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ORIGINAL ARTICLE

Anti-leishmanial activities of selenium nanoparticles and selenium dioxide on *Leishmania infantum*

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Abstract *Leishmania infantum* is one of the important causes of visceral leishmaniasis in many countries. There are different complications for treatment of leishmaniasis such as toxicity and drug resistant. So far, there isn't any information about the effects of selenium nanoparticles and selenium dioxide (chemical form of selenium) on *Leishmania* parasites; hence, the aim of the present study is to investigate in vitro effects of six dilutions of these drugs on *L. infantum*. Anti-leishmanial activities were studied by adding different dilutions of 2.5, 5, 10, 25, 50, and 100 μg/ml of the drugs into promastigote cultures. Promastigote cytotoxicity was tested using the colorimetric MTT assay. Anti-amastigote activity was assessed in peritoneal macrophages of BALB/c mice. Also, cytotoxic effect of these drugs was evaluated on uninfected macrophages. The results showed that both of drugs have dose-dependent anti-leishmanial activities. Selenium NPs have more growth-inhibitory effect on promastigotes than SeO_2; while the IC50 (50 % inhibitory concentration) was determined to be 25 and 50 μg/ml, respectively. The mean numbers of amastigotes per macrophage in selenium NPs-treated groups were less than SeO_2-treated and control groups. The IC50 of selenium NPs was 10 μg/ml and SeO_2 was 25 μg/ml for amastigotes. Also, the IC50 of selenium NPs and SeO_2 for uninfected macrophages were calculated to be 100 and 50 μg/ml, respectively. In addition, selenium NPs has less cytotoxic effect than SeO_2 on uninfected macrophages. These findings suggest that selenium NPs have more anti-leishmanial properties and less cytotoxic effects than SeO_2 against *L. infantum*.

Keywords Leishmaniasis · *Leishmania infantum* · Selenium nanoparticles · Selenium dioxide · Anti-leishmanial activities

Abbreviations

Selenium NPs	Selenium nanoparticles
SeO_2	Selenium dioxide

Introduction

Visceral leishmaniasis (VL) known as kala azar is a life-threatening parasitic infection that may cause death in less developed countries. *Leishmania infantum* is one of the significant causes of visceral leishmaniasis in many countries. VL is endemic in 62 countries, with a total of 200 million people at risk and estimated 500,000 new cases each year worldwide (Guerin et al. 2002; Chappuis et al. 2007; Murray et al. 2005).

The first-line treatments for VL in many areas are pentavalent antimonials—sodium stibogluconate and meglumine antimoniate. Amphotericin B is also as the first-line treatment

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for VL in some areas of the Bihar State of India. Miltefosine, which was initially developed as an anticancer drug, is the first effective oral drug for VL (Chappuis et al. 2007; Guerin et al. 2002).

There are various complications for treatment of leishmaniasis. Antimonials have several adverse side effects such as cardiac arrhythmia and acute pancreatitis (Chappuis et al. 2007; Guerin et al. 2002). Drug resistance in leishmaniasis is another significant complication. There are several cases of *Leishmania* being resistant to antimonials; resistance to miltefosine and paromomycin were reported in different countries (Maltezou 2010; Croft et al. 2006; Aït-Oudhia et al. 2011). Therefore, searching for new drugs to overcome these complications is needed.

Nanomedicine is defining the use of nanotechnology in medicine, which has been of great interest in recent years. The use of nanoparticles for therapeutics is one of the purposes of nanomedicine (Kim et al. 2010; Irache et al. 2011). In some experimental studies, nanoparticles such as nanogold and nanosilver have been used for treatment and or improvement of drug delivery in leishmaniasis (Mohebali et al. 2009; Allahverdiyev et al. 2011; Torabi et al. 2011; Zampa et al. 2009; Durand et al. 1997a; Durand et al. 1997b; Fusai et al. 1994; Torres-Santos et al. 1999).

