

Evaluation of Mixture Magnesium Oxide and Zinc Oxide Nanoparticles against Multi-drug-resistance Mycobacterium tuberculosis by Microplate Alamar Blue Assay

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Research note

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Abstract

Objective The current study is the first experimental study of which has evaluated the MICs and MBCs of ZnONPs, MgONPs, and mixture MgONPs-ZnONPs against H 37 Rv Mtb and MDR-Mtb. **Results** The MIC of MgONPs and ZnONPs were 0.195 and 0.468 $\mu\text{g}\cdot\text{ml}^{-1}$ against 10^4 of H 37 Rv Mtb. As well, 0.166 $\mu\text{g}\cdot\text{ml}^{-1}$ of MgONPs-ZnONPs was able to inhibit 10^{-4} H 37 Rv Mtb. The MIC of MgONPs against 10^4 concentrations of MDR-Mtb was 12.5 $\mu\text{g}\cdot\text{ml}^{-1}$. The MIC of MgONPs/ZnONPs against 10^4 concentrations of MDR-Mtb reached to 0.664 $\mu\text{g}\cdot\text{ml}^{-1}$. Based on the results, the MBC value of ZnONPs increased to 1.875 $\mu\text{g}\cdot\text{ml}^{-1}$ against 10^{-4} concentrations of MDR-Mtb. Testing showed that the MBCs of MgONPs/ZnONPs reached to 1.328 $\mu\text{g}\cdot\text{ml}^{-1}$ against 10^4 concentrations of MDR-Mtb. The half maximal inhibitory concentration (IC₅₀) against MDR-TB was 0.779 $\mu\text{g}\cdot\text{ml}^{-1}$ for ZnONPs and 0.883 $\mu\text{g}\cdot\text{ml}^{-1}$ for MgONPs-ZnONPs. The MgONPs-ZnONPs was not toxic to Vero cell lines however ZnONPs could inhibit the Vero and HepG 2 cell lines. We found that ZnONPs and mixture MgONPs-ZnONPs not only have higher bactericide behavior but might have also synergistic effects against MDR-TB.

Background

Multi-drug-resistant tuberculosis (MDR-TB) is a form of *tuberculosis* (TB) infection caused by *Mycobacterium tuberculosis* (Mtb) that are **resistant** to treatment with at least **isoniazid** (INH) and **rifampin** (RMP). It is estimated that *MDR-TB* caused 480,000 new TB cases and 250,000 deaths in 2015 [1].

Today, nanotechnology has attracted medical researchers for over a century and is now with heavy steps used in biomedical sciences [2]. The researchers have found, metallic NPs have shown anti-tubercular properties against Mtb [3]. In fact, there is a focus of interest related to using mixed metallic NPs because of their outstanding potential against MDR-TB [2, 4, 5].

Studies have shown that in the process pathogenesis of pulmonary tuberculosis, the metallic NPs can penetrate within the calcified granuloma and can attach to Mtb and kill it. The metallic NPs can also eliminate Mtb into the macrophage without any toxicity effect. Therefore, the metallic NPs especially mixed metallic NPs have potentially capable of electrostatic interactions with the Mtb cell membrane [6].

The anti-tubercular potency of metallic NPs such as MgONPs and ZnONPs are still unknown against MDR-Mtb. It is necessary to establish consistent conditions in order to directly compare the effects of the MgONPs and ZnONPs dosages on a clinical strain of MDR-Mtb. Under the consistent conditions, we investigated and compared the effects of different dosage of mixture MgONPs and ZnONPs on two different types of clinical isolates MDR-Mtb and their standard strain. According to the search, this is the first study to use the "Microplate Alamar Blue" method as a rapid and a safety test in order to quantify and compare anti-tubercular activities of mixture MgONPs and ZnONPs against two drug-resistant strains of Mtb clinical isolates with standard strain. Moreover, this is the first time to study the MgONPs-ZnONPs toxicity on African green monkey (Vero) and human liver cancer (HepG₂) cell lines.

