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THE POTENTIAL OF ZNO NPS TO PROMPT APOPTOSIS IN *L. DONOVANI* PROMASTIGOTES

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ABSTRACT

Visceral leishmaniasis is a form of leishmaniasis disease that is caused by Leishmania donovani. Pentavalent antimonial are used to treat leishmaniasis infection, however they are caused many side effects. The impact of different concentrations (0.1, 0.2, 0.4, 0.6, 0.8, and 1 µg/ml) of Zinc oxide nanoparticles (ZnO NPs) on L. donovani promastigotes was assessed through the apoptotic and necrotic analysis in comparison to the same concentrations of sodium stibogluconate (pentostam) or (Sb). The Flow cytometric analysis showed clearly apoptotic and necrotic effects on the parasite which emerged only in promastigotes that treated with ZnO NPs. While promastigotes treated with pentostam showed mild apoptotic and necrotic effects emerged in high concentrations (0.8 and 1 µg/ml) only. The percentages of early apoptosis were concentrations dependent. The highest concentration (1 µg/ml) of ZnO NPs and Sb showed 61.67% and 3.33% early apoptosis in promastigotes respectively. Also, the percentages of late apoptosis of L. donovani promastigotes treated with ZnO NPs after 72 hours showed direct correlation with increasing concentrations. The lowest concentration (0.1 µg/ml) revealed 2.83% and the highest concentration (1 µg/ml) revealed 31.33% of late apoptosis, while, the percentages of late apoptosis of promastigotes exposed to Sb drug were unsteady and didn´t reliance on increasing concentrations. It concluded that ZnO NPs can activate apoptosis in L. donovani promastigotes due to the using dose in vitro condition.

KEY WORDS

Cell death, Visceral leishmaniasis, Zinc oxide nanoparticles.

Introduction

Leishmaniasis is disease caused by single cell flagellates of the genus *Leishmania*, family Trypanosomatidae, and order Kinetoplastida [1]. They are transmitted through the bite of a female sand fly species of *Lutzomia* and *Phlebotomus* [2]. In some underdeveloped countries, leishmaniasis remains a major public health problem [3]. *Leishmania* life cycle started when the parasites are transmitted to their host through the bite of an infected female sand fly. The sand fly vectors are infected when taking a blood meal from an infected vertebrate host [4, 5]. During feeding, vertebrate macrophages that contain amastigotes are ingested by the sand fly. These parasites forms, round and non-motile, are move to the posterior abdominal midgut of sand fly, where they converted to promastigotes in the vector. These forms are motile, elongated and flagellated. The promastigotes then migrate to the anterior part of the alimentary tract of the sand fly and become the infection stage (metacyclic promastigotes). These metacyclic promastigotes are reserved by host macrophage, where they transform into the amastigote form. In visceral leishmaniasis infection, lymph nodes, spleen, liver, and bone marrow can be infected in addition to any other organs that contain macrophages [6]. Visceral leishmaniasis (VL) has a mortality rate of almost 100% if untreated [7].

The choice drug for treatment of veisceral leishmaniasis is antimonial compounds, whereas amphotericin B and pentamidine or miltefosin is being used as alternative

drugs [8, 9]. For 40 years, Pentavalent antimonials are the standard antileishmanial drugs [10, 11]. Despite that, its risky use due to its reported resistance, high toxicity, various side effects and the high cost, they still the most important antileishmanial drugs [12, 13]. In Iraq, several studies have been done previously on local isolates, showed that amphotericine B and miltefosine have a good antileishmanial drug and they were suggested as effective antileishmanial agents [9]. All of these medicines have different toxic effects and showed some resistance to the infection, therefore, the need to find a new antileishmaniasis drug with low toxicity and more effective is critical [8].

Nanomedicine is the use of nanotechnology in medicine, which has been of great attentiveness in latest years [14, 15]. An increasing percentage of nanomaterials are emerging and making advancement in different fields. Nanoparticles have exclusive physicochemical properties including tiny size, excessive surface area, and electrical charge and shape [16]. Metal oxide nanoparticles have different usage in the various sciences [17]. The nanoparticles are frequently used in medicine in drug delivery and cancer therapy [18].

