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Original Article

In Vitro Study of Leishmanicidal Activity of Biogenic Selenium Nanoparticles against Iranian Isolate of Sensitive and Glucantime-Resistant *Leishmania tropica*

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Abstract

Background: Sensitive and glucantime (MA) resistance *Leishmania tropica* are referred to those isolates, which are responsive, or non-responsive to one or two full courses of treatment by MA systematically and/or intra-lesionally, respectively. In this study, we evaluated the antileishmanial activity of biogenic selenium nanoparticles (Se NPs) alone and in combination with MA against sensitive and glucantime-resistant *L. tropica* on in vitro model.

Methods: The Se NPs were synthesized by employing the *Bacillus* sp. MSh-1. The antileishmanial effects of Se NPs alone and in combination with MA on promastigote and amastigote stages of sensitive and glucantime-resistant *L. tropica* strains have been investigated using a colorimetric MTT assay and in a macrophage model. In addition hemolytic activity in type O+ human red blood cells and infectivity rate of the promastigotes before and after treatment with the Se NPs was evaluated.

Results: In the promastigote stage, various concentrations of Se NPs significantly inhibited ($P<0.05$) the growth of promastigotes of both strains in a dose-dependent manner. Similarly, Se NPs especially in combination with MA significantly reduced the mean number of amastigotes of both strains in each macrophage. Se NPs showed no hemolytic effect on human RBCs at low concentrations. Moreover, infection rate of macrophages by promastigotes significantly ($P<0.05$) was reduced when promastigotes pre-treated with Se NPs.

Conclusion: The findings of this study suggest a first step in the search of Se NPs as a new antileishmanial agent. Further experiments are needed to investigate antileishmanial effects of biogenic Se NPs on *L. tropica* using a clinical setting.

Introduction

Leishmaniasis is one of the most important public health problems in tropical and sub-tropical countries. It is endemic in 98 countries and territories, affecting 12 million people and approximately threatens 350 million, worldwide (1). It consists several different presentations including cutaneous, mucocutaneous and visceral forms. In Iran, there are two epidemiological forms of cutaneous leishmaniasis (CL); Anthroponotic CL (ACL) and zoonotic CL (ZCL) caused by *Leishmania tropica* and *L. major,* respectively (2, 3).

At present, because there is not any effective vaccine; the effective control approach is prompt detection of the cases and early treatment against leishmaniasis (4, 5). Although, antimonial compounds such as meglumine antimoniate have been used as the treatment of choice, resistance has frequently been reported for *L. tropica* and *L. donovani*, the two Old World anthroponotic leishmaniases for many years (6-8). These species have developed resistance as reported from Africa, Indian sub-continent, South-east Asia particularly Iran (1). This problem indicates the urgent needs for development of new drugs or combination therapy for treatment of CL. However; extensive efforts have also been made to promote combination therapy of available drugs including verapamil, imiquimod and allopurinol that revealed synergistic effects with MA in the treatment of CL (9-11).

Selenium (Se) is a trace element of fundamental importance to human health. It is a component of selenoproteins, some of which have important enzymatic functions (12). Selenium has been used in various medical therapies such as cancer prevention including lung, esophagus, prostate and gastric-cardiac cancers, antiviral activities and antioxidant effects (13-15). Moreover, Se has additional important health activities relevant to the immune response and successful reproduction (15). It has currently been demonstrated that nanoparticles (NPs) due to their large surface-volume ratio show various unique properties such as enhancing interactions with biological molecules and microorganisms. NPs are also able to enter cells more frequently than other particles (16). NPs particularly Se NPs can effectively inhibit the growth of some bacteria such as *Staphylococcus aureus* and pathogenic *Escherichia coli* (16, 17). In addition, Se NPs significantly inhibited the growth of *Echinococcus granulosus* protoscoleces and *L. major* on *in vitro* and *in vivo* model (18, 19).

In this study, we synthesized the biogenic Se NPs by employing the *Bacillus* sp. MSh-1 as a potent bacterium in selenium reduction. In the next step, to separate and purify Se NPs from the whole-cell lysate, we used a liquid-liquid two-phase partitioning method. After characterization of the purified Se NPs, the effect of Se NPs on *L. tropica* promastigotes was studied by MTT assay. Finally, the antileishmanial activity of NPs on clinical stage was evaluated in an amastigote-macrophage model.