Methods

Study design and bacterial isolates

The multidrug resistance strain of Mtb isolated from a 55-year-old man who was proven tuberculosis by imaging, clinical findings, histological and cytological observations, previously. The patient had consumed first-line anti-tubercular treatment for 6 months but the symptoms of TB had reappeared about 7 months after completing the treatment. Spectrum sample of patients was cultured in LJ medium for 28 days. The susceptibility drug testing was done by the indirect proportion method to determining of MDR-Mtb [7].

Preparing Nanoparticles

In this study, MgONPs (average diameter of 20 nm) and ZnONPs (average diameter of 4 nm) were purchased from Tehran University of Medical Sciences (Tehran, Iran). Before each experiment, MgONPs and ZnONPs were sterilized through heating in an oven at 200 °C for one hour.

Antibiotic susceptibility test

To determine the minimum inhibition concentration (MICs) of NPs, the microplate Alamar blue (MABA) assay is used [8]. Two hundred microliter (200 µl) of sterile deionized water added to outer-perimeter wells of 96-well microplates. Then, 100 µl of 7H9 GC broth was added to each well. 100 µl of MgONPs (12.5 µg.ml⁻¹), ZnONPs (30 µg.ml⁻¹) and MgONPs-ZnONPs (42.5 µg.ml⁻¹) solutions were added to each well, and the serial dilutions were done. A hundred microliter (100 µl) of H37Rv Mtb (Razi serum and vaccine research institute, IRAN) was added to the wells [9]. The plates incubated at 37°C for 5 days. Fifty microliters (50 µl) of a freshly prepared 1:1 mixture of Alamar blue reagent and 10% Tween 80 added to well and reinsulated at 37°C for 24 hours. The blue color in the well interpreted as no growth and a pink color scored as growth. The MICs was defined as the lowest drug concentration which prevented a color change from blue to pink [9].

To determine the minimum bactericidal concentration (MBCs) of NPs against MDR-Mtb (Razi serum and vaccine research institute, IRAN), proportion method was also done. 10 ml of melted Löwenstein-Jensen (LJ) agar and 10 ml of NPs were poured and subsequently, serial dilution was performed. On the following day, 100 µl of 10⁻² and 10⁻⁴ McFarland of bacteria were added and then they are incubated at 37 °C. The colony-forming unit (CFU) of bacteria was counted after 28 days. The atomic force microscopy (AFM) (Institute for color science & technology, IRAN) of mixture MgONPs-ZnONPs fulfilled by commercial AFM system (Nanosurf, Switzerland) [10]. The TEM image was prepared at the Institute of Biochemistry and Biophysics (IBB) of Tehran University.

MTT assay

To MTT assay, Vero and HepG₂ cell lines (Institute for tuberculosis research, the University of Illinois at Chicago, USA) (1×10⁴ cells per well) were seeded in 96-well plates containing 100 µl of DMEM,

separately [11]. 100 μl of MgONPs, ZnONPs, and MgONPs-ZnONPs were inoculated to each well. Next, 5 mg MTT per ml added. Cell viability calculated using DMSO treated Vero and HepG₂ as the 100% viable control and measured (A540 of NPs \times treated sample/A540 of control) \times 100.

Statistical analysis

Statistical analyses were prepared by using of Kruskal-Wallis test and Mann–Whitney U test. The statistical significance threshold was resolute as P-value \leq 0.05.