Selenium (Se) is a trace element with a wide range of effects in human health, including cancer prevention, antiviral activities, and antioxidant and anti-inflammatory effects (Rayman 2012). In recent years, selenium has been fabricated in nanosize with different methods including green method in bacteria (Shakibaie et al. 2010). Different in vitro and in vivo studies have been conducted on the effects of selenium NPs and other forms of selenium (Shakibaie et al. 2010; Tran and Webster 2011; Chen et al. 2003; Huang et al. 2003; Wang et al. 2005; Zhang et al. 2005; Wang et al. 2007; Zhang et al. 2004; Kuppusamy et al. 2005). The main purpose for fabricating nano-selenium is increased efficacy and decreased cytotoxicity of selenium. As previously described, nano-selenium have less cytotoxic effect but have equal efficacy in comparison with other forms of selenium (Shakibaie et al. 2010;Wang et al. 2007; Zhang et al. 2005).

To our knowledge, there aren't any reports about the effects of selenium NPs and SeO_2 (chemical form of selenium) on *Leishmania* parasites; therefore, the aim of the present study is to evaluate the effects of different dilutions/times of these drugs on *L. infantum*. The second aim of the study is to compare the cytotoxic effect of selenium NPs and SeO_2 on uninfected macrophages.

Material and methods

Selenium NPs were provided from the Department of Pharmaceutical Biotechnology and Pharmaceutical Sciences Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences which were synthesized by green methods from SeO_2 (Shakibaie et al. 2010). SeO_2 (solubility 384 g/l at 14 °C) was prepared from Merck (Darmstadt, Germany).

Parasite culture

Promastigotes of *L. infantum* (WHO reference strain: MHOM/TN/80/IPT1) were prepared from the Pasteur Institute of Iran. For mass production, the promastigotes were cultured at 24 °C in RPMI1640 media (Gibco) supplemented with 10 % fetal calf serum (FCS) and antibiotics (100 IU/ml of penicillin and 100 μg/ml of streptomycin).

Anti-promastigote activity

Anti-promastigote assays were carried out using direct counting assay based on growth inhibition (Yousefi et al. 2009). The effects of selenium NPs and SeO_2 were evaluated in 24-well microtitre plates. The promastigotes were grown at an initial concentration equivalent to that of early log phase (2×10^6 promastigotes/ml) and then allowed to multiply for 72 h only in the RPMI medium with 10 % FCS as control group, and in the presence of different concentrations of selenium NPs and SeO_2 (2.5, 5, 10, 25, 50, and 100 μg/ml) as test groups without renewing the medium or drug. The promastigotes were counted at 24, 48, and 72 h in a hemocytometer with a light microscope. Each test was performed in triplicate and repeated in separate experiments.

Cell cytotoxicity by MTT assay

The 3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) test is a method that measures the ability of cells in transforming the yellow tetrazolium crystals (MTT) to insoluble blue formazan dye in normal cells; dead cells are incapable of this action (Paris et al. 2004). This test was carried out using a procedure as described earlier by Verma and Dey (2004) and Paris et al. (2004), with some modification. Briefly, 100 μl of promastigotes (containing 2×10^6 cells/ml) were added to RPMI medium as control group or in the presence of six dilutions of selenium NPs and SeO_2 (2.5, 5, 10, 25, 50, and 100 μg/ml) at 24±1 °C with 5 % CO_2 for 72 h, then 20 μl of MTT solution was added into each of wells and incubated again for 4-h. After incubation, the plates were centrifuged at 6,000×*g* for 10 min and supernatants were aspirated gently and discarded. Finally, 100 μl DMSO was added to each of wells

