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## The Cytotoxic Effect of ZnO Nps Against the Intracellular Amastigotes of *Leishmania Donovanii* in Vitro

Marwa M. Salih\*, Khawla H. Zghair

Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.

### Abstract

Leishmania parasite preferentially infect host phagocytic cells, primarily dendritic cells and macrophages. One of the main problems with Leishmania infections is the capability of these parasites to evade and subvert immune responses of the host. Leishmaniasis is treated with a small arsenal of drugs; all of them have disadvantages in terms of efficacy, high price, toxicity or treatment regimen. In this study, the effect of Zinc oxide nanoparticles (ZnO NPs) was evaluated against intracellular amastigotes *Leishmania donovani* in vitro conditions. The effect of different concentrations from ZnO NPs (0.18, 0.37, 0.75 and 1.5  $\mu\text{g} / \text{ml}$ ) was used to study on the viability of amastigotes and macrophages "following infection" using the colorimetric (MTT) assay. The results have been shown that the ZnO NPs have a cytotoxic effect on the proliferation of the amastigotes forms and have no effect. The IC50 of ZnO NPs on amastigotes was (0.610  $\mu\text{g}/ \text{ml}$ ).

This study concluded that the used concentrations of ZnO NPs have the ability to stimulate macrophages activity and promote the suppressive effects on *L. donovani* intracellular amastigotes in vitro following infection. These results may contribute to the production of an effective, non-toxic and cheap drug against Leishmania parasites

**Keywords:** Zinc oxide nanoparticles, Visceral leishmaniasis, Macrophages.

## التأثير السمي لدقائق أكسيد الزنك متناهية الصغر على الطور عديم السوط داخل الخلية للشمانيا الحشوية خارج الجسم الحي

مروة محمد صالح\* ، خولة حوري زغير

قسم علوم الحياة، كلية العلوم، جامعة بغداد، بغداد، العراق.

### الخلاصة

يصيب طفيلي الـشمانيا بشكل تفضيلي الخلايا الملتهمه وفي المقام الأول الخلايا الجذعية و البلعميه. أحد المشاكل الرئيسية مع عدوى الـشمانيا هو قدرة هذه الـشمانيا على التملص وتخريب الاستجابة المناعية للمضيف، تعالج امراض الـشمانيات بترسانه صغيره من الأويه، كلها ذات عيوب تشمل،الفعاليه،السعر المرتفع، السمية، وطرقيه العلاج. في هذه الدراسه،تم تقييم تأثير جزيئات أكسيد الزنك متناهية الصغر على الطور عديم السوط للشمانيا الحشوية في الظروف المختبريه.

تم دراسة تأثير تراكيز مختلفة من جزيئات اوكسيد الزنك متناهية الصغر (0.18، 0.37، 0.75، و 1.5 ميكروغرام / مل) على حيوية الطور المتكيس والبلاعم بعد العدوى الطفيلية باستخدام فحص اللونية (MTT assay). أشارت النتائج إلى فعالية (ZnO NPs) السمية على نمو الطور عديم السوط للشمانيا وعدم تأثيرها على الخلايا البلعمية. تم حساب قيمة ال IC50 لجزيئات ZnO NPs وكانت (0.610 µg/ml / مل).

## Introduction

Leishmaniasis is caused by an obligate intramacrophage protozoan of the genus *Leishmania*. In tropical regions protozoan parasitic diseases pose a main public health problem. After malaria, leishmaniasis stands second in having a high morbidity and mortality burden leading to economic loss. It widely manifests as cutaneous leishmaniasis (CL), mucocutaneous and visceral leishmaniasis (VL), or kala-azar. *Leishmania donovani* is the causative agent of VL in the Indian subcontinent and Africa; *L. infantum* causes VL in the Mediterranean basin [1]. VL is the most dangerous leishmaniasis form, it is characterized by fever, cachexia, hyper-gamaglobulinemia and hepatosplenomegaly, when untreated can be fatal [2]. The innate immune responses against *Leishmania* include phagocytes, Natural killer (NK) cells and cytokines [3]. Phagocytosis and anti-leishmanial activity of macrophages are the major factors in the elimination of *Leishmania* parasites. One route to estimate the infectivity of *Leishmania* and the anti-parasite immune response is to assess the germicidal activity of macrophages via the generation of reactive oxygen and nitrogen intermediates, particularly nitric oxide (NO) [4, 5]. The treatment for leishmaniasis is primarily dependent upon antimonial compounds as first-line drugs (e.g., meglumine antimoniate and sodium stibogluconate). Amphotericin B is a second-line drug exhibiting teratogenic effects and nephrotoxicity. Miltefosine is the only oral treatment available to cure leishmaniasis. Treatment failure rates are extremely high, and this fact motivates the scientific community to develop more efficient drugs [6]. These therapeutic protocols are usually expensive and cannot be used efficiently by poor countries. Moreover, these drugs are associated with increasing parasite resistance, severe toxicity, difficult route of administration and poor efficacy in endemic countries [7]. Nanoparticles are the key scientific tools that have been used in various pharmacological and biotechnological fields. The nanostructural zinc oxide has been used in the different biomedical applications in the modern world. Metal oxide nanoparticles are believed to be safe for applications because they are more stable and with salient properties [8]. ZnO nanoparticles increase antibacterial activity [9], so this study aimed to evaluate the cytotoxic effect of ZnO NPs against the intracellular amastigotes of *L. donovani* and macrophages *in vitro* conditions.