Results

The MIC of MgONPs was 0.195 $\mu\text{g.ml}^{-1}$ against 10⁴ of H₃₇Rv Mtb (Table 1A). According to the results, the MIC of ZnONPs was reported 0.937 $\mu\text{g.ml}^{-1}$ for 10⁻² H₃₇Rv Mtb and the MIC of ZnONPs reached to 0.468 $\mu\text{g.ml}^{-1}$ for 10⁻⁴ H₃₇Rv Mtb. As well, 0.332 $\mu\text{g.ml}^{-1}$ and 0.166 $\mu\text{g.ml}^{-1}$ of MgONPs-ZnONPs was able to inhibit 10⁻² and 10⁻⁴ H₃₇Rv Mtb, respectively (Table 1A). The MIC of MgONPs against both 10² and 10⁴ concentrations of MDR-Mtb were 12.5 $\mu\text{g.ml}^{-1}$ (Table 1A). The MIC of MgONPs/ZnONPs against 10² concentrations of MDR-Mtb was determined 0.166 $\mu\text{g.ml}^{-1}$ (Table 1A). The MIC of MgONPs/ZnONPs against 10⁴ concentrations of MDR-Mtb reached to 0.664 $\mu\text{g.ml}^{-1}$ (Table 1A).

The MBCs of MgONPs against 10² and 10⁴ concentrations of H₃₇Rv Mtb were 3.125 $\mu\text{g.ml}^{-1}$ and 1.875 $\mu\text{g.ml}^{-1}$. The MBCs of MgONPs against 10² and 10⁴ concentrations of MDR-Mtb were \geq 12.5 $\mu\text{g.ml}^{-1}$ (Table 1A). The MBC of ZnONPs was 0.937 $\mu\text{g.ml}^{-1}$ against 10⁴ concentrations of H₃₇Rv Mtb (Table 1A). Based on the results, the MBC value of ZnONPs increased to 1.875 $\mu\text{g.ml}^{-1}$ against 10⁻⁴ concentrations of MDR-Mtb. On the other hands, testing showed that the MBCs of MgONPs/ZnONPs reached to 1.328 $\mu\text{g.ml}^{-1}$ against 10⁴ concentrations of both H₃₇Rv Mtb and MDR-Mtb (Table 1A).

Dose-response curves showed ZnONPs and MgONPs-ZnONPs have high potency to eliminate 10⁻⁴ MDR-Mtb (Figure 1).

AFM image confirms that the MgONPs-ZnONPs are mono-disperse (Figure 2a). The sizes of the NPs were estimated at less than 50 nm. As we show in Figure 2a, some of NPs attached to the cell wall of Mtb (Figure 2a). It showed that agglomerated metallic NPs are able to attach to the cell wall and penetrates onto Mtb. The oval-shaped Mtb in the present of MgONPs-ZnONPs was demonstrated by TEM images (See also Figure 2b).

MTT assay results showed that MgONPs inhibited the growth of HepG₂ cell lines (Table 1B). Also, 3.579 $\mu\text{g.ml}^{-1}$ of ZnONPs was able to inhibit the growth of HepG₂ and Vero cell lines. The results showed

that the toxicity of mixture MgONPs-ZnONPs reached to $1.233 \mu\text{g.ml}^{-1}$ against Vero cells. The results indicated the IC_{50} of HepG₂ cell lines in exposure to MgONPs -ZnONPs reached to $0.414 \mu\text{g.ml}^{-1}$.

Discussion

The current study is the first experimental study of which has evaluated the MICs and MBCs of ZnONPs, MgONPs, and MgONPs-ZnONPs against H₃₇Rv Mtb and the MDR-Mtb. In this study, the toxicity effects of the NPs have evaluated, as well. This study reported the MIC of MgONPs against MDR-Mtb was $12.5 \mu\text{g.ml}^{-1}$ (Table 1A). Up to now, there has not been the report in the light of antibacterial effects of MgONPs against *Mycobacterium tuberculosis*. Previously, Nhu-YThi Nguyen has been reported that MgONPs was able to inhibit gram-positive, gram-negative, and endospore-forming bacteria [12]. Punjabi reported that $1.25 \mu\text{g.ml}^{-1}$ of ZnONPs inhibited clinical isolated of MDR-TB. 4 Patil et al., reported that ZnONPs has ability to inhibit Mtb at $12.5 \mu\text{g.ml}^{-1}$ [13]. We showed that the ZnONPs has the ability to eliminate MDR-TB at $1.875 \mu\text{g.ml}^{-1}$ (Table 1A). Mixture MnO₂NPs-ZnONPs also showed bactericidal effects on MDR-TB at $1.328 \mu\text{g.ml}^{-1}$ the dose-response curves also showed that anti-tubercular impact of NPs depends on the concentration of MDR-TB (Figure 1).

