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Cytotoxic Effect of ZnO Nanoparticles on the Viability of *Leishmania donovani* Promastigotes *in vitro*

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Abstract

Leishmaniasis is an endemic disease in Iraq, where both forms of the disease, cutaneous and visceral, are found. The effect of Zinc oxide nanoparticles (ZnO NPs) with mean particle size less than 100 nanometer (nm) on viability and growth rate of *Leishmania donovani* promastigotes was evaluated. The anti-leishmanial activity of different concentrations (0.1, 0.2, 0.4, 0.6, 0.8, and 1 µg/ml) of ZnO NPs was investigated on promastigotes growth rates and viability in comparison to promastigotes exposed to the same concentrations of sodium stibogluconate (Sb) (pentostam).The inhibitory concentrations (IC_{50s}) of ZnO NPs were calculated after 24 , 48 and 72 hr which were (0.871, 0.156 and 0.120 µg/ml) respectively with significant ($p< 0.05$) differences between them ,while the IC_{50} of pentostam cannot calculated because more than 50% of parasites number remained viable after 24 ,48 and 72hr.The IC_{50s} also calculated on the viability results to determine the most effective concentrations. They were 0.434, 0.361 and 0.182 µg/ml for promastigotes exposed to ZnO NPs after 24, 48 and 72hr respectively, while the same concentrations of Sb in the same periods didn't revealed the IC_{50s}. The results concluded that ZnO NPs affect on the growth rate and viability of *L. donovani* promastigotes by dose and time-depended manner *in vitro* condition.

Keywords: Nanoparticles, Visceral leishmaniasis, Pentostam.

تأثير جسيمات أوكسيد الزنك النانوية على حيوية الطور الأمامي السوط لطفيلي اللشمانيا الأحشائية في المختبر

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الخلاصه

داء اللشمانيات من الامراض المتوطنه في العراق بكلا شكليه الجلدي و الحشوي. في هذه الدراسة تم تقييم تأثير الجسيمات النانوية لأوكسيد الزنك مع متوسط حجم اقل من 100 نانومتر على نسبة النمو والحيوية للمسوط الامامي لطفيلي اللشمانيا الحشوية. تم التحري عن الفعالية المضادة للشمانيا للتراكيز المختلفة (0.1, 0.2,0.4, 0.6 , 0.8 و 1مايكروغرام/مل) من جسيمات أوكسيد الزنك النانويه ZnO NPs على معدل النمو و الحيوية للطور امامي السوط بالمقارنة مع الطفيليات المعرضة الى نفس التراكيز من عقار البنتوستام .

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تم حساب التركيز المثبط لنصف عدد الطفيليات (IC_{50}) لجسيمات أوكسيد الزنك النانويه بعد مرور 24 و48 و72 ساعة والتي كانت (0.871، 0.156 و 0.120)على التوالي ،بينما لم يتم حساب التركيز المثبط لنصف عدد الطفيليات (IC_{50}) لعقار البنتوستام وذلك لأن عدد الطفيليات بقي اعلى من 50 % بعد 24 و48 و72 ساعة .

وكذلك تم حساب ال IC_{50s} من نتائج الحيويه لتحديد اكثر التراكيز الفعالة ضد حيوية الطور امامي السوط للطفيلي. وقد كانت قيمتها (0.434 ، 0.36 و 0.182 مايكرو غرام/ مل) لل ZnO NPs بعد مرور 24، 48 و 72 ساعة على التوالي ، بينما نفس التراكيز من عقار البنتوستام ولنفس الفترات الزمنيه من المعامله لم تظهر قيمة ال IC_{50} .نستنتج من ذلك بأن جسيمات أكسيد الزنك النانويه لها القدرة على التأثير على معدل النمو و الحيوية للطورامامي السوط لطفيلي اللشمانيا الأحشائية اعتمادا على الجرعة والزمن في الظروف المختبرية .

Introduction

Leishmaniasis is a vector-born disease caused by protozoan flagellates of 20 species and subspecies of the genus *Leishmania*, family Trypanosomatidae, and order Kinetoplastida [1]. The annual incidence of new cases is estimated between 1.5 and 2 million, while 12 million people are currently infected worldwide [2].

Visceral leishmaniasis (VL) which is the severe form of the disease has a mortality rate of almost 100% if untreated. It is characterized by irregular bouts of fever, substantial weight loss, hepatosplenomegaly, and anemia. *Leishmania* species responsible for this form mainly belong to *L. donovani* complex [3].

The choice drug for treatment of veisceral leishmaniasis is antimonial compounds, whereas amphotericin B and pentamidine or miltefosin is being used as alternative drugs [4,5]. Used of pentavalent antimonials to treat leishmaniasis is associated with a range of cardiac, biochemical and blood factors adverse effects [6, 7].