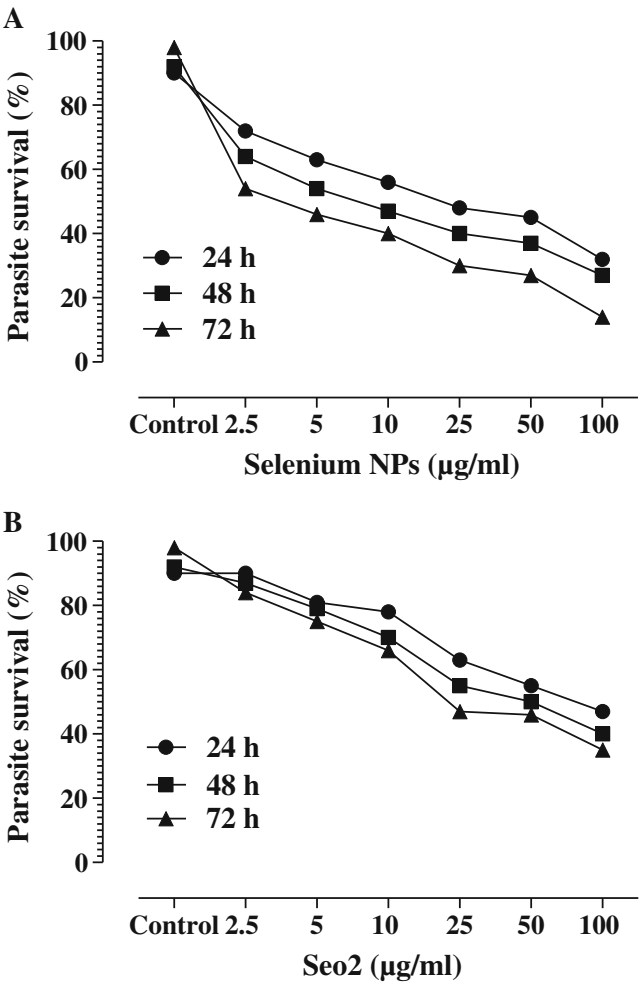

Fig. 1 Growth curve of *L. infantum* promastigotes treated with different dilutions of Selenium NPs (**a**) and SeO_2 (**b**) after 24, 48, and 72 h

to dissolve the formazan crystals and obtain a homogeneous blue solution suitable for measurement of the absorbance with an ELISA plate reader at 540 nm. The amount of color produced is directly relative to the number of viable cells. 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=(absorbance of treated cells/absorbance of control cells)×100.

Anti-amastigote activity

Briefly, 7 ml of RPMI medium injected into peritoneum of BALB/c mice and peritoneal macrophages were collected and stained with trypan blue for estimation of live macrophages. Afterward, isolated macrophages were seeded in 24-well plates and incubated at 37 °C with 5 % CO_2 for 24 h. Adhered macrophages were infected with stationary growth phase of promastigotes at a parasite/macrophage ratio of 10:1 and incubated for 24 h at 37 °C and 5 % CO_2 until promastigotes were phagocyte by macrophages. Then extracellular parasites were removed by washed with cold PBS. Infected macrophages were separated from the plates by cold method (10–15 min on ice) and stained by Giemsa. The percentage of infected cells and the number of amastigotes in each cell was assessed by light microscope. Then, infected macrophages were further incubated in the presence of six dilutions of selenium NPs and SeO_2 (2.5, 5, 10, 25, 50, and 100 µg/ml) or absence of these drugs for 24, 48, 72 h (37 °C with 5%CO_2) without replacing the culture medium. Finally, macrophages were washed with PBS, fixed in methanol, stained with Giemsa and the amastigotes inside the macrophage (200 macrophages per treatment) were counted under a light microscope (Verma and Dey 2004). All animals' experimentation protocols were approved by Animal Care and Use Committee of Medical Sciences Faculty of Tarbiat Modares University, Tehran, Iran.

The cytotoxicity assay

For evaluation of the cytotoxic effects of selenium NPs and SeO_2 on uninfected macrophages, these cells were incubated in the presence of six dilutions (2.5, 5, 10, 25, 50, and 100 µg/ml) of both drugs at 37 °C with 5 % CO_2 for 24, 48, 72 h. After 24, 48, 72 h, the percentages of live macrophages were calculated and compared among selenium NPs, SeO_2 and untreated control groups.