## Materials and methods

### *Leishmania* cultivation

*Leishmania donovani* strain (DUAA / IQ /2005 / MRU15) was kindly obtained from the Graduate Laboratory of Parasitology/ Department of Biology, College of Science, University of Baghdad. They were maintained and sub-cultured every 7 days in NNN media or every 5 days in M199.

Parasites were transferred from promastigots to metacyclic promastigots after counting them from planting six days old culture, centrifuged and washed two times with PBS (PH 7.2). The parasites were suspended by adding M199 and 5 % of fetal bovine serum then incubated at 37°C for 30 minutes.

### Preparation of macrophages culture

Mice were injected intraperitoneally with 5 ml of 3% thioglycollate broth. After four days, the mice were euthanized and peritoneal exudate cells were collected by lavage with Dulbecco's Modified Eagle Medium (DMEM) (without serum) and centrifuged for 10 min. Growth media DMEM were added (with serum) to the cells after discharged old ones. Macrophages incubated at 37°C and humidified atmosphere for 24 hrs. Cells were counted by haemocytometer and diluted by adding 2 ml of RBMI (with serum) for collecting cells.

### Prepared Zinc oxide Nanoparticles (ZnO NPs)

The stock of ZnO NPs (400 µg/ml) was dispersed in Dulbecco's Modified Eagle Medium (DMEM) (without serum) and sonicated at 100 W and 40 kHz for 40 min to obtain homogeneous suspension. ZnO NPs were then diluted two fold in DMEM and sonicated for 40 min. A stock solution of ZnO NPs was used to prepare, the following concentrations (0.18, 0.37, 0.75 and 1.5 µg/ml) immediately before used in each experiment.

### The cytotoxicity of ZnO NPs against *L. donovani* intracellular amastigotes and macrophages

The effect of different concentrations of ZnO NPs against amastigotes and macrophages was determined following macrophage ( $2 \times 10^3$  cells / well) contact with *L. donovani* ( $2 \times 10^4$  Parasite/ well). One hundred  $\mu\text{l}$  of macrophage culture was infected with 100  $\mu\text{l}$  of parasites at a ratio of 1:10 macrophage / Parasite and they were incubated at  $37^\circ\text{C}$  for about 4 hrs. After incubation, 50  $\mu\text{l}$  serially diluted ZnO NPs was introduced to each culture and incubated for 24 hrs and 48 hrs.

Viability percentage of parasites and macrophages following infection were detected by MTT assay, twenty eight  $\mu\text{l}$  of 2 mg/ml MTT solution ( Bio-World, USA) was added to each well and incubated for 4 hrs at  $37^\circ\text{C}$ . MTT solution was discharged, 130  $\mu\text{l}$  of DMSO (Dimethyl Sulphoxide) was added and incubated at  $37^\circ\text{C}$  for 15 min to solubilize the remaining crystals in the wells, .The absorbency was determined by a microplate reader at 584 nm.

#### Data Analysis

Differences between means have analyzed by least significant differences( LSD ) at ( $p \leq 0.05$ ) and expressed as ( Mean  $\pm$  SEM ),  $\text{IC}_{50}$  values were calculated at each experiment, x-axis represent logarithm of concentrations and y-axis represent relative density, by the interpolation method find  $\text{IC}_{50}$  value [10].