Nanomedicine is defining the use of nanotechnology in medicine, which has been of great interest in recent years. The use of nanoparticles (NPs) for therapeutics is one of the purposes of nanomedicine [8, 9]. In last years, an increasing percentage of nanomaterials are emerging and making advancement in different fields. Nanoparticles have unique physicochemical properties such as tiny size, great surface area, and electrical charge and shape [10]. Metal oxide nanoparticles have different usage in the various sciences [11]. The nanoparticles are commonly used in medicine in drug delivery and cancer therapy [12].

Zinc Oxid Nanoparticls (ZnO NPs) is one of the five zinc compounds that are currently listed as generally recognized as safe by the U.S. Food and Drug Administration. This nanoparticle has an antibacterial effect on gram-positive and gram-negative bacteria such as *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* [13, 14].

The present study investigates the anti-leishmanicidal activity of ZnO NPs against promastigotes growth rate and viability of *L. donovani* in comparison to pentostam activity *in vitro* conditions.

Materials and Methods

Drug concentrations

Sodium stibogluconate (Sb) or pentostam

Pentostam (100 mg/ml) was manufactured by (Glaxo Operation UK Limited Barnard Castle, Member of the Glaxo Smith Kline group companies). The drug stored below 25°C and protects from light. A stock solution of sodium stibogluconate was used to prepare the concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1 µg/ml) immediately before used.

Zinc oxide Nanoparticles (ZnO NPs)

ZnO NPs powder was purchased from Spsnda Company (Iran). NPs were prepared according to the manufacturer's procedure as follow:

The stock of ZnO NPs was dispersed in deionized 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. A stock solution of ZnO NPs was used to prepare the concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1 µg/ml) immediately before used.

Parasite culture *Leishmania donovani* strain (DUAA / IQ /2005 / MRU15) was obtained from the Medical Unit at College of Medicine, Al-Nahrain University. Promastigotes were cultivated in M199 medium containing 100 units/ml penicillin, streptomycin 100 µg/ml and 10% (Fetal Bovin Serum)FBS fore 72 hr in a 26 ℃ incubator.

Large amount of promastigotes form was harvested in log phase, they were counted using Neubauer improved bright-line haemocytometer (Weber) to calculate the numbers of parasite that required in final concentration [15]. Promastigotes were added (1×10^4 parasite/ml) to each vial contain (10 ml) M199 media with estimated concentrations of ZnO NPs and Sb drug in triplicate, then it was counted daily for 3 days using hemocytometer, prism GraphPad was used to determine the IC_{50}.

Colorimetric MTT Assay

Leishmania donovani promastigotes and test compounds (ZnO NPs and pentostam drugs at different concentrations) were prepared in 96-well microtiter plate with a final volume (100 µl/ well).

The microtiter plate was Incubated at 25 ℃ for three days, (10 µl) of MTT solution per well was added to achieve a final concentration of 0.5 mg/ml.

After another incubation period at 25℃ for 4 hours, Media was removed and 100 µl of DMSO solution was added to each well to dissolve formazan crystals products. Microtiter plate was mixed to ensure complete solubilization and left for 15 minutes. Absorbance was recorded at 630 nm by microplate reader.

Relative numbers of live cells were determined based on the optical absorbance of the treated and untreated samples and blank wells using the following formula:

Viable cells (%) = (AT-AB) / (AC-AB) ×100

Where AT is the absorbance of the treated samples, AC is the absorbance of the untreated, and AB is the absorbance of the blank [16].

Statistical analysis

Results were expressed as the concentration that inhibited parasite growth by 50% (IC_{50}) after 24, 48 and 72hr. The Statistical Analysis System- SAS (2012) program was used to effect of difference factors in study parameters [17]. Least significant difference –LSD test was used to significant compare between means in this study.

Results and Discussion

The effect of ZnO NPs on promastigotes growth rate

Growth inhibitory effect of six concentrations (0.1, 0.2, 0.4, 0. 6, 0.8 and 1 µg/ml) of ZnO NPs on the promastigotes was evaluated after 24, 48 and 72 hr. The results showed that the growth inhibitory effect is dose dependent where the 0.1µg/ml concentration of ZnO NPs after 24hr incubation and the concentration of 1 µg/ml after 72hr incubation show minimum and maximum percent of mortality rate respectively. Statistical significant ($P<0.05$) differences were found between the growth of parasites exposed to (0.1, 0.2, 0.4,0.6,0.8 and 1µg/ml) of ZnO NPs after 24 hr; it was (7.43, 6.93, 4.80, 1.26, 1.50 and 0×10^4 cell/ml) respectively, and the number of promastigotes exposed to the same concentrations of (Sb) was (8.20, 8.06,7.90,7.50,7.33 and 7.13×10^4 cell/ml) in comparison to 8.33×10^4 cell/ml for untreated promastigotes (control) . After 48 and 72 hr of exposure, promastigotes growth was decreased clearly and particularly in the high concentrations of ZnO NPs from 0.4 to 1 µg/ml as shown in Figure-1.

