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### Original Article

## In Vitro Study on Cytotoxic Effects of ZnO Nanoparticles on Promastigote and Amastigote Forms of *Leishmania major* (MRHO/IR/75/ER)

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#### Abstract

**Background:** Although pentavalent antimony compounds are used as antileishmanial drugs but they are associated with limitations and several adverse complications. Therefore, always effort to find a new and effective treatment is desired. In this study, the effect of ZnO nanoparticles with mean particle size of 20 nanometers (nm) on *Leishmania major* promastigotes and amastigotes was evaluated.

**Methods:** Viability percentage of promastigotes after adding different concentrations of ZnO nanoparticles (30, 60, 90 and 120 µg/ml) to the parasite culture was evaluated by MTT assay. In the flow cytometry study, Annexin V-FITC Apoptosis detection Kit was used to study the induced apoptosis and necrotic effects.

**Result:** IC<sub>50</sub> after 24 hours of incubation was 37.8 µg/ml. ZnO nanoparticles exert cytotoxic effects on promastigotes of *L. major* through the induction of apoptosis. A concentration of 120 µg/ml of ZnO nanoparticles induced 93.76% apoptosis in *L. major* after 72 hours.

**Conclusion:** ZnO NPs can induce apoptosis in *L. major* by dose and time-dependent manner in vitro condition.

### Introduction

Cutaneous Leishmaniasis is a major public health problem caused by the genus *Leishmania* and is transmitted by the bite of a sand fly (1). It is endemic in 98 countries and the incidence of this disease is

0.7 to 1.2 million cases annually in the world (2). Cutaneous lesions resulted from development of nodules that converse to ulcerative lesions (3). The drugs which World Health Organization (WHO) recommends for cuta-

neous leishmaniasis are antimonial compounds (4). These drugs have different side effects; also the recrudescence may occur (5). Also, drug resistance to pentavalent antimonials which have been recommended for the treatment of leishmaniasis has been reported in endemic countries (6). Whereas, there is not any effective vaccine against leishmaniasis; so, investigation to find effective drugs is taken into consideration (5). During the last two decades, many attempts have been made to develop effective new compounds for treatment of cutaneous leishmaniasis (CL) that would be economical, applicable topically to lesions and could avoid development of resistance (7). Cosmetically unacceptable lesions, chronic lesions, large lesions, lesions in immunosuppressed patients, lesions over joints, multiple lesions, nodular lymphangitis and worsening lesions are reasons to treatment of cutaneous leishmaniasis (7).

Nanoparticles have unique physicochemical properties such as tiny size, great surface area, electrical charge and shape (8). Metal oxide nanoparticles have different usage in the various sciences (9). The nanoparticles are commonly used in medicine in drug delivery and cancer therapy (10). ZnO is one of the five zinc compounds that are currently listed as generally recognized as safe by the U.S. Food and Drug Administration (11). This nanoparticle has an antibacterial effect on gram-positive and gram-negative bacteria such as *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (11,12). Antibacterial activity of ZnO is attributed to the generation of reactive oxygen species (ROS) on the surface of these oxides (13,14). Zinc oxide increases fat oxidation in prokaryotic and eukaryotic cell membranes (11) and it is effective on resistant microorganisms (15). Wang et al. (2008) have investigated the fatality rate of zinc oxide, aluminium oxide and titanium oxide on *Caenorhabditis elegans* nematode eggs (8). Torabi et al. (2011) and Mohebbali et al. (2009) have investigated antileishmanial activity of gold and silver na-

noparticles on Iranian strain of *L. major* (16,17).

The present investigation was aimed to evaluate the antileishmanial activity of ZnO nanoparticles on *L. major* in vitro condition.

## Materials and Methods

### Nanoparticle

ZnO nanoparticle powder with mean particle size of 20 nanometers (nm) was purchased from Selechem Company, USA. The stock of ZnO nanoparticle was dispersed in ultrapure water by sonication at 100W and 40 kHz for 40 min for forming homogeneous suspensions. The NPs were then serially diluted in sterile ultrapure water and additionally sonicated for 40 min. Small magnetic bars were placed in the suspensions for stirring during dilution to avoid aggregation and deposition of particles.

### Parasite culture

*L. major* parasites (MRHO/IR/75/ER) prepared from Razi vaccine and serum research institute of Iran. The promastigotes were cultivated in RPMI 1640 medium containing 100 units/ml penicillin, streptomycin 100 µg/ml and 20% FBS in a 25 ± 1 °C incubator. The infectivity of the parasites was maintained by serial subcutaneous passage in BALB/c mice.