The size of NPs may also affect their anti-tubercular properties. The previous study showed that the ZnONPs with smaller size exhibited higher efficiencies against Mtb [14]. The bigger size of MgONPs - about 20 nm- in comparison with ZnONPs -about 4 nm- might be effective on weakly anti-tubercular activities of MgONPs. Moreover, researchers believed that metallic NPs have a strong tendency to agglomerate in the broth suspension because of their high surface energy, which could have affected the interactions and penetrations of these nanoparticles with Mtb.

Limitations

We believe that there are no standards of regular experimental techniques for testing the anti-tubercular properties of NPs. In fact, NPs with varying sizes, physicochemical characterizes, concentrations, and different initial seeding densities have been used in the previous studies, which do affect the results, significantly. We found that ZnONPs and mixture MgONPs-ZnONPs not only have higher bactericide behavior but might have also synergistic effects against MDR-TB. Actually, even though the current study does prove that MgO and ZnONPs have anti-tubercular properties against MDR-Mtb, the anti-tubercular potency of mixture MgONPs/ZnONPs is still unknown and incomparable.

Abbreviations

Mtb: *Mycobacterium tuberculosis*

MDR-TB: Multidrug-resistant *Mycobacterium tuberculosis*

ZnONPs: Zinc oxide nanoparticles

MgONPs: Magnesium oxide nanoparticles

IC50: The half maximal inhibitory concentration

HepG₂: Human liver cancer cell line

Vero: African green monkey cell line

MICs: Minimum inhibition concentration

LJ: Löwenstein-Jensen agar

CFU: Colony-forming unit

AFM: Atomic force microscopy

IBB: Institute of Biochemistry and Biophysics

Declarations

Ethics approval and consent to participate

This study was in accordance with the declaration of Helsinki and an ethical approval was sought from the institutional Ethics Committee of Guilan University of Medical Sciences (Approval No. IR.GUMS.REC.1396.481). However, because we only used leftovers from clinical specimens, the local ethics committee waived the need for informed consent.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

M. Shahriari and A. Jafari: conceived the study. M. Shahriari and A. Jafari: participated in the design of the study and performed the statistical analysis. A.A. Foumani and S. Falahatkar: interpreted the data. A. Jafari: obtained ethical clearance and permission for study. S. Falahatkar: Supervised data collectors. F. Movahedzadeh and A. Jafari: Drafting the article or revisiting it critically for important intellectual content. A.A. Foumani: was project leaders and primary investigators of the study. All authors read and approved the final manuscript.

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Tables

Table 1: The MICs, MBCs (A) and MTT assay (B) results of MgONPs, ZnONPs and mixture MgONPs-ZnONPs

(A)

Nanoparticle	Highest concentration ($\mu\text{g.ml}^{-1}$)	<i>H₃₇Rv</i>				<i>MDR-TB</i>			
		MIC ($\mu\text{g.ml}^{-1}$)		MBC ($\mu\text{g.ml}^{-1}$)		MIC ($\mu\text{g.ml}^{-1}$)		MBC ($\mu\text{g.ml}^{-1}$)	
		10^{-2}	10^{-4}	10^{-2}	10^{-4}	10^{-2}	10^{-4}	10^{-2}	10^{-4}
MgO ₂	12.5	0.390	0.195	3.125	1.875	12.5	12.5	12.≤ 5	12.≤ 5
ZnO	30	0.937	0.468	7.5	0.937	1.875	0.937	7.5	1.875
MgONPs/ZnO NPs	42.5	0.332	0.166	2.656	1.328	1.328	0.664	5.312	1.328