Materials and Methods

Chemicals

Meglumine antimoniate (MA, Glucantime) as control drug was prepared from Rhône, Poulenc, France. Penicillin and streptomycin were obtained from Alborz Pharmacy, Karaj, Iran and were stored at room temperature (25°C) until testing. MTT powder [3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide)], fetal calf serum (FCS) and RPMI-1640 medium with L-glutamine were purchased from Sigma-Aldrich, St Louis, MO. Selenium dioxide (SeO_2), nutrient broth, nutrient agar, *n*-octyl alcohol, sodium dodecyl sulfate (SDS) and Tris base were purchased from Merck Chemicals (Germany). All other chemicals and solvents were of analytical grade. The microorganism used in this study was identified as *Bacillus* sp. MSh-1 by the methods described earlier (12). The organism was continuously maintained on nutrient agar plates

complemented with 1.26 mM SeO_2 using continuous sub-culturing every 14 days.

Biosynthesis and characterization of the Se NPs

Se NPs were prepared according to the method described elsewhere (12). Briefly, a sterile nutrient broth (NB) medium was supplemented with the Se^{+4} ions (100 mg/L; equal to 1.26 mM SeO_2 solution) and 100 mL of this medium was transferred to a 500-mL Erlenmeyer flask. The medium was inoculated with 1 mL of the fresh inoculums (OD_{600}, 0.1) of *Bacillus* species MSh-1 and was incubated aerobically at 30°C in a shaker incubator (150 rpm). After 14 h, the bacterial cells and Se NPs were removed from the culture medium by using centrifugation at 4000 *g* for 10 min. The pellets were washed with 0.9% NaCl solution using centrifugation, transferred to a mortar and then it was frozen by adding liquid nitrogen and was then disrupted by a pestle. The resulting slurry was ultrasonicated at 100 W for 5 min and washed three times by sequential centrifugation (10000 *g* for 5 min), with a 1.5 M Tris-HCl buffer (pH 8.3) containing 1% SDS and deionized water. The next step involved extracting and purifying the Se NPs through an organic-aqueous partitioning system (n-octyl alcohol-water). For transmission electron microscopy, an aqueous suspension containing the Se NPs was dispersed ultrasonically, and a drop of the suspension was placed on carbon-coated copper TEM (transmission electron microscope) grids and dried under an IR lamp. The crystalline structure of the Se NPs was evaluated by the X-ray diffraction (XRD) technique using an X-ray diffractometer (Philips PW1710) with CuKα radiation (λ = 1.5405 Å) over a scanning range of Bragg angles from 20 to 80°C.

Parasite strains and culture

Glucantime-resistant strain of *L. tropica* was prepared from a CL patient in Bam, southeastern Kerman province of Iran. This isolate was identified by nested-PCR as *L. tropica* and further determined by conventional PCR for *MDR1* gene (8). Subsequently the DNA extract was sequenced and recorded in Gen Bank under HM854717 Accession Number. Sensitive (MHOM/IR/2002/Mash2) strain of *L. tropica* was kindly provided by the Center for Research and Training in Skin Diseases and Leprosy (Tehran, Iran). The parasite was cultured in RPMI-1640, supplemented with penicillin (100 IU/mL), streptomycin (100 µg/mL), and 15% heat-inactivated fetal calf serum (FCS).

Preparation of murine macrophages

In this study, murine macrophage cells were collected from male BALB/c mice (5-8 weeks old) by injecting 3-5 mL of cold RPMI-1640 medium into mouse peritoneal cavity and aspirated macrophages were washed twice and resuspended in RPMI-1640 medium. The experimental procedures carried out in this study complied with the guidelines of the Kerman University of Medical Science (Kerman, Iran) for the care and use of laboratory animals.

Leishmanicidal activity against promastigotes

Leishmanicidal activity of Se NPs on promastigote forms of sensitive and glucantime-resistant strains of *L. tropica* was evaluated by colorimetric cell viability MTT assay using the method described by Mahmoudvand et al. (20). Initially, 100 µL of the promastigotes (10^6cells/ mL) harvested from logarithmic growth phase was added into a 96-well microtiter plate. Then 100 µL of different concentrations of Se NPs alone (0-25 µg/mL) or MA alone (0-25 µg/mL) and along with Se NPs (2 µg/mL) was added to each well and incubated at 25°C ± 1°C for 72 h. After incubation 10 µL of MTT solution (5 mg/mL) was added into each wells and were incubated at 25°C for 4 h. Promastigotes cultured in complete medium with no drug used as positive control, and complete medium with no promastigotes and drugs as blank. All experiments were repeated in triplicate. Finally, absorbance was measured

by an ELISA reader (BioTek-ELX800) at 490 nm. We also calculated the 50% inhibitory concentrations (IC_{50} values) by Probit test in SPSS software.