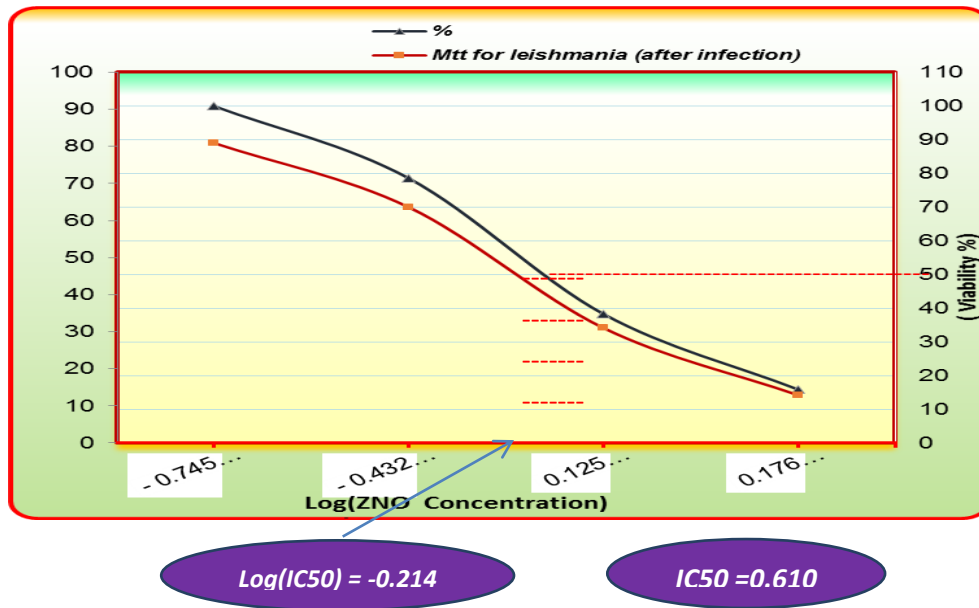
#### Results and Discussion

The cytotoxic effect of different concentrations of ZnO NPs against *L. donovani* parasites and the macrophages were determined using MTT assay following macrophages parasitic infection. The percentage of viability for *L. donovani* amastigotes following macrophages infection showed significant ( $P < 0.05$ ) differences among all used concentrations of ZnO NPs .The lowest concentration of ZnO NPs (0.18  $\mu\text{g}/\text{ml}$ ) revealed ( $80.9 \pm 0.899$ ) percentage of viable cells, however the highest one (1.5  $\mu\text{g}/\text{ml}$ ) revealed ( $12.7 \pm 0.393$ ) percentage of viable cells, Table-1. The  $\text{IC}_{50}$  of ZnO NPs against *L. donovani* amastigotes after 48 hrs of macrophage infection was 0.610  $\mu\text{g}/\text{ml}$ ; Figure-1.

**Table 1-** The effect of ZnO NPs on *L. donovani* following macrophage infection

ZnO NPs $\mu\text{g}/\text{ml}$	Percentage of viability
Control	$100.00 \pm 0.000$ a
0.18	$80.93 \pm 0.899$ b
0.37	$63.56 \pm 0.463$ c
0.75	$30.93 \pm 0.897$ d
1.5	$12.78 \pm 0.393$ e
<b>LSD <math>P \leq 0.05</math></b>	<b>1.983</b>

On the other hand, the percentage of viable macrophages, which treated with ZnO NPs “following” the parasitic infection also showed significant ( $P < 0.05$ ) differences between all used concentrations.



**Figure 1**-The  $IC_{50}$  of ZnO NPs against *L. donovani* amastigotes following infection.

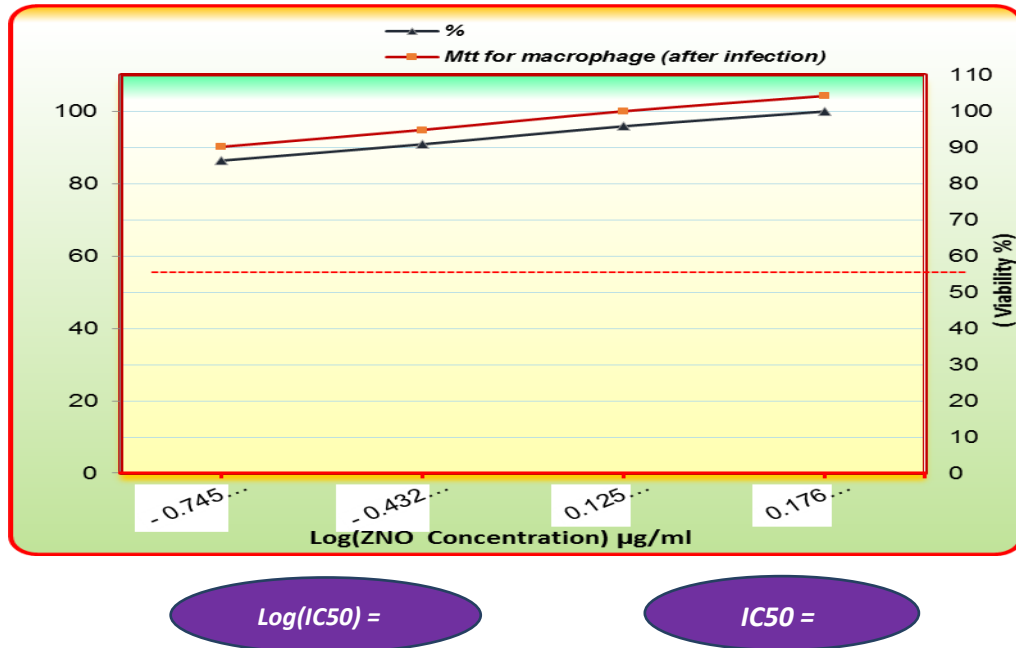
The lowest ones displayed ( $90.1 \pm 0.514$ ) and ( $94.81 \pm 0.514$ ) percentages of viable cells, while the highest one ( $1.5 \mu\text{g/ml}$ ) displayed ( $104.2 \pm 1.210$ ) percentage of viable macrophages, Table-2.