(B)

Nanoparticle	Initial concentration ($\mu\text{g.ml}^{-1}$)	Stock ($\mu\text{g.ml}^{-1}$)	IC ₅₀ of <i>HepG</i> ₂ cell lines ($\mu\text{g.ml}^{-1}$)	IC ₅₀ of <i>Vero</i> cell lines ($\mu\text{g.ml}^{-1}$)
MgO ₂	25	12.5	0.279	1.134
ZnO	60	30	3.579	3.579
MgO NPs/ZnO NPs	42.5	21.25	0.414	1.233

Figures

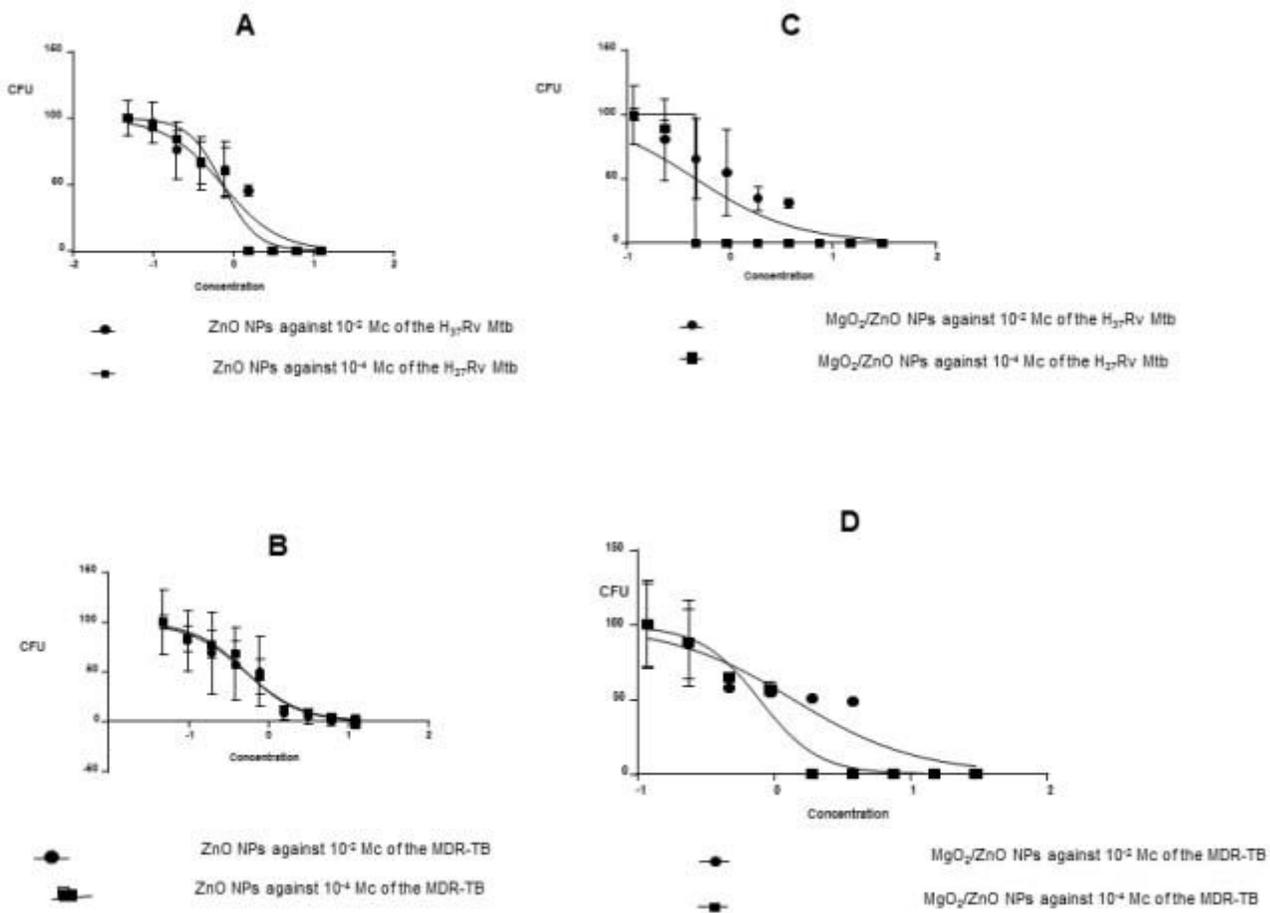


Figure 1

