h exposure to ceftazidime and ciprofloxacin alone or in combination with zinc oxide nanoparticles (ZnO-NPs).

ZnO concentration (mg/mL)	Ceftazidime concentration (μg/mL)				Ciprofloxacin concentration (μg/mL)			
	0	16	32	64	0	4	8	16
0	0	1.24 ± 0.01	2.31 ± 0.02	1.99 ± 0.04	0	1.02 ± 0.014	2.1 ± 0.081	1.95 ± 0.032
0.25	19.6 ± 0.071	4.82 ± 0.075	31.41 ± 0.41	7.7 ± 0.37	20.9 ± 0.075	10.98 ± 0.03	45.84 ± 0.023	20.9 ± 0.75

Table 2
Mean zone of inhibition of ceftazidime and ciprofloxacin with and without zinc oxide nanoparticles (ZnO-NPs) against *Acinetobacter baumannii*.

Antibiotic	Zone (mm)	
	Without ZnO-NPs	With ZnO-NPs
Ceftazidime	0	33
Ciprofloxacin	13	38

These results indicated that *A. baumannii* isolates obtained from Ghaem Hospital are resistant to ciprofloxacin and ceftazidime. A suitable approach to overcoming antibiotic resistance is combining antibiotics with nanoparticles. In this study, the resistance of *A. baumannii* to ciprofloxacin and ceftazidime was reduced in the presence of ZnO-NPs. These results are consistent with the observations of Thati et al. who demonstrated that ZnO-NPs (80 nm) significantly improved the antibacterial efficacies of cephalosporin and aminoglycoside antibiotics against *Staphylococcus aureus* [15]. Combined activity of ZnO-NPs (20–45 nm) with different antibiotics against *S. aureus* and *Escherichia coli* was also studied by Banoee et al. [16]. The exact mechanism of antibacterial action of ZnO-NPs with antibiotics has not yet been clearly elucidated. The synergistic activity between conventional antibiotics and NPs has been suggested to be due to inhibition of the export of antibacterial agents by blocking efflux pumps or by enhancing the entrance of antibiotics into the cell by disrupting the bacterial membrane [16].

Several previous studies concluded that ZnO-NPs may induce genotoxicity indirectly by promoting oxidative stress or by directly passing through the cellular membrane and interacting with the DNA to damage all four bases or producing thymine–tyrosine cross-linking [17]. Here, no DNA fragmentation or degradation with ZNO-NPs was observed in *A. baumannii* cells.

The flow cytometry data of this study revealed that combined treatment of *A. baumannii* cells with ZnO-NPs and the antibiotics ciprofloxacin and ceftazidime significantly increased the intensity of SSC (granularity of bacterial cells) compared with each of them alone. We may conclude that exposure of bacterial cells to ZnO-NPs damages the cell membrane and enhances the cellular uptake of antibacterial agents. This is in agreement with the results of Kumar et al. who reported the remarkable internalisation of antibacterial agents in the presence of ZnO- and TiO_2-NPs in *E. coli* and *Salmonella* Typhimurium [14]. The combination of ZnO-NPs with antibiotics may form a complex that can disturb the cell membrane of *A. baumannii* and facilitate the uptake of antibacterial agents. The complex may also block efflux pumps to decrease the export of antibiotics and nanoparticles [16]. Therefore, the increase in granularity of *A. baumannii* cells can be due to membrane damage and accumulation of antibacterial agents in the cells.

SEM analyses showed that rod-shaped cells of *A. baumannii* changed to cocci or filaments after exposure to combinations of ZnO-NPs and antibiotics. There are several research reports indicating that some antibiotics cause changes in cell morphology. For example, Horii et al. showed that ceftazidime induces filament formation in five Gram-negative species [18]. Soon et al. [19] also reported morphological differences between colistin-susceptible and -resistant cells of *A. baumannii*.

In this work, it was shown that the sensitivity of *A. baumannii* to antibiotics is increased by affecting the cell morphology and membrane permeability. It is suggested that ZnO-NPs increase the concentration of antibiotics in the cell by blocking antibiotic efflux or by enhancing antibiotic entry into the cells. The increase in antibiotics appears to induce morphological changes. These theoretical explanations require further study to be supported by experimental data.

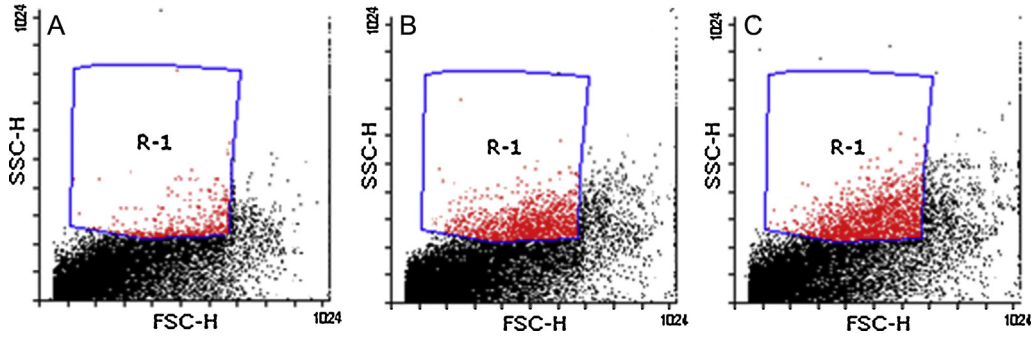

Fig. 2. Scatter analysis of zinc oxide (ZnO) nanofluid and antibiotic uptake in *Acinetobacter baumannii* after 60 min: cells treated with (A) ZnO nanofluid, (B) a combination of ZnO nanofluid with ciprofloxacin and (C) a combination of ZnO nanofluid with ceftazidime. SCC, side scatter; FSC, forward scatter.