### Anti-promastigote assay

Promastigotes of *L. major* was cultured in RPMI culture medium supplemented with 15% FBS in 96-well plates in density of 2 × 10<sup>6</sup> cell/ml as triplicate in the presence of 30, 60, 90 and 120 µg/ml of ZnO nanoparticles and were incubated at 24 °C. In order to evaluate the parasite survival, the multiplication of the promastigotes was determined by counting the cells by hemocytometer chamber (Neubauer chamber) before and after adding the nanoparticles after 24, 48 and 72 h of incubation. In negative control group, promastigotes were cultured as triplicate without ZnO nanoparticles. GraphPad prism5 was used to determine the IC50.

### **MTT assay**

The promastigotes viability was estimated by MTT assay. *Leishmania major* promastigotes were cultured in 96-well plates ( $2 \times 10^5$  parasites/well) in the presence of 30, 60, 90 and 120  $\mu\text{g/ml}$  of ZnO nanoparticles and were incubated at 24 °C. Promastigotes without NPs with RPMI 1640 supplemented with 15% FBS were considered as a control group. Three wells also were considered as blank wells which only contained 100  $\mu\text{l}$  culture medium. After 24, 48 and 72h incubation periods of the wells, 20  $\mu\text{l}$  MTT (5mg/ml) reagent was added per well, then they incubated for 4h at 24 °C in dark room. The cells were centrifuged at 3000 rpm for 10 min and 100  $\mu\text{l}$  DMSO (dimethyl sulfoxide) was added to pellets and incubated again. After 10 min optical density (OD) of plate was read by an ELISA reader at 540 nm. Viability percentage was calculated by:

$$[(AT-AB) / (AC-AB)] \times 100.$$

Where, AB is OD of blank well, AC is OD of negative control and AT is OD of treated cells.

### **Anti-amastigote assay**

The peritoneal macrophages were isolated from the peritoneum of BALB/c mice by injection the cold phosphate-buffered saline (PBS) and re-aspiration. Isolated macrophages were seeded on a glass coverslip in tissue culture 12-well plates (18) and incubated at 37 °C with 5% CO<sub>2</sub> for 24 h.

Non-adherent cells were removed by two washes PBS. Adherent macrophages were infected with the stationary growth phase of promastigotes at a parasite/macrophage ratio of 10:1, then plate incubated for 24h at 37 °C with 5% CO<sub>2</sub>. Non-internalized promastigotes were removed by washing with cold PBS. Infected macrophages were further incubated in the presence or absence (negative control group) concentrations of the ZnO NPs for 24 h and 48 h. Infected macrophages in coverslip were stained with Giemsa stain, and the amastigotes inside the macrophage (100 mac-

rophages per cover slip) were counted under a light microscope.

### **Flow cytometry analysis for inducing apoptosis**

The Annexin-V FLUOS Staining Kit (Biovision, USA) was used for the detection of apoptotic and necrotic cells. The promastigotes were cultured in 24well plates ( $3 \times 10^5$  parasites/well) in the absence (negative control group) and the presence of 30, 60, 90 and 120  $\mu\text{g/ml}$  of ZnO nanoparticles and were incubated at 24 °C. According to the kit instruction, the promastigotes were collected after 24, 48 and 72h incubation and centrifuged at 3000 rpm for 5 min, then supernatant was discharged, and 500 $\mu\text{l}$  binding buffer, 5 $\mu\text{l}$  annexin and 5 $\mu\text{l}$  propidium iodide (PI) were added to the residue. The samples incubated at room temperature and dark situation for 5min. Then they were obtained by BD FACSCanto II and were analysed by FlowJo Software.

### **Data Analysis**

One-way ANOVA were used to analyse the obtained results with SPSS (version 16) software, and a probability (*P*) value of <0.05 was considered as statistically significant.

## **Results**

### **The effect of ZnO NPs on promastigotes growth**

Growth inhibitory effect of four concentrations (30, 60, 90 and 120  $\mu\text{g/ml}$ ) of ZnO NPs on the promastigotes was evaluated at 24, 48 and 72 h (Table 1). The result showed that the growth inhibitory effect is dose dependent where the concentration of 30 $\mu\text{g/ml}$  after 24h incubation and the concentration of 120  $\mu\text{g/ml}$  after 72h incubation show minimum and maximum percent of mortality rate respectively. Statistically significant difference was found between test and control groups. The IC<sub>50</sub> of ZnO NPs was measured 37.8  $\mu\text{g/ml}$ .

**Table 1:** Mean number (Cell/ml) and percentage of viable parasites after adding different concentrations of ZnO NPs

ZNO NPs (µg/ml)	After 24h		Viable parasites		After 72h	
	No.	%	No.	%	No.	%
30	653750	52.3	637050	46.5	600000	40
60	473750	37.9	465800	34	435000	29
90	387500	31	369900	27	352500	23.5
120	350000	28	315100	23	292500	19.5
Control	1250000		1370231		1505000	

Numbers of promastigotes were counted by hemocytometer chamber (Neubauer chamber) before and after adding the nanoparticles. Viability percentage obtained using number of parasites in test and control groups.