ZnO is as safe particles due to 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* [19, 20]. Antibacterial activity of ZnO is related to the stimulation of reactive oxygen species (ROS) [21, 22]. Zinc oxide rises fat oxidation in the cell membranes of the prokaryote and eukaryote cells [20]. This study aimed to investigate the effectiveness of ZnO Nps on the *L. donovani* promastigotes in vitro conditions.

Materials and Methods

Leishmania cultivation

Leishmania donovani strain (DUAA/ IQ/ 2005/ MRU15) was gained from the Biology Department, University of Baghdad. They were maintained and sub-cultured every 10 days in Novy- MacNeal- Nicolle (NNN) media or every 5 days in M199. Medium M199 **(**Sigma-Aldrich (USA)) was prepared according to the manufacturer's procedure.

Drug concentrations

Pentostam or Sodium stibogluconate (Sb)

Pentostam present in liquid form as an injectable ampoule (100 mg/ml), it 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. It was gained from Al-Yarmok Teaching Hospital. A stock solution of Sb was used to prepare the following concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1 µg/ml) immediately before used.

Zinc oxide Nanoparticles (ZnO NPs)

ZnO nanoparticles powder is nano-sized particles of ZnO with a size less than 100 nm was purchased from Spsnda Company (Iran). NPs were prepared according to the manufacturer's procedure. The stock of ZnO NPs was dissolved in ultrapure (deionized) water by sonication at 100W and 40 kHz for 40 min. The ZnO NPs were then serially diluted in sterile ultrapure water. Small magnetic bars were placed in the suspensions for stirring during dilution to avoid aggregation of particles. A stock solution of ZnO NPs was used to prepare the following concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1 µg/ml) immediately before used.

Annexin V (FITC) Apoptosis detection BioAssay

The Annexin-V FLUOS Staining Kit (Usbiological, USA) was used for the detection of apoptotic cells [24]. It was used for detecting early/middle stages of apoptosis and to differentiate apoptosis from necrosis. The one-step staining procedure takes only 10 minutes. The kit can perform the differences between apoptosis and necrosis by using both Annexin V-FITC and propidium iodide (PI) staining. The promastigotes were cultured in 24 well plates (1 x 10^4 parasites/ well) in the absence of ZnO NPs (negative control group) and the presence of 0.1, 0.2, 0.4, 0.6, 0.8 and 1 µg/ml of ZnO NPs. The plate was incubated at 26 °C. According to the kit instruction, the promastigotes were collected after incubated 72 hr and centrifuged at 3000 rpm for 5 minutes. Supernatant was discharged, and 500µl binding buffer, 5µl annexing and 5µl propidium iodide (PI) were added to the residue. The samples incubated at room temperature and dark situation for 5min. Then they were analyzed by CyFlow® Cube 6 (flow cytometer). The same procedure was repeated for promastigotes exposed to the known concentrations of Sb drug.

Statistical analysis

The Statistical Analysis System- SAS program was used to study the effect of difference factors in the

experiment parameters. Least significant difference – LSD test was used to compare the significant values between means in this study.

Results and Discussion

Induced apoptosis by ZnO NPs

Flow cytometric analysis was used in the treatment of *L. donovani* promastigotes with different concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1 µg/ml) of ZnO NPs and (Sb) drug. Necrotic and apoptotic effects on the parasite were emerged only in promastigotes that treated with ZnO NPs as shown in figure (1). Promastigotes treated with (Sb) showed apoptosis and necrotic effects emerged in high concentrations (0.8 and 1 µg/ml) only, figure (2).