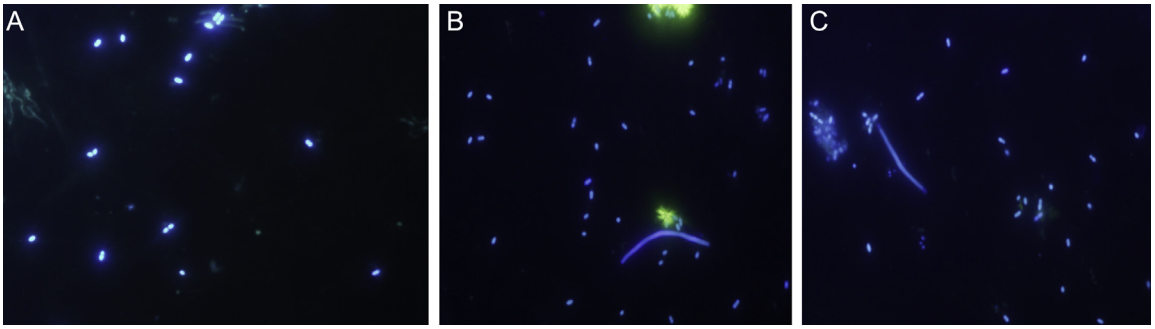

Fig. 3. Epifluorescence images of DAPI-stained *Acinetobacter baumannii*: cells treated with (A) zinc oxide (ZnO) nanofluid, (B) a combination of ZnO nanofluid with ciprofloxacin and (C) a combination of ZnO nanofluid with ceftazidime. DAPI, 4′,6-diamidino-2-phenylindole.

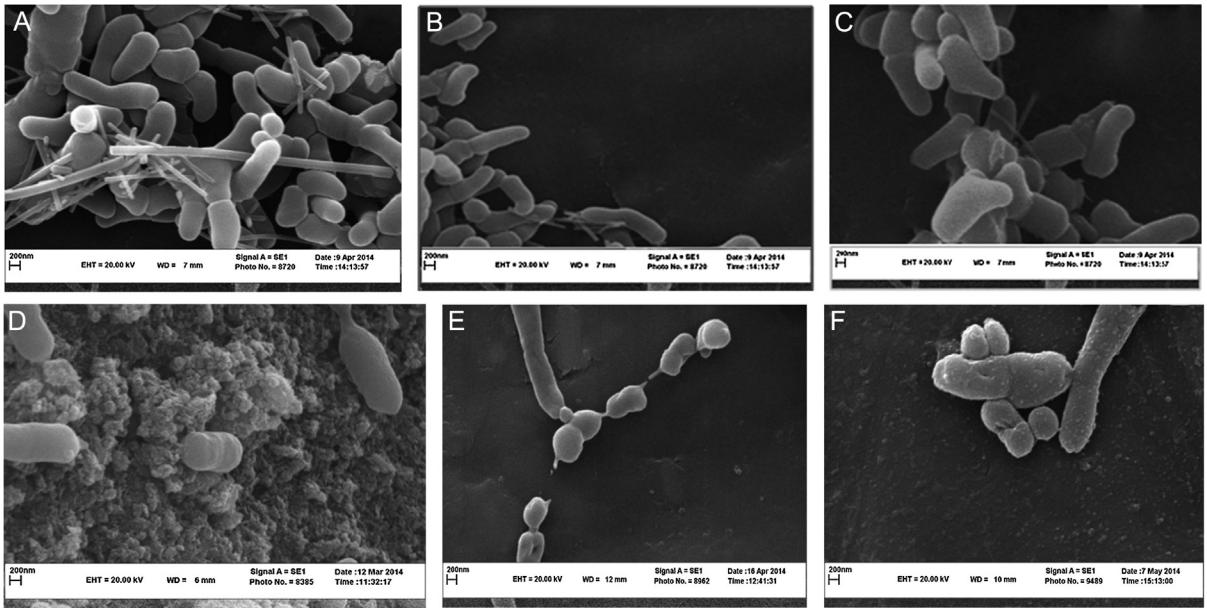

Fig. 4. Scanning electron microscopy (SEM) images of *Acinetobacter baumannii*: (A) untreated cells and (B–F) cells treated with ciprofloxacin (B), ceftazidime (C), zinc oxide (ZnO) nanofluid (D), a combination of ZnO nanofluid with ciprofloxacin (E) and a combination of ZnO nanofluid with ceftazidime (F).

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Competing interests

None declared.

Ethical approval

Ethical approval for this study was received from Ferdowsi University of Mashhad.

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