### Cytotoxic effect of ZnO NPs on *L. major* by MTT assay

The cytotoxic effect of ZnO NPs on promastigotes of *L. major* was evaluated at four concentrations (30, 60, 90 and 120 µg/ml) for 24, 48 and 72 h. The concentration of 120 µg/ml of ZnO NPs after 72h shows maximum cytotoxic effect on promastigotes of *L. major*. Result shows, by increasing the NPs concentration, the viability of promastigotes will decrease (Table 2).

### Antiamastigote assay

The anti-amastigote activity of ZnO NPs was evaluated in infected macrophage. Result shows, by increasing the NPs concentration, the number of amastigotes in infected macrophage will decrease. Mean number of amastigotes /macrophage after 48h in the negative control group were 4.8 and in 30 µg/ml and 120 µg/ml of ZnO NPs groups

were 1.5 and 0.8 respectively (Table 3). These results showed the significant reduction of mean number of amastigotes in test groups compared to the control groups.

### Induced apoptosis by ZnO NPs

Flow cytometric analysis shows, following treatment of promastigotes with four concentrations (30 , 60 , 90 and 120 µg/ml) of ZnO NPs for 24, 48 and 72 h, necrotic and apoptotic effects in the parasite were emerged. The percent of apoptosis (Early and Late Apoptosis) in promastigotes induced by 30, 60, 90 and 120 µg/ml of ZnO NPs after 24 h was 31.57%, 47.2%, 63.8% and 71.92% respectively. Induced apoptosis (Early and Late Apoptosis) in promastigotes after 48h was 37.9% , 56% , 67% and 79.58% respectively and after 72h was 46.4% , 62.8% , 71.5% and 93.76% respectively (Fig. 1).

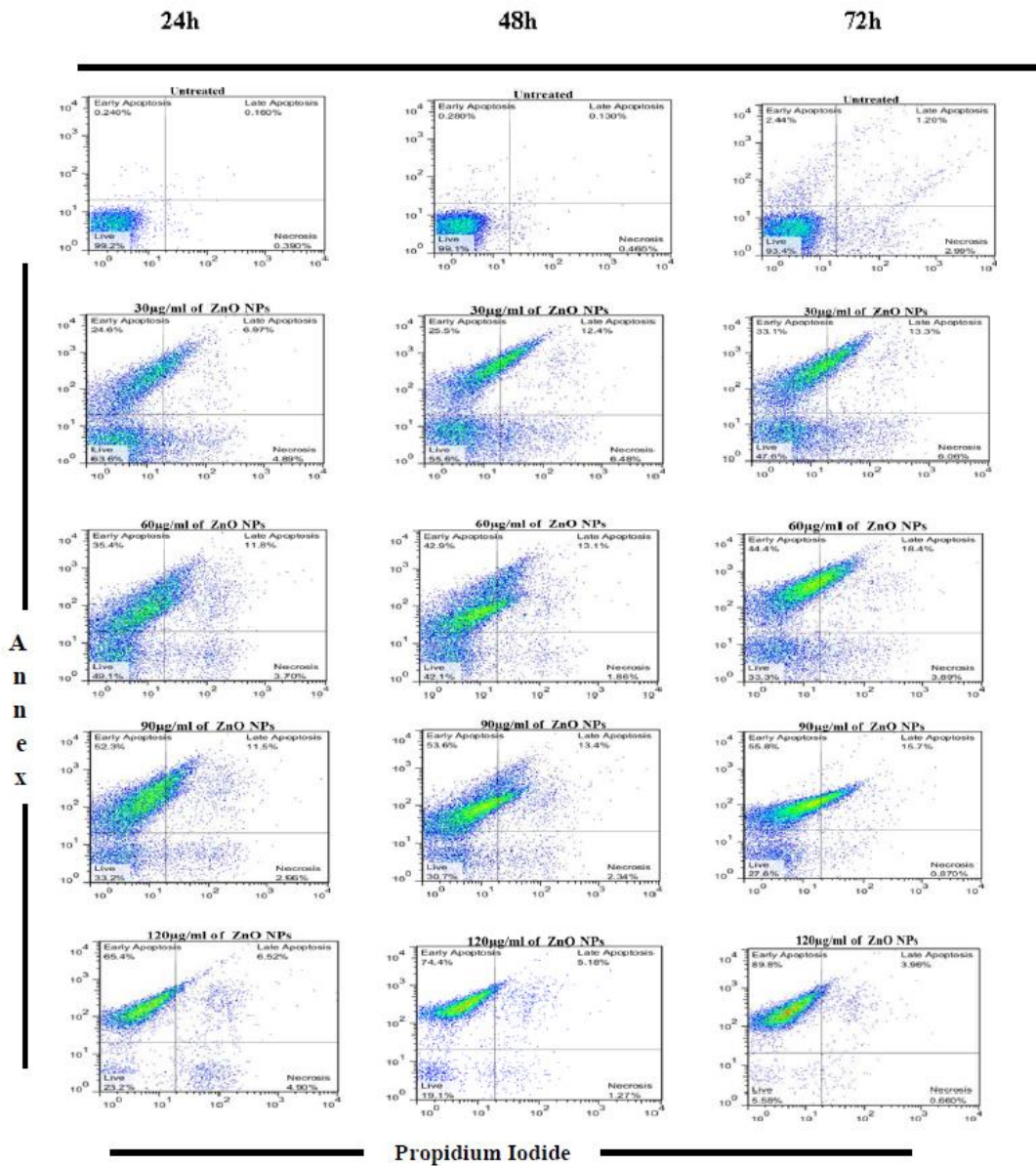
**Table 2:** Viability percentage of promastigotes following treatment with different concentrations of ZnO NPs