Table (1) showed early apoptosis percentages for *L. donovani* promastigotes treated with all concentrations of ZnO NPs after 72 hr. There were a significant ($P<0.05$) differences in early apoptosis between them and with all Sb concentrations. When using ZnO NPs, the percentages of early apoptosis were concentrations dependent, while the percentages of early apoptosis in promastigotes exposed to Sb were not affected by increasing concentrations. The lowest concentration (0.1µg/ml) exhibited 2% and 4% of early apoptosis in promastigotes exposed to ZnO NPs and (Sb) respectively, while the highest concentration (1 µg/ml) showed 61.67% and 3.33% of early apoptosis in promastigotes exposed to the same treatments respectively.

Also, the percentages of late apoptosis of *L. donovani* promastigotes treated with ZnO NPs after 72 hr showed direct correlation with the increasing concentrations, thus the lowest concentration (0.1 µg/ml) revealed 2.83% and the highest one (1 µg/ml) revealed 31.33% of late apoptosis. The percentages of late apoptosis of promastigotes exposed to Sb drug were unsteady and didn't reliance on the increasing concentrations as shown in Table (2). There were a significant ($p<0.05$) differences between both treatments.

Living percentages of *L. donovani* promastigotes treated with ZnO NPs and (Sb) after 72 hr was shown in Table (3). There were a significant ($P<0.05$) differences in living percentages between the two treatments. The lowest concentrations (0.1 µg/ml) showed 89.0% and 92.33% living percentages promastigotes which exposed to ZnO NPs and (Sb) respectively, while the highest concentration (1µg/ml) showed 1.33% and 89.67% living percentages of promastigotes exposed to the same treatments respectively. Also, all Sb drug concentrations recorded convergent percentages of living promastigotes.

The percentages of necrosis in *L. donovani* promastigotes treated with ZnO NPs and Sb after 72hrs were displayed in Table (4). Both treatments showed low necrosis percentages in most of used concentrations. There was a significant ($p< 0.05$) difference only between the percentages of necrosis in promastigots exposed to the highest concentrations (1 µg/ml) of both treatments.