**Table 2**-The effect of ZnO NPs on macrophages following the infection with *L. donovani*

ZnO NPs $\mu\text{g/ml}$	Percentage of viability
Control	$100.00 \pm 0.000$ b
0.18	$90.10 \pm 0.514$ d
0.37	$94.81 \pm 0.514$ c
0.75	$100.00 \pm 0.541$ b
1.5	$104.25 \pm 1.210$ a
<b>LSD <math>P \leq 0.05</math>      2.371</b>	

ZnO NPs did not record  $IC_{50}$  value on treated macrophages “ following ” infection with *L. donovani*, Figure-2. These results showed that the viability of *L. donovani* amastigotes was decreased more when exposed to high concentrations of ZnO NPs. These findings are agreed with prior study confirmed the affectivity of ZnO NPs *in vitro* against *Leishmania* species [11, 12]. ZnO NPs have many effects on the great amount of microorganisms including bacteria, fungi, parasites and viruses [13]. Nano-ZnO-inhibited the growth of *Bacillus subtilis* and *E. coli* in a concentration-dependent manner, showing complete bacteriocidal effect for concentrations over than  $0.1 \text{ mg/ml}$  [14]. The  $IC_{50}$  of ZnO NPs against *L. donovani* intracellular amastigotes was ( $0.646$ ), it was lower than those obtained by Delavarim *et al.* [11], which was  $37.8 \mu\text{g/ml}$  for promastigotes of *L. major*, and higher than those obtained by Enad and Zghair [12] for *L. donovani* promastigotes which was  $0.361$ . The difference in the  $IC_{50}$ s of the drug may be due to the sensitivities of different species, and strains of *Leishmania* parasites against these drugs, also, the differences of the parasite stage susceptibilities. ZnO NPs did not record  $IC_{50}$  values against macrophage cells because the viability level stayed over than 50%. These results are agreed with a previous study confirmed that the ZnO showed no toxicity on human macrophages even at the high concentrations ( $0.625, 1.25, 2.5, 5.0, 10, 20$ ), and the

attracting attention that the high concentrations show over growth in macrophages [14]. ZnO NPs produce dissolved Zn<sup>2+</sup> ions, after then enter the cell and cause production of intracellular Reactive Oxygen Species [15].



**Figure 2-**The IC 50 of ZnO NPs exposed to macrophages following infection with *L. donovani*

Macrophages produce high amount of ROS in order to kill microbial agents such as parasites, fungi and viruses [16]. Higher amounts of ROS could be produced even when only small amounts of ZnO NPs or CuO are incorporated into cells [17]. Moreover, ROS generation is inhibited by *Leishmania* parasites through enzymatic pathways, and leads to *Leishmania* survival within macrophages as the host cells [18]. ROS-inducing nanoparticles may overcome the inhibition of ROS generation in macrophages by *Leishmania* isolates [19]. Most often, the harmful effects of ROS may be manifested by oxidations of poly-unsaturated fatty acids in lipids, oxidations of amino acids in proteins and DNA damage [20]. Through the production of toxic oxygen and nitrogen products such as NO, macrophages are the major factor in the elimination of intracellular *Leishmania* [21].

This study concluded that the used concentrations of ZnO NPs have the ability to stimulate macrophages activity and promote the suppressive effects on *L. donovani* intracellular amastigotes in vitro following infection. These results may contribute to the production of an effective, non-toxic and cheap drug against *Leishmania* parasites.

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