Effects on intramacrophage amastigotes

For evaluation of antileishmanial activity of Se NPs on *L. tropica* intracellular amastigotes, murine macrophages collected from male BALB/c mice were used according to the method described elsewhere (21). Briefly, $1cm^2$ cover slips were placed in the wells of 4-chamber slides (Lab-Tek, Nalge Nunc International NY, USA) and then 200 µL of the murine macrophages (10^6cells/mL) was placed in each well. After 2 h, promastigotes of each strain were added to each well and were incubated at 37°C in a CO_2 incubator (5% CO_2 and 95% relative humidity). After 24 h free parasites were removed by washing with RPMI-1640 medium and infected macrophages were treated with different concentrations of Se NPs alone (0-50 µg/mL) or MA alone (0-50 µg/mL) and along with Se NPs (5 µg/mL) for 72 h. Finally, dried slides were fixed with methanol, stained by Giemsa and studied under a light microscope. In this step, macrophages containing amastigotes without treatment and macrophages with no parasite and treatment were considered as positive and negative controls, respectively. Antileishmanial activity of treatments and the mean number of amastigotes were evaluated by counting the number of amastigotes in 100 macrophages (9). All experiments were carried out in triplicate. In addition, the IC_{50} values were calculated by Probit analysis.

Inhibition of infection in macrophage cells

The inhibitory effect of the Se NPs on the *Leishmania* invasion of macrophages was investigated in promastigote forms of sensitive and Glucantime-resistant strains of *L. tropica*. Firstly, promastigotes in the stationary phase were pre-incubated with Se NPs (1 µg/mL) for 1 h at 37°C. In the next step, promastigotes were washed with RPMI-1640 medium and further incubated for 4 h with murine macrophages at a ratio of 10 *Leishmania* per 1 macrophage. After washing, the macrophages were fixed with methanol, stained by Giemsa and studied by a light microscope and frequency of infected macrophages was measured by counting 100 macrophages (22).

Hemolytic activity of Se NPs

The hemolytic activity was evaluated by incubating the various concentrations of Se NPs (0- 200 µg/mL) with a 5% O+ human red blood cells (RBC) suspension for 1 h at 37°C. The erythrocyte suspension was centrifuged (1000 g for 10 min), and cell lysis was determined spectrophotometrically (540 nm), as described (23). The absence (blank) or 100% presence of hemolysis (positive control) was determined by replacing the Se NPs for an equal volume of PBS 1x or distilled water, respectively. The results were calculated by the percentage of hemolysis compared to the negative and positive controls.

Statistical analysis

SPSS Software ver. 17 (SPSS Inc., Chicago) was used for data entry and statistical analysis and the differences between groups were determined by using one way analysis of variance (ANOVA) test. Moreover, to compare the IC_{50} values of Se NPs and control drug *t*-test performed. *P*-value of less than 0.05 was considered statistically significant.

Results

Characterization of Se NPs

Figure 1 shows the TEM image of Se NPs. The TEM micrograph clearly illustrates individual Se NPs with a small amount of aggregation and the NPs have a spherical shape. Size distribution measured from manual counting of 400 individual particles from different TEM images showed that the size of nanoparticles was 80–220 nm and NPs with the size of 105 to 130 nm had the most frequency. The XRD pattern of Se NPs showed

the presence of broad peaks without any clear lattice parameters (results not shown).Thus the obtained Se NPs are amorphous.

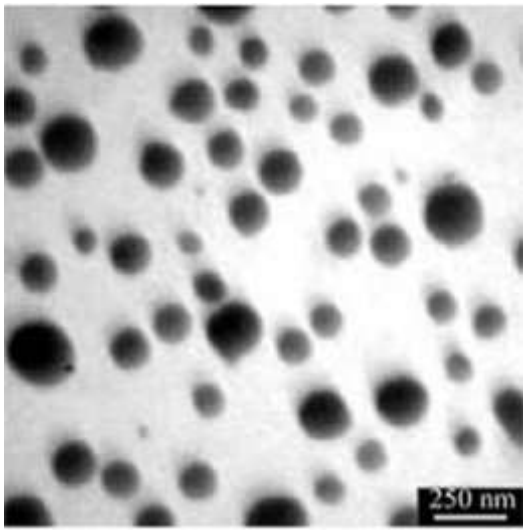

Fig. 1: Transmission electron micrograph of the purified selenium nanoparticles (Se NPs) synthesized by *Bacillus* sp. MSh-1