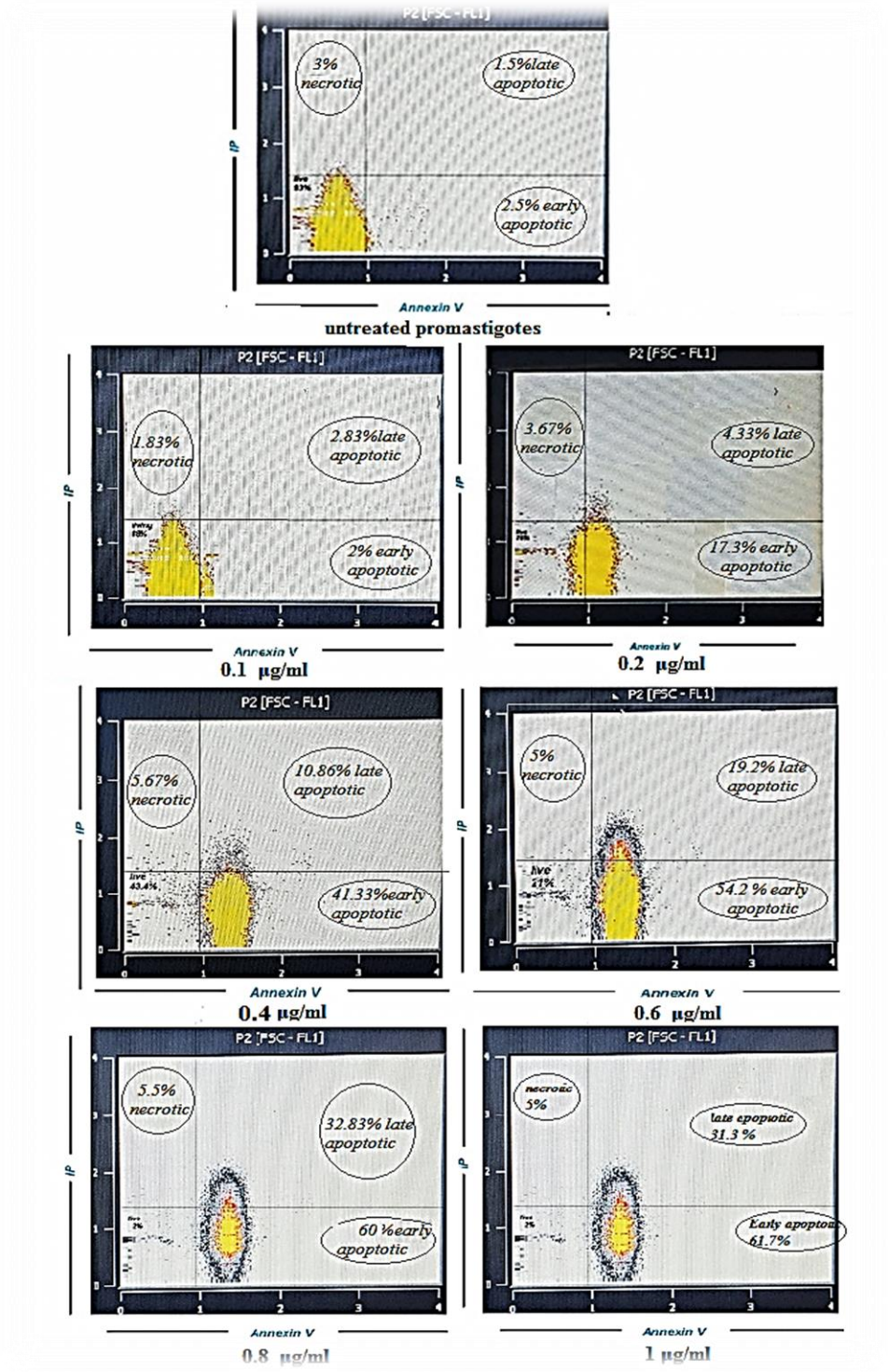

Figure 1: Flow cytometry results, promastigotes stained with Annexin V and Propidium Iodide after 72 hr of treatment with different concentrations of ZnO NPs.