The dose – response curves of ZnONPs against H37Rv Mtb (a) and against MDR-Mtb (b). The dose – response curves of MgONPs-ZnONPs against H37Rv Mtb (c) and against MDR-Mtb (d) in 10⁻² and 10⁻⁴ McFarland.

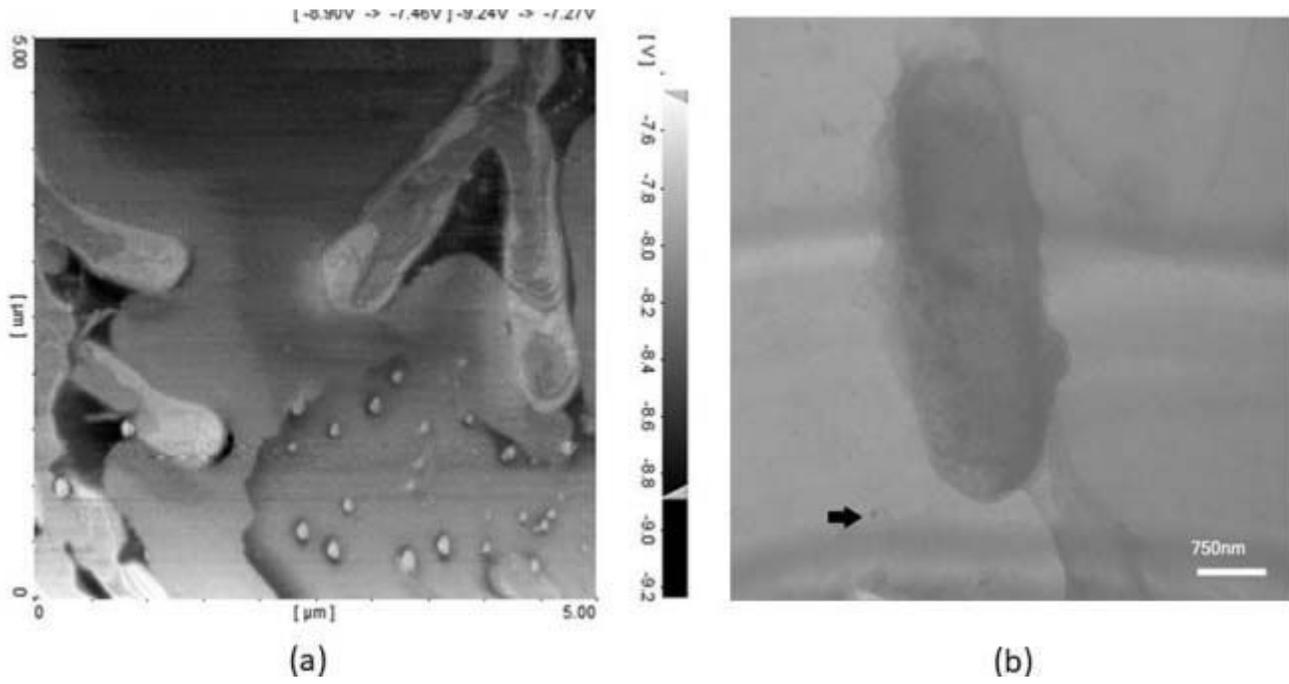


Figure 2

AFM image of H37Rv Mtb and mixed MgONPs-ZnONPs (a) on silicon surface and TEM image (b) of NPs around of H37Rv Mtb on the scale of 750 nm.