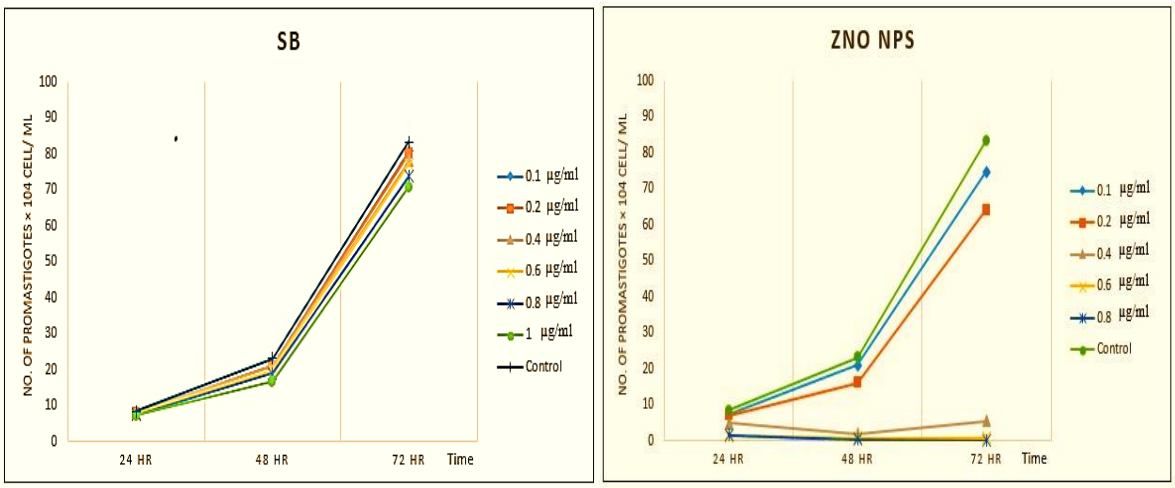

Figure 1- The effect of different concentrations of ZnO NPs and pentostam drug on growth rate *L. donovani* promastigotes**.**

These results showed high efficiency of ZnO NPs on *L. donovani* promastigotes often in high concentrations, these results were agreed with a previous study confirmed the affectivity of ZnO NPs *in vitro* against *leishmania* species [18].

Pentostam drug in the same concentrations of ZnO NPs showed lower efficiency on *L. donovani* promastigotes numbers in all using concentrations. This may be due to the fact that the pentavalent antimonials are pro-drugs that require biological reduction to the trivalent form (Sb III) for antileishmanial activity.

The half maximal inhibitory concentration (IC_{50}) for ZnO NPs were 0.871, 0.156 and 0.120 µg/ml after 24, 48 and 72hrs respectivly, with a significant ($p< 0.05$) differences between them, as shown in Table-1.

Table 1- The calculated $IC_{50\ S}$ of ZnO NPs on *L. donovani* promastigotes viability by growth rate assay.

Time (hour)	IC_{50}
24	0.871
48	0.156
72	0.120
LSD value	0.286 *
* (P<0.05).	

While IC_{50} for (Sb) couldn't be calculated as the promastigotes number exposed to the used concentrations reside above 50% after 24, 48 and 72 hr.

The current study showed that ZnO NPs have dose-dependent antileishmanial activities with lower efficiency of the used concentrations of pentostam on *L. donovani* promastigotes.

Based on the previous studies, ZnO NPs have many effects on the vast amount of microorganisms including bacteria, parasites, fungi and viruses. Furthermore, ZnO NPs as one of the multifunctional inorganic nanoparticles has many significant features such as physical and chemical stability, high catalysis activity, with varied applications as semiconductors, solar cells, sensors, ect. [13]. However, antileishmanial mechanism of ZnO NPs is yet unknown [18].

Cytotoxic effect of ZnO NPs on *L. donovani* promastigote *in vitro* by MTT assay

The MTT assay is based on the capacity of mitochondrail enzyme, succinate-dehydrogenase of viable cells to transform the MTT tetrazolium salt into a blue colored product; MTT formazan and proportional to the number of living cells present [19]. The result showed that, by increasing the ZnO NPs concentrations, the viability of promastigotes will decrease. The concentration of 1 µg/ml of

ZnO NPs after 72hr showed maximum cytotoxic effect on *L. donovani* promastigotes. Statistically, there were significant (P<0.05) differences found between viability of promastigotes exposed to ZnO NPs in the used concentrations, while, no significant (p<0.05) differences in the mean between those treated with Sb drug in all used concentrations after 24,48 and 72 hr as shown in (Tables -2 and 3).