ZNO NPs Concentrations (µg/ml)	Percent viability of promastigotes after adding Nanoparticles		
	24h	48h	72h
30	55	50	47.8
60	45.3	43	38.9
90	37.1	36	31
120	33	29.5	22



**Table 3:** Mean numbers of amastigote /macrophage at 24 and 48h after treatment with different concentrations of ZnO NPs

ZNO NPs Concentrations( $\mu\text{g/ml}$ )	Mean number of amastigote/macrophage	
	24h	48h
30	1.8	1.5
60	1.47	1.3
90	1.2	1.12
120	0.95	0.8
Control	4.3	4.8



**Fig.1:** Flow cytometry result. Promastigotes staining with Annexin V and Propidium Iodide after treatment with different concentrations of ZnO NPs in three periods of time (24, 48 and 72 h)

## Discussion

Antimonial compounds are the drugs of choice for treatment of cutaneous leishmaniasis, whereas amphotericin B and pentamidine or miltefosin is being used as alternative drugs (4, 19). Use of pentavalent antimonials to treat leishmaniasis is associated with a range of cardiac, biochemical and blood factors adverse effects (20, 21). The result of a study conducted on an Iranian strain of *L. major* showed that miltefosine has a good activity against *L. major* and it is suggested as effective anti-leishmanial agent (19). Also, miltefosine effectiveness in treating cutaneous leishmaniasis caused by *L. major* compared to meglumine antimoniate was evaluated in a clinical trial (22) in 2007. The results showed that miltefosine is apparently at least as good as meglumine antimoniate in cutaneous leishmaniasis treatment (22). But, all of these medicines have different toxic effects and show some resistance to the infection. Therefore, there is an urgent need to find a new anti-leishmaniasis drug (4).

Since the nanoparticles have potential in the search for new agents against different microorganisms. In the present study, we examined leishmanicidal activity of ZnO NPs on the Iranian strain of *L. major*. To our knowledge, based on a search on the literature, no studies have been conducted on the cytotoxicity effects of ZnO NPs on *L. major* in vitro. In this work, a relevant viability test (MTT) was used to evaluate the cytotoxic effect of ZnO NPs on *L. major* promastigotes.

Results of this study showed that ZnO NPs have dose-dependent anti-leishmanial activities. Counting number of parasites indicated statistically significant difference between test and control groups. Other nanoparticles such as nanogold, nanosilver and nanoselenium similarly have been reported possess anti-leishmanial activities on *L. major* and *L. infantum* (16,17,23). Based on the previous studies, ZnO NPs have many effects on the vast

amount of microorganisms including bacteria, viruses, fungi and parasites. Furthermore ZnO nanoparticles as one of the multifunctional inorganic nanoparticles has many significant features such as chemical and physical stability, high catalysis activity, with varied applications as semiconductors, sensors, solar cells, etc. (12).

Some other anti-*Leishmania* drugs like Miltefosine can induce apoptosis in *Leishmania*. Miltefosine induces apoptosis in *L. donovani* through increasing cytochrome c releasing, followed by mitochondrial membrane permeability reduction (24). But antileishmanial mechanism of ZnO NPs is yet unknown. According to other studies, the drug has lethal effects against cancer cells and fibroblasts. According to the Ahamed et al. results, ZnO nanorod induces apoptosis in human alveolar adenocarcinoma cells via p53, surviving and Bax/bcl-2 pathways (9). However, ZnO NPs have been reported that can induce apoptosis in human dermal fibroblasts (25) and cell death in human mesothelioma and rodent fibroblasts (26).

## Conclusion

ZnO NPs can induce apoptosis in *L. major* by dose and time-dependent manner in vitro condition. Search for the antileishmanial mechanism of ZnO NPs can be an interesting subject for study in the future.

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