Statistical analysis

In vitro anti-leishmanial activity expressed as IC50 (50 % inhibitory concentration), was determined by linear regression analysis.

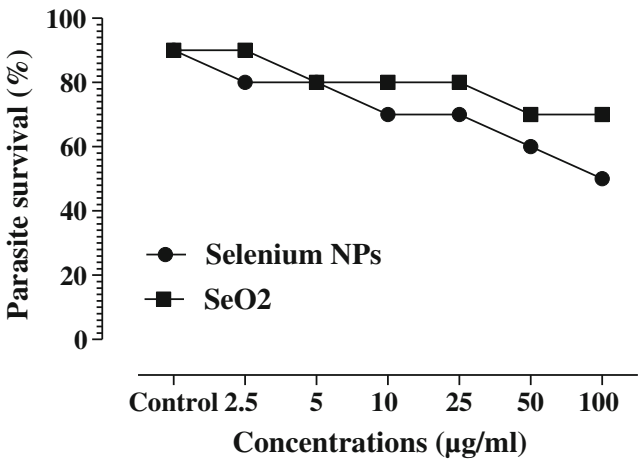

Fig. 2 The viability of *L. infantum* promastigotes in the presence of different dilutions of Selenium NPs and SeO_2 assessed by MTT after 72 h

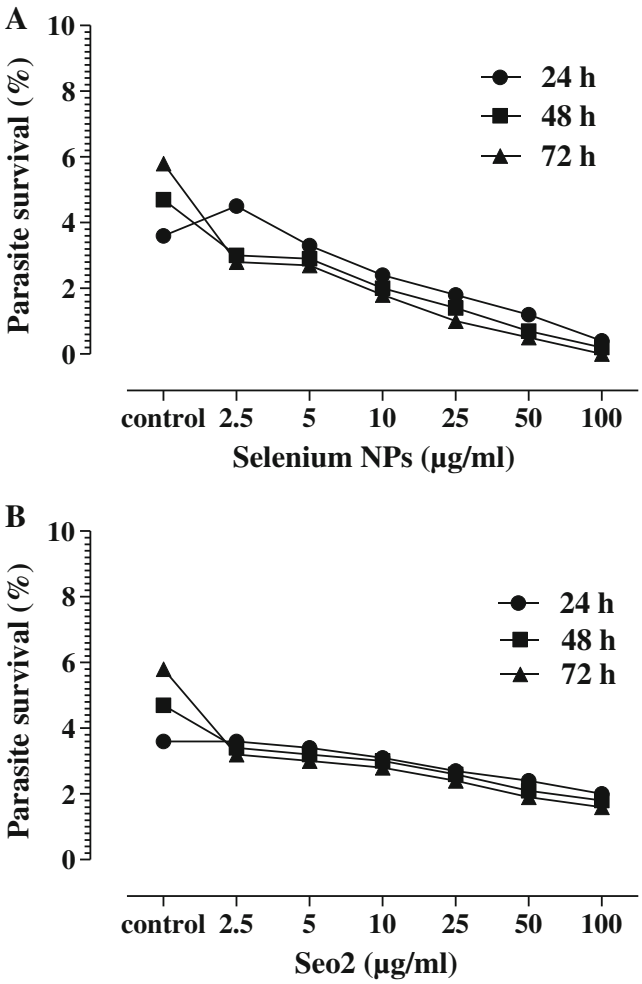

Fig. 3 Mean number of amastigote/macrophage following exposure to different dilutions of Selenium NPs (**a**) and SeO_2 (**b**) after 24, 48, and 72 h

Result

Anti-promastigote activity of selenium NPs and SeO_2

For evaluation of anti-promastigote activities, six dilutions of selenium NPs and SeO_2 incubated with in vitro cultures of *L. infantum* promastigotes at 24, 48 and 72 h. As shown in Fig. 1, a dose-dependent growth inhibition was observed at 24, 48, and 72 h after incubation; while maximum reductions of parasite proliferation were observed at 100 µg/ml of both drugs after 72 h of incubation. The IC50 (50 % inhibition concentration of cell growth) was determined to be 25 and 50 µg/ml for selenium NPs and SeO_2, respectively. Selenium NPs have more growth-inhibitory effects than SeO_2, while the percentages of viable promastigotes in selenium NPs were less than SeO_2 at all doses/times after incubations.