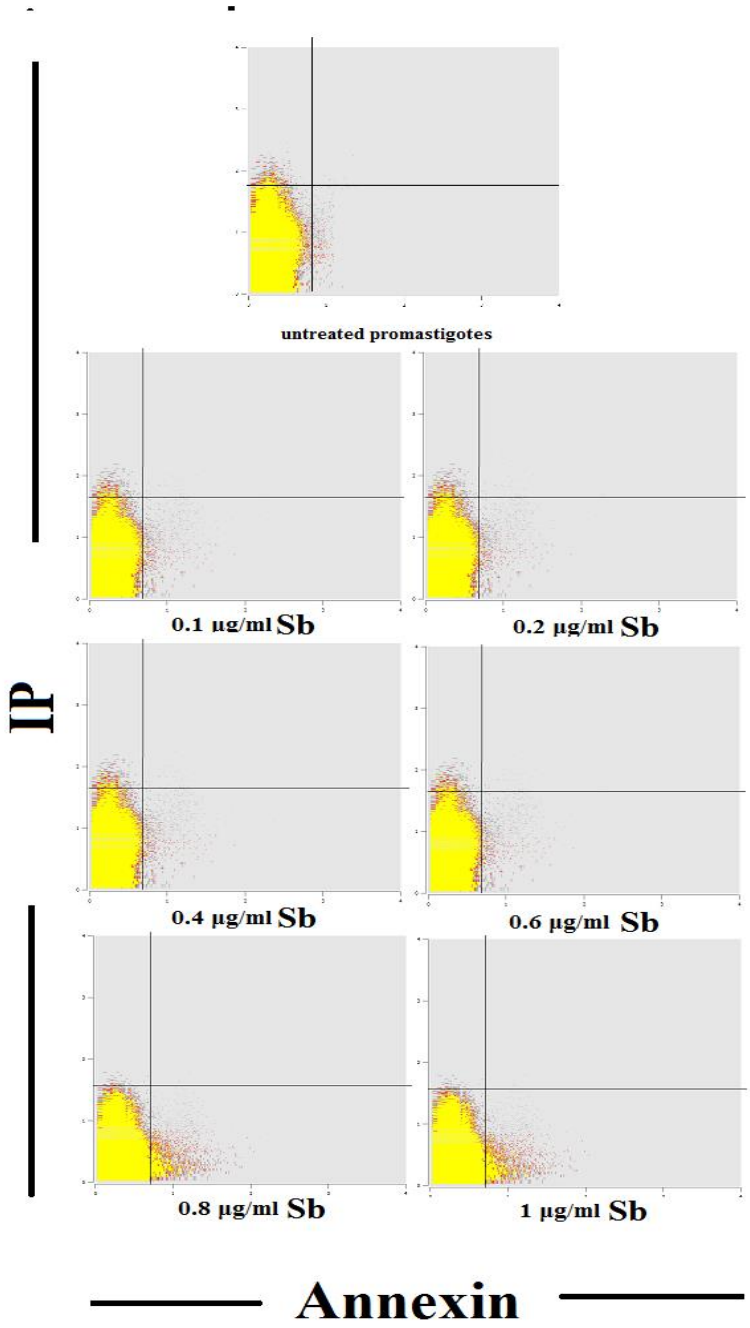

Figure 2, Flow cytometry results, promastigotes stained with Annexin V and Propidium Iodide after 72hr of treatment with different concentrations of Sb.

Table 1: Early apoptosis percentage in *L. donovani* promastigotes treated with ZnO NPs and pentostam after 72hrs.

Concentration (µg/ml)	Early apoptosis percentage %		LSD value
	ZnO NPs	Pentostam	
0.1	2.00 ± 0.57	4.00 ± 0.00	1.603 *
0.2	17.33 ± 1.20	3.33 ± 0.23	3.462 *
0.4	41.33 ± 0.67	4.00 ± 1.00	3.336 *
0.6	54.20 ± 0.58	2.50 ± 0.76	2.658 *
0.8	60.00 ± 0.57	4.16 ± 0.83	2.814 *
1	61.67 ± 1.66	3.33 ± 0.67	4.983 *
Control	2.5 ± 0.88		
LSD value	2.910 *	1.929 NS	--
	*** (P<0.05).**		

Table 2: Late apoptosis percentages in *L. donovani* promastigotes treated with ZnO NPs and pentostam after72hr.

Concentration (µg/ml)	Late apoptosis percentage %		LSD value
	ZnO NPs	Pentostam	
0.1	2.83 ± 0.17	1.83 ± 0.16	0.654 *
0.2	4.33 ± 0.33	1.33 ± 0.23	1.308 *
0.4	10.86 ± 0.86	3.33 ± 0.23	2.578 *
0.6	19.20 ± 2.60	1.33 ± 0.23	7.277 *
0.8	32.83 ± 1.58	1.83 ± 0.16	4.438 *
1	31.33 ± 1.85	2.00 ± 0.00	5.152 *
Control	1.50 ± 0.28		
LSD value	4.244 *	0.714 *	---
	*** (P<0.05).**		

Table 3: living percentage in *L. donovani* promastigotes treated with ZnO NPs and pentostam after72hrs.

Concentration (µg/ml)	Living apoptosis percentage%		LSD value
	ZnO NPs	Pentostam	
0.1	89.00 ± 0.57	92.33 ± 0.33	1.851 *
0.2	74.33 ± 0.33	92.67 ± 0.33	1.308 *
0.4	42.47 ± 0.74	90.33 ± 0.33	2.259 *
0.6	21.60 ± 0.60	92.83 ± 0.17	1.728 *
0.8	2.67 ± 0.33	89.33 ± 0.33	1.308 *
1	1.33 ± 0.16	89.67 ± 0.88	2.491 *
Control	93.33 ± 0.88		
LSD value	1.728 *	1.281 *	---
*** (P<0.05).**			

Table 4: Necrosis percentage in *L. donovani* promastigotes treated with ZnO NPs and pentostam after72hrs.