Table 2- The viability of *L. donovani* promastigotes exposed to ZnO NPs by MTT assay after 24, 48 and 72 hr.

Concentration (µg/ml)	Time (hr.)			LSD value
	24	48	72	
0.1	86.50 ± 0.76	80.63 ± 0.44	75.50 ± 0.29	3.282 *
0.2	77.00 ± 0.57	65.70 ± 0.47	45.33 ± 0.67	2.876 *
0.4	66.17 ± 0.60	42.73 ± 0.54	27.17 ± 0.17	3.042 *
0.6	62.67 ± 0.88	14.80 ± 0.46	11.53 ± 0.29	4.189 *
0.8	57.17 ± 0.60	12.00 ± 0.57	8.43 ± 0.26	3.652 *
1	50.33 ± 0.88	10.50 ± 0.28	7.36 ± 0.18	3.507 *
* (P<0.05).				

Table 3- The viability of *L. donovani* promastigotes exposed to Pentostam by MTT assay after 24, 48 and 72 hr**.**

Concentration (µg/ml)	Time (hr.)			LSD value
	24	48	72	
0.1	89.50 ± 0.28	89.66 ± 0.67	89.86 ± 0.13	2.048 NS
0.2	87.33 ± 0.33	86.67 ± 0.33	87.80 ± 0.42	2.755 NS
0.4	86.50 ± 0.50	86.83 ± 0.44	86.33 ± 0.33	2.091 NS
0.6	86.00 ± 0.28	85.90 ± 0.37	86.20 ± 0.20	3.159 NS
0.8	85.67 ± 0.33	84.86 ± 0.13	85.67 ± 0.88	2.240 NS
1	85.67 ± 0.88	84.46 ± 0.29	84.50 ± 0.28	1.967 NS
NS: Non-0significant.				

According to the results of MTT assay the IC_{50} was calculated to determine the most effective concentrations on the viability of *L. donovani* promastigotes. The IC_{50s} of ZnO NPs after 24, 48 and 72 hr were 0.434, 0.361 and 0.182 µg/ml respectively, there was a significant (p< 0.05) difference between them as shown in Table-4, in comparison to Sb drug which doesn't revealed the IC_{50} in all used concentrations and periods.

Table 4- The calculated IC_{50}s of ZnO NPs on the viability of *L. donovani* promastigotes.

Time (hr.)	IC_{50}
24	0.434
48	0.361
72	0.182
LSD value	0.173 *
* (P<0.05).	

The using concentrations of ZnO NPs affected on *L. donovani* promastigotes viability. However, in a mimic study done by [18] used much higher concentrations (30, 60, 90 and 120 µg/ml) of ZnO NPs against *L. major* promastigotes than the present study, the viability was decreased with increasing NPs concentrations. After 72 hr the highest concentration of ZnO NPs (120 µg/ml) showed maximum cytotoxic effect on *L. major* promastigotes. Also, the number of amastigotes in the infected macrophages was decreased by the increasing of NPs concentrations.

Several studies on the metal derived ZnO NPs have revealed that this nanoparticles exhibited strong tendency to generate reactive oxygen species (ROS) in pathogens and develop oxidative stress, which causes membrane damage by electrostatic binding [20,21].

They have been widely used as antibacterial agent to treat the resistant strains [22]. The activity of ZnO NPs was evolved against *E. coli, P. aeruginosa,* and *S. aureus*. The minimum inhibitory concentration (MICs) of ZnO NPs was found to be 500 µg/mL, 500 µg/mL, and 125 µg/mL, respectively [14].

Several previous studies have been assessed the effectiveness of ZnO NPs against the viability of different cell types. Acute myeloblastic leukemia (HL60) cells and normal PBMCs were assessed for the toxicity of ZnO NPs by MTT assay. ZnO NPs showed dose-dependent cytotoxicity against HL60 cells with a 50% cytotoxic concentration (CC50) of 52.80 µg/mL, whereas the CC50 value against normal PBMCs was 741.82 µg/mL. Cancer cell line HL60 displayed strikingly greater sensitivity to ZnO toxicity, compared with the normal PBMCs. In the same assay system, bulk ZnO with the highest concentration of 1000 µg/mL did not show cytotoxicity against HL60 cells [23, 14].

The present results concluded that ZnO NPs were more effective on *L. donovani* promastigotes than pentostam drug at the same concentrations *in vitro.* The potent leishmanicidal effect of the active synthesized ZnO NPs may provide promising leads for the development of new and safer drugs against visceral leishmaniasis in near future.

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