Promastigotes cytotoxicity by MTT assay

Cytotoxic effects of selenium NPs and SeO_2 on *L. infantum* promastigotes was tested using the MTT assay after 72 h. As shown in Fig. 2, both of the drugs have cytotoxic effects dependent on their doses. The IC50 of selenium NPs and SeO_2 were obtained 25 and 50 µg/ml, respectively. The viability of the promastigotes was decreased with increasing of the doses of drugs. The maximum cytotoxic effects were observed following exposure to 100 µg/ml concentration; however, the cytotoxic effects of selenium NPs was more than SeO_2 at different doses.

Anti-amastigote activity of selenium NPs and SeO_2

The anti-amastigote activity of selenium NPs and SeO_2 were evaluated in *L. infantum*-infected macrophage cultures. The

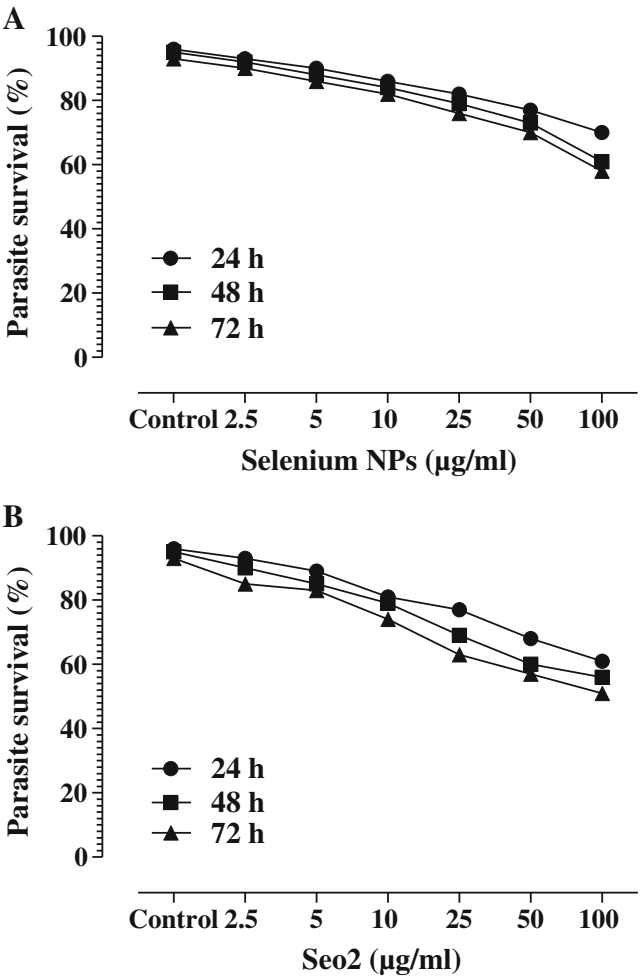

Fig. 4 Percentages of viable macrophages following exposure to different dilutions of Selenium NPs (**a**) and SeO_2 (**b**) after 24, 48, and 72 h

mean numbers of amastigotes/macrophage was shown in Fig. 3. The IC50 of selenium NPs was 10 μg/ml and SeO_2 was 25 μg/ml for amastigotes. Selenium NPs and SeO_2 have a dose-dependent effect on amastigotes. The mean numbers of amastigotes/macrophage in selenium NPs-treated groups were more than SeO_2-treated groups; these numbers were 2, 1.8, and 1.6 for SeO_2-treated groups and were 0.4, 0.2, and 0 for selenium NPs-treated groups.