Leishmanicidal activity against promastigotes

In contrast to controls, various concentrations of Se NPs alone or combined with MA significantly inhibited ($P<0.05$) the growth rate of promastigotes of both strains in a dose-dependent manner. The measured IC_{50} values of Se NPs for sensitive and glucantime-resistant strains were 2.7 and 3.6 µg/mL, respectively. Similarly, the IC_{50} values of MA plus Se NPs for sensitive and glucantime-resistant strains were 1.5 and 2.8 µg/mL, respectively. In contrast, the IC_{50} values of MA for sensitive and glucantime-resistant strains were 11.6 and 43.6 µg/mL, respectively, which are significantly ($P<0.05$) higher than the measured IC_{50} values for Se NPs alone and along with MA against both strains of *L. tropica*, reflecting more effective leishmanicidal effects of Se NPs alone and along with MA as compared with MA alone upon promastigotes of both strains of *L. tropica* (Table 1).

Table 1: Comparison of the mean IC_{50} values of meglumine antimoniate (MA), biogenic selenium nanoparticles (Se NPs) alone and combined with MA upon the growth rate of promastigote and amastigote forms of sensitive and glucantime-resistant strains of *L. tropica*. Data are expressed as the mean ± SD (n = 3)

Chemicals	IC_{50} (µg/mL)			
	Promastigotes		Amastigotes	
	SS[a]	RS[b]	SS	RS
MA	11.6 ± 1.55	43.6 ± 3.05	39.2 ± 2.52	151 ± 4.35
Se NPs	2.7 ± 0.3	3.6 ± 1.4	6.4 ± 2.01	7.3 ± 1.15
MA plus Se NPs	1.5 ± 0.43	2.8 ±1.15	3.3 ± 1.0	5.7 ± 1.15

a: sensitive strain of *L. tropica*/b: glucantime-resistant strain of *L. tropica*

Leishmanicidal effects on amastigotes

Biogenic Se NPs prepared by *Bacillus* sp. MSh-1 indicated a potent antileishmanial effect especially in combination with MA to the intramacrophage amastigotes of both strains after 72 h exposure. It could be observed that the anti-amastigote effects of Se NPs was based on a dose-dependent response, so that Se NPs induced the inhibition of the growth rate of amastigote forms within macrophage cells at concentrations of higher than 5 µg/mL when the mean number of amastigotes in each macrophage was assessed. However, when Se NPs combined with MA, the proliferation rate of amastigotes was significantly reduced ($P<0.05$) at concentration of higher than 2.5 µg/mL for both indices of amastigote-macrophage model as compared with positive control. The measured IC_{50} values for Se NPs alone, MA alone and along with Se NPs against this parasitic form of sensitive and glucantime-resistant strains of *L. tropica* were also shown in Table 1.

Inhibition of infection in macrophages

Assessment of infectivity rate of the sensitive and glucantime-resistant strains of *L. tropica* promastigotes to the murine macrophage before and after treatment with the Se NPs indicated that promastigotes of both strains of *L. tropica* treated with no Se NPs were able to infect 78% and 81% of the macrophages, respectivelly. When promastigotes of both strains were pre-incubated with 1 µg/mL Se NPs for 2 h, they were able to infect only 16.3 and 21.6% of the murine macrophage cells respectively, in turn presenting reductions in their infectivity in the order of 79.3 and 73.3% for sensitive and glucantime-resistant strains of *L. tropica*, respectively (Table 2). Based on these results, it can be deduced that pre-treatment with the Se NPs was able to inhibit significantly the infection of *Leishmania* promastigotes into the murine macrophages.

Table 2: Inhibition of the infection in murine macrophages after treatment of sensitive and glucantime-resistant strains of *L. tropica* promastigotes with the meglumine antimoniate(MA) and biogenic selenium nanoparticles (Se NPs). Data are expressed as the mean ± SD (n = 3)

Chemicals	Percentage of infected macrophages by non-treated promastigotes		Percentage of infected macrophages by treated promastigotes		Infectiveness Reduction (%)	
	SS[a]	RS[b]	SS	RS	SS	RS
MA	78.6 ± 3.05	81 ± 3.6	34.6 ± 2.52	27.3 ± 2.15	56	66.3
Se NPs	78.6 ± 3.05	81 ± 3.6	16.3 ± 2.15	21.6 ± 2.52	79.3	73.3

a: sensitive strain of *L. tropica*/b: Glucantime-resistant strain of *L. tropica*

Hemolytic activity of Se NPs

In evaluation of the hemolytic activity of Se NPs in O+ human RBCs, it could be seen no significant hemolytic activity at concentations lower than 25 µg/mL, As compared with the positive control. However, Se NPs at concentrations of 25 and higher indicated significant hemolytic activity in O+ human RBCs.