Concentration (µg/ml)	Necrosis percentage%		LSD value
	ZnO NPs	Pentostam	
0.1	1.83 ± 0.60	1.83 ± 0.16	1.731 NS
0.2	3.67 ± 1.45	2.67 ± 0.33	4.138 NS
0.4	5.67 ± 2.16	5.00 ± 1.52	7.360 NS
0.6	5.00 ± 2.08	3.00 ± 1.00	6.411 NS
0.8	5.50 ± 2.46	4.67 ± 1.20	7.617 NS
1	5.0 ± 0.88	2.00 ± 0.33	2.617 *
Control	3 ± 0.44		
LSD value	4.938 NS	2.570 *	---
* (P<0.05).			

The present results revealed the effect of lowest concentrations (0.1, 0.2, 0.4, 0.6, 0.8 and 1 µg/ml) of ZnO NPs on *L. donovani* promastigotes that induced apoptosis which conflicted with the result of the only study of ZnO NPs effects on *leishmania* parasite that carried out by Delavari *et al.* [20]. They used high concentrations (30, 60, 90 and 120 µg/ml) of ZnO NPs that induced apoptosis in *L. major* promastigotes. The apoptosis percentages of *L. major* promastigotes have induced after 72 hr were 46.4%, 62.8%, 71.5% and 93.76% respectively; these results of apoptosis were higher than in this study and this may be due to the high concentration of ZnO NP they used.

Another study on *L. donovani* was conducted by Zahir *et al.* [21]. In this study the researcher used Ag NPs and they demonstrate that the activity of different concentrations of synthesized Ag NPs was assessed by measuring cellular uptake of propidium iodide in *L. donovani* promastigotes after 24 hr treatment. Propidium iodide (PI) application was used to quantify the cells in which membrane integrity was gone, resulting in cell death. The results are consistent with those of other studies and they suggested that the cytotoxic effect induced by biosynthesized Ag NPs involved apoptotic changes and the nuclear condensation was studied by the PI staining method [25].

Treatment of promastigotes with synthesized Ag NPs for 24 hr revealed a gradual decrease in ROS generation. The decreased ROS is responsible for necrotic cell death mechanism [26]. It was reported that the Annexin V- FLUOS staining induced apoptotsis in promastigotes of *L. major*. Antimony sulfide NPs can persuade apoptosis in *L. infantum* promastigote stage at 50 and100 µg/mL concentrations [27, 28]. Other study was performed by [20] to reveal if ZnO NPs stimulate cytotoxicity via an apoptotic pathway. ZnO started the apoptosis process with fragmentation of DNA [29]. Together, these studies reveal that a primary mechanism of ZnO nanoparticles cytotoxicity might proceed by inducing the generation of ROS, which then were responsible for the induction of apoptosis [20] ZnO also cause DNA strand breakage and oxidative DNA damage in bone marrow [30]. A study done by [31] revealed that the threshold dose of ZnO NPs was lethal to stem cells. Cellular ROS levels induce by ZnO NPs lead to the dissipation of the mitochondrial membrane potentials and caused the cellular apoptosis [32]. A study showed that ZnO NPs induced apoptosis in human dermal fibroblast cells [33]. It has been well documented that extensive DNA damage triggers apoptosis [34]. In another study, ZnO NPs induced up-regulation of two important molecular markers, p53 and phospho-p38. P53 arrests the cell cycle, allowing DNA repair enzymes to act, and if the damage is beyond repair, initiates apoptosis to prevent the mutation from being passed on through subsequent cell divisions. With the increased levels of p53, the up-regulation of phospho-p38 indicates that ZnO NP exposure resulted in cellular stress [35].

Conclusion

The present study has given deep insight into the potential of ZnO NPs to prompt apoptosis in *L. donovani* promastigotes.

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