Cytotoxic effect of selenium NPs and SeO_2 on macrophages

The results show that selenium NPs and SeO_2 have a dose-dependent cytotoxic effect on macrophages; while by increasing drugs concentrations, the viability was decreased (Fig. 4). Remarkably, selenium NPs have less cytotoxic effect than SeO_2 on macrophages, while the IC50 of selenium NPs was 100 μg/ml and SeO_2 was 50 μg/ml. Also, the viability of selenium NPs-treated macrophages were more than SeO_2 at all dilutions/times after treatment; for example, after 72 h exposure to 100 μg/ml of both drugs, the percentages of viable macrophages were 70, 61, and 58 in selenium NPs-treated group and were 61, 56, and 51 in SeO_2-treated group.

Discussion

Our results revealed that both of drugs have dose-dependent anti-leishmanial activities. In comparison of these two drugs, selenium NPs have a more anti-leishmanial activities and less cytotoxic effects (on uninfected peritoneal macrophages) than SeO_2, while the IC50 of selenium NPs for promastigote, amastigote, and macrophage was determined 25, 10 and 100 μg/ml, respectively. The IC50 of SeO_2 was calculated to be 50, 10, and 50 for promastigote, amastigote, and macrophage respectively. We observed selenium NPs have less cytotoxic effect on uninfected macrophages but have more anti-amastigote and anti-promastigote activities than SeO_2.

There are some evidences that other nanoparticles such as nanosilver and nanogold have anti-leishmanial activities on *Leishmania major* and *Leishmania tropica* (Mohebali et al. 2009; Allahverdiyev et al. 2011; Torabi et al. 2011). Also Zampa et al. (2009) observed that the nanostructure form of dermaseptin 01(antimicrobial peptides) has in vitro anti-leishmanial activities on *Leishmania chagasi*. In addition, the use of nanoparticles for improving drug delivery was evaluated in the mouse model of visceral leishmaniasis by some workers. They showed that pentamidine bounded with nanoparticles are more potent than the free drugs for treatment of *L. infantum*-infected mice (Durand et al. 1997a, b; Fusai et al. 1994).

Previous studies revealed that selenium at nano-size can serve as antioxidant (Huang et al. 2003; Wang et al. 2007) with lower toxic effects than selenium (Wang et al. 2007; Zhang et al. 2005; Shakibaie et al. 2010). In this regard, Shakibaie et al. (2010) observed green synthesis of nano-selenium have lower toxicity than other forms of selenium in human fibrosarcoma cells. Wang et al. (2007) showed that selenium at nano-size have lower toxicity than selenomethionine but possesses equal efficacy in activities of glutathione peroxidase and thioredoxin reductase compared with selenomethionine. Also, Zhang et al. (2005) compared toxicity of nano-selenium and selenite in mice. They showed, over a short term and high dose treatment in mice, that selenite could cause more oxidative stress and liver injury than nano-selenium.

According to GuanYi et al. (2010), nano-selenium could induce apoptosis in HeLa cell line (GuanYi et al. 2010). Moreover, SeO_2 could induce apoptosis and inhibiting the telomerase activity in lung cancer cells (Chen et al. 2003). Also melatonin selenium nanoparticles could inhibit oxidative stress induced by lipopolysaccharide in mice (Wang et al. 2005). Tran and Webster (2011) also observed in vitro proliferation of *Staphylococcus aureus* inhibition by selenium nanoparticles. Other studies revealed that SeO_2 could inhibit in vitro proliferation of breast and lung cancer cells (Kuppusamy et al. 2005; Chen et al. 2003).

In conclusion, the results of our study revealed that selenium NPs and SeO_2 have dose-dependent anti-leishmanial activities. Also, selenium NPs have more anti-leishmanial activities with less cytotoxic effects than SeO_2.

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