Discussion

Leishmaniasis is a major public health problem in many countries around the world. The first line drugs, antimonials have been used for more than 65 years. In addition, other new drugs approved for the oral treatment of ACL and/or ZCL including miltefosine, ketoconazole, itraconazole, allopurinole, dapsone, and different physical therapies such as cryotherapy, surgical excision and heat are used (24-26). At present, the use of these drugs are limited due to high cost, toxicity, long-term treatment, partial effectiveness and the emergence of drug resistance (6-8). Recently, combination therapies with intra-lesional or systemic MA have been used for CL with different drugs, such as verapamil, allopurinol and imiquimod that proved their synergistic effects along with MA or sodium stibogluconate in treatment of CL (9, 11). Furthermore, in recent years, the use of nano drugs such as silver and gold nanopaticles to treat CL has beenproven (27, 28).

Selenium compounds have extensive applications in medicine as anticancer, antioxidant and antimicrobial agents and their effects are related to immune system (6-8). Nanoparticles have been used for various medical applications due to their small nanoscale sizes and their high surface-to-volume ratios that allow more active sites for interacting with biological molecules such as microorganisms and other bioactive entities. On the other hand, nanoparticles provide a valuable feature to penetrate easily tissues with tumors and can eliminate cancerous cells without affecting the

normal tissues (6-8). Se NPs are able to reduce the development of HIV progression to AIDS and can inhibit the progression of hepatitis B and C to liver cancer (13, 29-31). In addition, it has been shown that Se NPs inhibit the growth of *Staphylococcus aureus* and pathogenic *Escherichia coli* (16, 17).

In this study, we evaluated the antileishmanial activity of biogenic Se NPs alone and in combination with MA against sensitive and glucantime-resistant *L. tropica* on *in vitro*. The main reason for selection of this species was that the control of *L. tropica* is only by chemptherapy, which makes it vulnerable to resistance (7, 8). In the promastigote stage, the mean optical density (OD) and consequently IC_{50} values for Se NPs along with MA against promastigote forms of both strains of *L. tropica* were lower than the Se NPs or MA alone.

This showed that the Se NPs were toxic to promastigotes and it might cause some morphological and biochemical changes such as DNA fragmentation during apoptotic cell death in the parasite as previously demonstrated elsewhere (19). Similarly, the Se NPs significantly reduced the viability of intramacrophage amastigotes of both strains and the mean infection rate of macrophages a compared with each drug alone. Furthermore, we demonstrated that the infection rate of macrophages significantly decreased when promastigotes of both strains of *L. tropica* were pre-treated with Se NPs.

It has previously been proven that some protozoan including *Trypanosoma* and *Leishmania* and other higher microorganisms, need trace amounts of Se ions (32). However, we showed higher in vitro concentrations of Se NPs were toxic for both stages of sensitive and glucantime-resistant strains of *L. tropica*. Also in the present study it is found that promastigote forms were more susceptible to Se NPs (alone or in combination with MA) than amastigote forms. This difference in susceptibility of promastigote and amastigote stages against various concentrations of Se NPs might be related to structural, biochemical and morphological features (9, 33) and also other factors, including the phagolysosomal membrane of the macrophage, which reduce the entry of Se NPs into the macrophage cells and decrease the concentrations of Se NPs around the intramacrophage amastigotes (12, 19). In this study we reported that Se NPs at lower concentration had no hemolytic effect, whereas, the higher concentration of Se NPs showed more hemolytic effect on O+ human red blood cells.

Similar results were found by Beheshti et al. and Shakibaie et al. that demonstrated Se NPs at the low doses had no cytotoxic effects and could be considered safe (12, 19). Recently Shakibaie et al. have found that no biochemical changes were observed from the orally administration of 2.5, 5 and 10 mg/kg of Se NPs to male mice for two weeks, but a dose of 20 mg/kg of Se NPs indicated signs of toxicity including lower body weight and changes in clinical chemistry and hematological parameters (34). Moreover, they showed that the biogenic Se NPs were less toxic than synthetic Se NPs and much less (26-fold) toxic than the SeO_2.

Conclusion

High potency and synergistic effect of Se NPs alone and combined with MA in inhibiting growth of promastigote and amastigote stages of sensitive and glucantime-resistant strains of *L .tropica*. The results of this study suggest a first step in the search of Se NPs as a new antileishmanial agent. Further clinical studies are required to investigate the activity of the Se NPs as a new therapeutic agent against leishmaniasis.

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